Multisite Thrombus Imaging and Fibrin Content Estimation With a Single Whole-Body PET Scan in Rats

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Objective—Thrombosis is a leading cause of morbidity and mortality worldwide. Current diagnostic strategies rely on imaging modalities that are specific for distinct vascular territories, but a thrombus-specific whole-body imaging approach is still missing. Moreover, imaging techniques to assess thrombus composition are underdeveloped, although therapeutic strategies may benefit from such technology. Therefore, our goal was to test whether positron emission tomography (PET) with the fibrin-binding probe $^{64}$Cu-FBP8 allows multisite thrombus detection and fibrin content estimation.

Approach and Results—Thrombosis was induced in Sprague-Dawley rats (n=32) by ferric chloride application on both carotid artery and femoral vein. $^{64}$Cu-FBP8-PET/CT imaging was performed 1, 3, or 7 days after thrombosis to detect thrombus location and to evaluate age-dependent changes in target uptake. Ex vivo biodistribution, autoradiography, and histopathology were performed to validate imaging results. Arterial and venous thrombi were localized on fused PET/CT images with high accuracy (97.6%; 95% confidence interval, 92–100). A single whole-body PET/MR imaging session was sufficient to reveal the location of both arterial and venous thrombi after $^{64}$Cu-FBP8 administration. PET imaging showed that probe uptake was greater in younger clots than in older ones for both arterial and venous thrombosis ($P<0.0001$). Quantitative histopathology revealed an age-dependent reduction of thrombus fibrin content ($P<0.001$), consistent with PET results. Biodistribution and autoradiography further confirmed the imaging findings.

Conclusions—We demonstrated that $^{64}$Cu-FBP8-PET is a feasible approach for whole-body thrombus detection and that molecular imaging of fibrin can provide, noninvasively, insight into clot composition. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.306055.)

Key Words: autoradiography • fibrin • positron-emission tomography • thrombosis • whole body imaging

Thrombosis is often the underlying cause of major cardiovascular diseases, including heart attack, stroke, and venous thromboembolism, which are leading causes of morbidity and mortality. Current imaging modalities have good sensitivity and specificity, and have been accepted as gold standards for thrombus detection depending on the anatomic location. Particularly, transesophageal echocardiography and contrast-enhanced magnetic resonance imaging (MRI) are used to detect thrombi in the cardiac chambers, Doppler ultrasound has high sensitivity for deep vein thrombosis, and computed tomography is the gold standard in the diagnosis of pulmonary embolism and is used to detect the early signs of stroke. However, thrombus imaging would benefit from a whole-body approach capable of detecting multisite thrombi instead of several examinations, especially for those cardiovascular events (eg, thromboembolism) where the identification of both culprit embolus and source thrombus is necessary. Moreover, thrombus detection often relies on anatomic and mechanical vascular abnormalities or on blood flow deficit rather than directly imaging the target, which may negatively affect the specificity of detection. Furthermore, none of these modalities are informative about the composition of the thrombus, although therapeutic strategies may benefit from such information.

Direct targeting of the thrombus components using molecular imaging offers instead a noninvasive solution with high sensitivity and specificity, as well as potential whole-body applications. Several components of the coagulation cascade have been targeted to date with molecular probes, including some specific and abundant thrombus constituents as activated platelets and fibrin. In particular, fibrin is an ideal target for molecular imaging of thrombosis because it is present at high concentrations in both venous and arterial clots but not in circulating blood, resulting in potential high sensitivity and specificity of detection. We recently evaluated several positron emission tomography (PET) probes for noninvasive thrombus detection based on fibrin-targeting peptides with high specificity for fibrin over fibrinogen and other plasma proteins. The fibrin-binding probe $^{64}$Cu-FBP8 emerged as the best candidate for further translation because...
Nonstandard Abbreviations and Acronyms

MRI  magnetic resonance imaging
PET  positron emission tomography

of its high target affinity, fast blood clearance, and high metabolic stability.

Here, we aim to assess the feasibility of $^{64}$Cu-FBP8-PET imaging for whole-body thrombus detection in both the venous and arterial circulation at different stages of thrombus evolution after a single administration of probe. Moreover, we also tested whether $^{64}$Cu-FBP8-PET could inform thrombus composition by noninvasive evaluation of fibrin content.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Detection of Arterial and Venous Thrombi With $^{64}$Cu-FBP8-PET

Thrombi were generated in the common carotid artery and femoral vein of 32 Sprague-Dawley rats using the ferric chloride model, and thrombus location (right versus left) was kept undisclosed until the end of the study to assess in a blinded fashion the accuracy of $^{64}$Cu-FBP8-PET imaging. Contra lateral incisions were performed to create a sham surgical wound, hiding the actual location of the clot. At 1, 3, or 7 days after thrombus induction, rats were imaged according to the scheme depicted in Figure 1. Sequential PET scans of the hindlimbs and the neck region revealed the presence of isolated hot-spots in every animal. The localization of the radioactivity in PET images was suggestive of the common carotid artery and femoral vein visualized with computed tomography angiography. Fused PET/computed tomography images confirmed that the isolated hot-spots were located in the thrombosed vessels. Some radioactivity was detected at the surgical wound and the excretory organs, as previously reported. Blinded analysis of the accuracy of $^{64}$Cu-FBP8-PET imaging to detect the thrombus location was performed by 2 independent readers on a total of 21 animals. One reader correctly detected the location of 40 thrombi of 42 (1 day, 14/14; 3 days, 14/14; and 7 days, 12/14), whereas the other correctly detected the location of all clots (42/42). The mean overall accuracy was 97.6% (82/84), with an exact 95% confidence interval ranging from 0.916 to 0.997.

To further demonstrate that $^{64}$Cu-FBP8-PET is suitable for multisite thrombus detection with a single whole-body scan, we imaged a small cohort of rats in a clinical PET/MR scanner 1 day after thrombosis, as previously described. Rats were imaged for 60 minutes starting 1 hour after $^{64}$Cu-FBP8 injection (Figure 2). The location of both arterial and venous thrombi was clearly identified in this wide field of view after

Figure 1. Molecular imaging of arterial and venous thrombosis with $^{64}$Cu-FBP8-PET/CT. Experimental scheme showing probe administration and imaging time points (A). Computed tomography (CT), positron emission tomography (PET), and fused images from a representative animal injected with a single dose of $^{64}$Cu-FBP8 3 days after the induction of arterial (B) and venous (C) thrombosis. Contrast-enhanced CT angiography was used to detect the common carotid artery (B, arrowhead) and the femoral vein (C, arrowhead). The thrombus location appears as a hot-spot on the ipsilateral carotid artery (B, arrows) and femoral vein (C, arrows). Blinded assessment of PET accuracy to detect the location of the clot (right versus left) performed by 2 independent investigators (D). n=7 per group. CI indicates confidence interval.
a single administration of $^{64}$Cu-FBP8. Background activity was only detected in the liver, surgical wounds, and excretory organs.

$^{64}$Cu-FBP8 Uptake in Arterial and Venous Thrombi Depends on Thrombus Age

$^{64}$Cu-FBP8-PET imaging revealed that the radioactive signal for both arterial and venous thrombi was dependent on thrombus age. Signal intensity was the greatest in 1-day old thrombi and gradually declined with thrombus age, with a statistically significant difference among the 3 groups ($P<0.0001$, 1-way ANOVA; Figure 3). Particularly, we observed 0.65±0.19%ID/cc SD (1 day), 0.35±0.16%ID/cc SD (3 days), and 0.06±0.02%ID/cc SD (7 days) for the arterial clots, and 0.55±0.15%ID/cc SD (1 day), 0.36±0.05%ID/cc SD (3 days), and 0.12±0.04%ID/cc SD (7 days) for the venous thrombi. Contralateral carotid artery and femoral vein did not show any radioactive signal that could suggest the presence of a thrombus. Some activity was detected at the surgical wound, excretory organs, and genital area, the latter consistent with urine contamination. The high thrombus uptake combined with the low off-target background resulted in high target/background ratios. For the arterial thrombus, we found 20- to 30-fold at 1 day after surgery, 10- to 20-fold at 3 days, and 2- to 4-fold differences at 7 days, whereas for the venous clots, we observed 10- to 20-fold, 5- to 10-fold, and 2- to 4-fold differences at 1, 3, and 7 days after thrombosis, respectively. Because the arteries were imaged >1 hour after the veins, and because $^{64}$Cu-FBP8 progressively clears from background tissues but shows steady levels at the target site, this difference in target/background ratios between arterial and venous thrombi is expected based on the timing of the imaging.

Ex Vivo Biodistribution, Autoradiography, and Histopathology

Animals were euthanized at the end of the imaging experiments, and tissues were harvested and processed for biodistribution, autoradiography, and histopathology. The presence and the location (left versus right) of the thrombi in the carotid artery and femoral vein were confirmed in every animal. Biodistribution analysis showed that probe uptake was highest in the younger thrombus for both arterial and venous clots (Figure 4). In particular, the uptake of the arterial thrombi was 1.17±0.39%ID/g SD at 1 day, 0.66±0.36%ID/g at 3 days, and 0.20±0.19%ID/g at 7 days, whereas the activity at the level of the venous thrombi...
was 1.46±0.76 at 1 day, 0.96±0.65 at 3 days, and 0.21±0.10 at 7 days. Pearson’s analysis showed a positive correlation between the probe uptake detected with $\gamma$-counting and PET quantification, a further validation of the imaging findings. Ex vivo autoradiography showed results comparable with PET imaging and $\gamma$-counting. A hypointense region was detected in the thrombosed vessels, but not contralaterally. Autoradiography performed on histological slices confirmed the high activity of ipsilateral vessels, corresponding to the hematoxylin and eosin–stained thrombus, and low activity in contralateral arteries and veins. Quantitative assessments revealed high ipsilateral/contralateral activity ratios for arterial and venous thrombi, with a trend comparable with PET imaging and biodistribution. We observed higher ipsilateral/contralateral ratios for the younger thrombi (artery: 15-fold at 1 day and 10-fold at 3 days; vein: 35-fold at 1 day and 15-fold at 3 days) than for the older ones (artery: 3-fold at 7 days; vein: 5-fold at 7 days).

Histopathology revealed time-dependent changes of thrombus size and composition for both arterial and venous clots ($P<0.001$, 1-way ANOVA; Figure 5). Martius Scarlet Blue$^3$ staining showed that thrombi were conspicuous at 1 and 3 days after surgery, whereas at 7 days, the clots comprised a lesser extent of the vessel. Fibrin was a major constituent of both arterial and venous thrombi, confirmed by histochemical and immunofluorescence stainings. Younger thrombi (1 and 3 days) were rich in fibrin (purple-red) and erythrocytes (yellow), whereas some collagen fibers (blue) were also present at 7 days. Color segmentation was performed on Martius Scarlet Blue–stained sections to quantitatively assess the thrombus composition.$^{24–26}$ The fibrin content peaked at 1 day post thrombosis for both carotid artery and femoral vein and gradually decreased over time along with the thrombus size. At 7 days after thrombosis, the fibrin content in venous thrombi was greater than in arterial clots, consistent with the difference in uptake observed with PET imaging. In particular, the fibrin content was 0.74±0.25 mm$^3$ SD at 1 day, 0.39±0.18 mm$^3$ SD at 3 days, and 0.08±0.03 mm$^3$ SD at 7 days for arterial thrombi, and 0.83±0.26 mm$^3$ SD, 0.49±0.09 mm$^3$ SD, and 0.19±0.06 mm$^3$ SD at 1, 3, and 7 days for the venous clots, respectively. A positive correlation was found between

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**Figure 3.** $^{64}$Cu-FBP8-PET reveals age-dependent changes in thrombus uptake. Fused positron emission tomography (PET)/computed tomography images of representative rats injected with $^{64}$Cu-FBP8 showing age-dependent decrease in arterial (A) and venous (B) thrombus uptake. No uptake was detected in the contralateral vessels (contra). PET quantification reveals statistically significant differences in thrombus uptake among groups and high target/background tissue ratios for both arterial (C) and venous (D) thrombi. Contra indicates contralateral; and ipsi, ipsilateral. Yellow arrow indicates thrombus; red thin arrow, contralateral vessel; blue arrowheads, surgical wound; and green arrowheads, urine. Error bars are SEM. ***$P<0.001$ and **$P<0.01$, 1-way ANOVA followed by Tukey post hoc test. n=7 per group.
we assessed whether $^{64}$Cu-FBP8-PET can provide insights on thrombus age and composition by evaluation of fibrin content. Our findings showed that probe uptake was higher for the younger clots and lower for the older ones and that such age-dependent uptake was consistent with the amount of fibrin in these thrombi. Taken together, these results provide a proof-of-concept for a new, sensitive approach based on PET imaging for whole-body thrombus detection and to noninvasively assess changes in thrombus composition by directly targeting its main component: fibrin.

Despite the recent advances in noninvasive thrombus detection, current imaging modalities still have some limitations that challenge both diagnosis and therapy monitoring. A main diagnostic drawback is the absence of a single-session approach with high sensitivity and specificity to detect thrombosis in different anatomic locations. In fact, sensitivity and specificity of current imaging modalities depend on the anatomic location of the thrombus. However, in the case of thromboembolism, both diagnosis and therapeutic strategy would benefit from a single approach with whole-body detection capabilities to reveal the location of the culprit embolus (eg, middle cerebral artery in case of stroke, pulmonary artery in case of pulmonary embolism) and the source thrombus (eg, atherosclerotic carotid plaque, aortic arch, left atrial thrombus, deep vein thrombosis). Indeed, a potential application for $^{64}$Cu-FBP8-PET is secondary stroke prevention. The diagnosis of stroke pathogenesis is crucial for secondary prevention because the risk of recurrent events is associated with the underlying pathology. Current stroke diagnostic workup includes ultrasonography, transesophageal echocardiography, MR angiography, and computed tomography angiography to detect common embolic sources (eg, aortic arch and intra/extracranial atheroma, atrial thrombus), resulting in a time consuming and expensive process that can delay therapeutic intervention. Despite these different diagnostic tools, a third of ischemic strokes are classified as cryptogenic (ie, the source thrombus is unknown). A whole-body approach would be beneficial in this large population. Earlier work with $^{64}$Cu-FBP7, a close analog of $^{64}$Cu-FBP8, detected carotid thrombi and intra/extracranial emboli with high target/background ratios, and we anticipate similar efficacy with $^{64}$Cu-FBP8. Therefore, if translated to a clinical setting, $^{64}$Cu-FBP8-PET may help identify the location of both the culprit embolus (eg, middle cerebral artery) and the source thrombus (eg, carotid artery, aortic arch) in stroke patients.

Thrombus evolution is a dynamic process, and changes in fibrin content can affect clot stability. Human thrombi are characterized by platelets and fibrin in the early phase, whereas smooth muscle cells, proteoglycans, and collagen are main constituents of healing clots. Fresh clots are usually softer, unstable, and are more likely to be lysed than mature, organized thrombi. Therefore, noninvasive assessment of clot composition can be useful for thrombus staging and to facilitate therapeutic choices (eg, thrombolysis versus thrombectomy). Several attempts have been made to characterize thrombus composition using noninvasive imaging. Ultrasound elastography has shown thrombus staging capabilities ex vivo, but its feasibility still needs to be addressed in vivo.
Changes in T₁ relaxation time, magnetization transfer, and diffusion-weighted MRI have been associated with iron metabolism and protein turnover of venous thrombi, showing diagnostic potential to assess thrombus age and predict successful clot lysis. However, noncontrast MRI offers limited target sensitivity and specificity, and therefore detecting small thrombotic events may be challenging especially for whole-body applications. Molecular MRI using the fibrin-binding probe EP-2104R has shown good correlation with fibrin thrombus content. However, molecular MRI requires pre and postcontrast imaging sessions, which can delay the diagnostic workflow. Moreover, previous experience with EP-2104R in patients with thrombosis revealed that the clots appear more conspicuous at later time points after injection (24 hours), which may complicate the analysis of pre and postprobe images and further delay decision making and treatment. Scintigraphy with the ⁹⁹ᵐTechnetium-labeled activated platelets, inhibitor DPM-444 and ⁹⁹ᵐTechnetium-rtPA have shown good feasibility identifying fresh clots over organized thrombi, but at the same time the low efficacy in detecting mature clots reduced their clinical applications. Fluorodeoxyglucose-PET has been recently used to stage thrombosis by imaging neutrophilic infiltration in fresh venous thrombi. However, fluorodeoxyglucose uptake is also increased in other pathologies associated with high cellularity and hypermetabolism (eg, cancer, chronic inflammatory conditions), which may limit the specificity of this imaging approach. The findings shown here demonstrate that ⁶⁴Cu-FBP8-PET can detect differences in fibrin content in both arterial and venous clots, even when thrombi were small and fibrin levels low (ie, 7-day old thrombi). Because the high target specificity of molecular imaging and the great sensitivity and absolute quantification capabilities of PET, thrombus imaging with ⁶⁴Cu-FBP8 may overcome the shortcomings of previous imaging modalities and thus provide a novel approach for whole-body clot detection and fibrin content estimation.

This work has some limitations, including the small sample size. We tested our hypothesis in animal models that only partially mimic the human pathology; therefore, our results need...
to be validated in clinical settings. Moreover, this approach may not be suitable for diagnosis of thrombosis in the emergency department, where minutes of delay negatively affect the clinical outcome. However, $^{64}$Cu-FBP8-PET may be beneficial when the life-threatening culprit is under control, but the source thrombus is still unknown and the risk of recurrence is high. In these circumstances, a thrombus imaging approach with whole-body clot detection capabilities may provide useful insight for both diagnosis (eg, etiologic assessment) and intervention. PET imaging has higher costs compared with other modalities and exposes the patient to ionizing radiation. However, the high sensitivity of PET allows using micrograms of tracer compared with other contrast-based imaging modalities where grams of probe are required (eg, MRI), thus reducing potential chemical toxicity. Despite the relatively long half-life of $^{64}$Cu (12.7 hours), the rapid whole-body clearance and low retention of $^{64}$Cu-FBP8 suggest that radiogenic adverse effects should not limit its clinical translation, and recent radiation dosimetry studies in rats support this conclusion. Moreover, the longer half-life may allow synthesis of the molecular probe in advance, and even on demand shipment to remote sites far from a cyclotron. Finally, because of its straightforward radiolabeling procedure, $^{64}$Cu-FBP8 is also amenable to kit formulation which may further facilitate bench-to-bedside translation.

Conclusions

$^{64}$Cu-FBP8-PET is a feasible approach for whole-body thrombus detection and for noninvasive evaluation of fibrin content and represents a novel and valuable diagnostic strategy for translation in clinical imaging of thrombosis.

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Disclosures

Dr Caravan has equity in Factor 1A, LLC, the company which holds the patent rights of the fibrin-binding peptide used in this study. The other authors report no conflicts.

References


**Significance**

Thrombosis is the underlying cause of deadly diseases, such as stroke, pulmonary embolism, deep vein thrombosis, and heart attack, which affect millions of people worldwide. Current thrombus imaging techniques are specific for selected anatomic locations, but whole-body detection of thrombi with a single scan is not feasible yet. Therefore, the diagnosis of cardiovascular diseases where multiple thrombi are hidden, and ready to generate deadly emboli, requires several diagnostic assessments, which may delay both diagnosis and therapeutic intervention. Molecular imaging of thrombosis using positron emission tomography and the fibrin-specific probe FBP8 allows sensitive and specific whole-body detection of thrombi with a single scan providing a novel and powerful tool that may facilitate diagnosis, guide therapeutic choices, and monitor treatment efficacy.
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MATERIAL AND METHODS

Fibrin-binding probe $^{64}$Cu-FBP8

$^{64}$Cu-FBP8 was synthesized in quantitative yield (purity >99% by HPLC) as previously reported$^1$, with a specific activity of 6-12 GBq/µmol. Briefly, the cyclic disulfide peptide precursor FHCHypY(3-Cl)DLCIL-PXD (Hyp=L-4-hydroxyproline, Y(3-Cl)=L-3-chlorotyrosine, PXD=para-xylenediamine) was conjugated with the chelator NODAGA (1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid), followed by labeling with $^{64}$CuCl$_2$. The peptide in $^{64}$Cu-FBP8 comes from a family of peptides that have been widely studied in the past, and we previously showed that these peptides have high specificity for fibrin and the DD(E) fragment when compared to either fibrinogen or serum albumin, and similar affinity to human, rat, mouse, pig, and dog fibrin$^2, 3$. $^{64}$Cu-FBP8 has high affinity for the soluble fibrin fragment DD(E) ($K_i = 430$ nM) and binds to fibrin immobilized in a well plate. Moreover, $^{64}$Cu-FBP8 is remarkably stable in blood after intravenous administration (>90% intact probe up to 4 hours post injection), and clears prevalently by the renal pathway (plasma half-life 14 min)$^1$.

Experimental model of arterial and venous thrombosis

All animal experiments were performed in accordance with the NIH “Guide for the Care and Use of Laboratory Animals” and were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. Adult male Sprague-Dawley rats (n = 32, 200-250 g, Charles River Laboratories) were anesthetized with isoflurane (4% induction, 2-2.5% maintenance, in medical air), and arterial and venous thrombosis was induced by using the ferric chloride model$^4$. A small piece of filter paper was soaked for 1 min in a solution of ferric chloride (Sigma, 25% w/v, in sterile saline), and then applied for 5 min on the common carotid artery and the femoral vein of each animal to induce thrombosis. At the end of the procedure, the surgical site was rinsed with sterile saline to remove the excess of ferric chloride, and the formation of the clot was confirmed by visual inspection. Thrombus location (right vs. left) was kept undisclosed until the end of the study to assess in a blinded fashion the accuracy of $^{64}$Cu-FBP8-PET imaging. Contralateral incisions were performed to create a sham surgical wound, hiding the actual location of the clot.

Probe administration

1, 3, or 7 days after thrombosis induction, rats were anesthetized with isoflurane (4% induction, 2-2.5% maintenance) and then positioned in a small-animal PET/SPECT/CT scanner (Triumph; TriFoil Imaging) equipped with respiratory monitoring, heating pad system and inhalation anesthesia. Each rat was injected via tail vein with ~10 MBq in a volume of ~300 µL, followed by saline flush. The injected dose was calculated by the difference of the radioactivity in the syringe before and after the administration, as measured by a dose calibrator (CRC-25PET, Capintec)$^1, 5, 6$.

Micro-PET/CT imaging

$^{64}$Cu-FBP8 was allowed to clear from the blood for 60 min before starting the PET acquisition. Since the field of view of the PET covered just 6-7 cm of the animal, we first imaged the hindlimbs (isocenter: femoral vein), followed by the neck (isocenter: carotid artery), with each scan lasting 60 min. At the end of each PET imaging session, a CT scan was performed over
4.27 min with 512 projections and 2 frames per projection (peak tube voltage, 70 kV; tube current, 177 mA). A polyethylene glycol-coated gold nanoparticle contrast agent (300 mg/Kg, i.v.) was injected before the CT scan for angiography. This gold nanoparticle contrast agent was used as it is long-circulating and provides good contrast for both arterial and venous trees. PET and CT images were reconstructed using the LabPET software package (TriFoil Imaging). The PET data were corrected for decay, scatters, randoms and dead time, and reconstructed using a maximum-likelihood expectation-maximization algorithm run over 30 iterations. CT data were used to provide attenuation correction. Reconstructed data were quantitatively evaluated using AMIDE, by drawing volumes of interest (VOIs) on thrombosed and contralateral arteries and veins (4.2 mm³), muscle (acromiotorapezius and calf, 65.4 mm³), and bone (spinal process of cervical vertebrae and tibia, 4.2 mm³). All VOIs were placed on standardized anatomical locations based on the CT-only image. However, since the thrombus location showed some variability among the animals, and the actual location of the clot was indistinguishable in CT images, VOIs were first placed on the vessel using CT-only images and then centered on the “hot spot” using fused PET/CT images, as we previously reported. PET data are expressed as percentage of injected dose per cubic centimeter (%ID/cc).

**Whole-body PET/MR imaging**

A small cohort of rats (n = 2) was scanned 1 day after thrombus induction in a clinical PET/MR scanner, as previously described. Rats were imaged for 60 min starting 1 hour after ⁶⁴Cu-FBP8 injection. PET data were acquired using the BrainPET, a MR compatible PET scanner that operates inside of a Magnetom TIM Trio 3T MRI (Siemens) and provides an axial field of view of ~20 cm, covering the whole rat body length. The list-mode emission data were rebinned in the sinogram space for fast reconstruction. The uncorrected PET volume was first reconstructed and binary segmented based on an empirically determined threshold in soft tissue and air. A uniform linear attenuation coefficient (0.096 cm⁻¹, corresponding to water at 511 keV) was assigned to all soft tissue voxels and the resulting attenuation map (combined with the coil attenuation map) was forward projected to derive the attenuation correction factors in sinogram space. The scatter sinogram is obtained using a fully 3D scatter calculation method based on the single scatter estimation method. First the normalization and attenuation corrected emission volume is reconstructed. Second, a scatter estimate is obtained by Monte Carlo simulations from this volume and the attenuation map. Third, this scatter estimate is scaled axially to fit the tails of the normalized true data which accounts for the out of field of view scatter. The normalization was calculated from a 64 hr scan of a plane-source rotated in the FOV. The images were reconstructed with the ordinary Poisson ordered subsets expectation maximization (OP-OSEM) algorithm using 16 subsets and 6 iterations. The reconstructed volume consists of 153 slices with 256×256 pixels (1.25×1.25×1.25 mm³). The spatial resolution at the center of the field of view is approximately 2.5 mm. MR imaging was performed simultaneously to the PET acquisition. 3D gradient echo T1-weighted time-of-flight (TOF) MR angiography sequences were performed with an echo time of 3.86 ms, repetition time of 21 ms, flip angle of 18°, field of view = 9 × 18 cm, bandwidth = 178 Hz/pixel, 2 averages, and an acquisition time of 15.3 min. TOF data was reconstructed into 256 256 × 128 matrices with an in-plane pixel spacing of 0.703 × 0.703 mm², and a slice thickness of 0.7 mm.
Ex vivo studies
Animals were euthanized at the end of the imaging experiments, and tissues harvested and processed for biodistribution, autoradiography and histopathology.

Biodistribution
For biodistribution studies (n = 7/group), the radioactivity of the thrombosed carotid artery and femoral vein, the contralateral vessels, muscle and bone (calf and tibia, obtained from the contralateral paw) were quantified with a gamma-counter (Wizard\textsuperscript{2}, PerkinElmer) to determine the percentage of injected dose per gram of tissue (%ID/g\textsuperscript{1,5,6}).

Autoradiography
Ipsilateral and contralateral carotid arteries and femoral veins were further analyzed by autoradiography (n = 5/group) using a multipurpose film and phosphor imaging system (Cyclone Plus, PerkinElmer). A subset of vessels was fresh-frozen, cryosectioned and then exposed on a multipurpose film for 4 hours. Adjacent slices were stained with Hematoxylin and Eosin to detect the intraluminal thrombus. Regions of interest were drawn around thrombosed and contralateral vessels (OptiQuant, PerkinElmer), and ipsi:contra activity ratios were obtained by dividing matched ipsilateral and contralateral raw values from each animal\textsuperscript{1,5,6}.

Histopathology
Histopathology (n = 5/group) was performed to evaluate thrombus morphology and composition. Preliminary experiments showed that the morphology of the vessels was not preserved after autoradiography. Therefore, 2 out of 7 animals per group were randomly assigned to the histopathological analysis, and 3 additional rats were added to each group only for histology. Thrombus size and composition were comparable between the animals that were imaged and those that were not. Arteries and veins were rinsed in phosphate buffer, fixed in neutral buffered formalin, embedded in optimal cutting temperature mounting medium (OCT, Tissue-Tek), and then snap-frozen. Vessels were cryosectioned to sample the entire length of the thrombus, and stained using the trichrome method Martius Scarlet Blue (MSB) which allows to differentiate between erythrocytes (yellow), fibrin (purple-red) and collagen (blue\textsuperscript{11}). Color segmentation was performed using the ImageJ software (NIH), and the sum of the areas occupied by fibrin was integrated with the interval between adjacent sections (500 \(\mu\)m) to obtain the total volume (mm\textsuperscript{3})\textsuperscript{12-14}. This method has shown high correlation with fibrin quantification obtained with western blot analysis\textsuperscript{12-14} and, differently from western blot, provides volumetric information about the thrombus size. To confirm the specificity of MSB to detect fibrin, adjacent slices were stained with H&E, MSB and an anti-fibrin antibody (Abcam, U45, 10 \(\mu\)g/mL\textsuperscript{5}). Negative control experiments were performed by omitting the primary antibody. Images were acquired using a microscope equipped with epifluorescence illumination (TE-2000, Nikon).

Statistics
Data were expressed as mean ± SEM. Differences between groups were analyzed using 1-way ANOVA followed by Tukey post-hoc test. The Pearson correlation coefficient was computed to assess quality of linear correlations and a t-statistic was calculated based on the null hypothesis that the correlation coefficient was zero. A p-value <0.05 was considered significant.
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