Supplemental data for

Multimodal molecular imaging reveals high target uptake and specificity of ^{111}In and ^{68}Ga labeled fibrin-binding probes for thrombus detection

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Supplemental methods

Reagents

All chemicals were purchased commercially and used without further purification. NODAGA-NHS (2,2'-(7-(1-carboxy-4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)-1,4,7-triazonane-1,4-diyl)diacetic acid) was purchased from Chematech (Dijon, France). $^{68}GaCl_3$ was eluted from a $^{68}Ge/^{68}Ga$ generator supplied by iThemba Labs/IDB Holland BV (50 mCi). $^{64}CuCl_2$ (specific activity 1.3 ± 0.2 mCi/nmol) was obtained from the University of Wisconsin, Medical Physics Department, $^{111}InCl_3$ and $Na^{99m}TcO_4$ were purchased from Nordion and Triad Isotope, respectively, and $Na^{125}I$ was bought from Perkin-Elmer.

High Performance Liquid Chromatography (HPLC)

Preparative HPLC purification was performed on a Varian Prostar system with two Prostar 210 pumps and a Prostar 325 UV/Vis detector, using a Phenomenex Luna C18 column (250 x 21.2 mm, 10 μm). Liquid chromatography-electrospray mass spectrometry (LC-MS) was performed using an Agilent 1100 Series apparatus with a Phenomenex Luna C18 column (100 × 2 mm, 5 μm), equipped with an LC-MSD trap and a Daly conversion dynode detector. Radio-HPLC analyses were obtained on an analytical Agilent 1100 Series system using a Phenomenex Luna C18 (150 x 4.6 mm, 5 μm; for analysis of the ^{64}Cu, ^{68}Ga and ^{111}In probes), a Phenomenex Kromasil C4 (250 x 4.6 mm, 5 μm; for analysis of the ^{99m}Tc probe), or a MetaChem Polaris C18 column (150 x 4.6 mm, 5 μm; for analysis of blood samples). Five different HPLC methods were used depending on whether HPLC was being used for purification (Method 1 and 2), for LC-MS analysis (Method 3), to check radiochemical yields and purity (Method 4) or to analyze blood metabolites (Method 5). All Methods used as mobile phase A H_2O with 0.1% trifluoroacetic acid (TFA) and as mobile phase B CH_3CN with 0.1% TFA. Method 1: flow rate of 15 mL/min, gradient = 0 – 35 min, 5 to 55% B; 35 – 40 min, 55 to 95% B; 40 – 41 min, 95% B; 41 – 41.1 min, 95 to 5% B; 41.1 – 45 min, 5% B. Method 2: flow rate

of 15 mL/min, gradient = 0 - 1.5 min, 5% B; 1.5 – 8 min, 5 to 50% B; 8 – 10 min, 50 to 95% B; 10 – 14 min, 95% B; 14 – 14.1 min, 9 to 5% B; 14.1 – 16 min, 5% B. Method 3: flow rate of 0.7 mL/min, gradient = 0 – 1 min, 5% B; 1 – 10 min, 5 to 95% B; 10 – 12 min, 95% B; 12 – 12.5 min, 95 to 5% B; 12.5 – 15 min, 5% B. Method 4: flow rate of 1 mL/min, gradient = 0 – 13 min, 5 to 95% B; 13 - 14.75 min, 95% B; 14.75 – 15.0 min, 95 to 5% B; 15.0 – 17.0 min, 5% B. Method 5: flow rate of 1 mL/min, gradient = 0 – 16.5 min, 5 to 60% B; 16.5 – 18 min, 60 to 95% B; 18 – 19 min, 95% B; 19 – 19.1 min, 95 to 5% B; 19.1 – 22 min, 5% B.

Synthesis of L-Cys-Pep and D-Cys-Pep

The peptides L-Cys-Pep and D-Cys-Pep (Pep = FHC*HypY(3-Cl)DLCHIL-PXD, C* = L-Cys or D-Cys, Hyp=*L*-4-hydroxyproline, Y(3-Cl)=*L*-3-chlorotyrosine, PXD=*para*-xylenediamine) were synthesized on a 0.5 mmol scale using microwave assisted solid phase peptide synthesis (CEM Liberty Microwave Peptide Synthesizer) using 1,4-bis-(aminoethyl)-benzene trityl resin (1.3 mmol/g Novabiochem, CA), Fmoc protected amino acids (EMD Chemicals, NJ), and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) coupling chemistry. The protected amino acids were dissolved in dimethylformamide (DMF, 0.2 M), the activator HBTU solution was prepared also in DMF (0.45 M) and the activator base diethylisopropylamine (DIEA) in N-methyl-2-pyrrolidone (NMP, 2 M). The deprotection solution was prepared as a 20 % v/v solution of piperidine in DMF with 0.1 M 1-hydroxybenzotriazole (HOBt) hydrate. Trityl resin was subjected to the deprotection solvent (7 mL) and irradiated by microwave at 75 °C for 300 seconds (s). The resin was filtered and washed with DMF (15 mL) and again subjected to another cycle of deprotection (7 mL of 20 % piperidine/HOBt; irradiation at 75 °C and 20 watts for 300 s). The solution was filtered, washed with DMF (4 x 15 ml) and filtered again. The first Fmoc-protected amino acid was added (10 eq., 25 mL), followed by HBTU (10 eq., 11.1 mL) and finally the base DIEA (10 eq., 2.5 mL). The reaction vessel was heated to 75 °C (only 50 °C for histidine/cysteine) using microwave irradiation at 20 watts for 300 s. The resin was filtered and washed with DMF (3 x 15 mL). Each amino acid was added sequentially from the C-terminus to the N-terminus using the same procedure. Upon completion of the sequence, the resin was

filtered and deprotected with 5 mL of a cocktail of TFA:methanesulfonic acid (MSA, 85% solution):triisopropylsilane (TIS): dodecanethiol (DDT) (86.5:4.5:4.5:4.5) for 3 h. The solution was isolated by filtration, triturated with diethylether and centrifuged. The filtrate was removed by decanting, and the pellet dried under high vacuum to remove residual solvents. The peptides were cyclized using 15% DMSO, 10% acetonitrile, and 75% water (10 mL/mmol crude) over 48 h. The crude mixture was purified using Method 1.

Synthesis of $(NODAGA)_2$-L-Cys-Pep and $(NODAGA)_2$-D-Cys-Pep

$(NODAGA)_2$-L-Cys-Pep and $(NODAGA)_2$-D-Cys-Pep were prepared following the procedure depicted in Figure 1. The cyclic fibrin-binding peptide D-Cys-Pep (7 mg, 4.58 μmol) was dissolved in DMF, followed by 3 eq. of ^{t}Bu-NODAGA-NHS activated ester (8.80 mg, 13.7 μmol) and DIEA until pH 6 (5 μL). The reaction was allowed to proceed until all of the free peptide had reacted as detected by LC-MS. The protecting groups were removed by reaction with a TFA cocktail (see above) for 3h. The solution was then precipitated with cold diethylether, centrifuged, the filtrate removed by decanting, and the pellet purified by HPLC (Method 1). Lyophilization of the desired peak provided 2.3 mg of white powder. Retention time (analytical HPLC, Method 3): 6.0 min. LC-ESI-MS: theoretical MW for $C_{101}H_{142}ClN_{23}O_{29}S_2 = 2241.9$; observed 1121.5 $[M + 2H]^{2+}$.

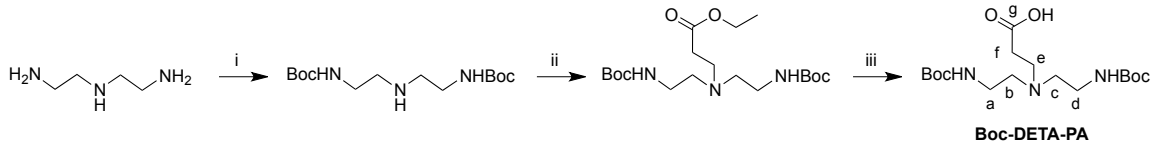
Synthesis of $(DOTA)_2$-L-Cys-Pep

DOTA (80 mg, 197 μmol) was dissolved in water and activated with tetrafluorophenol (33 mg, 197 μmol) dissolved in acetonitrile and DCC (41 mg, 197 μmol) in pyridine as described in (*1*). After 1 hour of reaction analysis, of the mass-chromatogram showed the presence of about 50% of unreacted DOTA and about 17% of a side product corresponding to the bis-activated DOTA. Longer reaction times did not improve the yield. Purification by preparative HPLC using Method 2 gave 16.3 mg of a white powder (29.4 μmol, 15% yield) after lyophilization of the desired peak. Retention time (analytical HPLC, Method 3): 4.3 min. LC-ESI-MS: theoretical MW for $[M+H]^+ = 553.4$, observed 553.2. The activated ester dissolved in 200 μL of DMF (2.28 mg, 4.12 μmol, 3 eq.) was

slowly added to L-Cys-Pep (2.1 mg, 1.37 μmol, dissolved in 400 μL of DMF with 5 μL of DIEA) during 8 h. $(DOTA)_2$-L-Cys-Pep (1.2 mg of a white powder) was isolated by prep-HPLC using Method 1. Retention time (analytical HPLC, Method 3): 6.1 min. LC-ESI-MS: theoretical MW for $C_{103}H_{148}ClN_{25}O_{29}S_2 = 2300.0$; observed 767.4 $[M + 3H]^{3+}$.

Synthesis of (DETA-PA)$_2$-L-Cys-Pep

The tridentate ligand Boc-DETA-PA (Boc-diethylenetriamine propanoic acid) was synthesized as depicted in supplemental scheme 1 following a slightly modified procedure published in literature (*2*). Briefly, Boc protection of the primary amines of diethylenetriamine, alkylation of the secondary amine with ethyl 3-bromopropanoate and subsequent ester hydrolysis with sodium hydroxide afforded the Boc-protected bifunctional ligand. The final compound was characterized by ^1H and ^{13}C NMR and LC-MS. ^1H-NMR [$CDCl_3$, 500 MHz, δ(ppm)]: 5.317 (N*H*Boc, br s, 2H), 3.310 (4H, m, C$\underline{H}_2^a$ and C$\underline{H}_2^d$), 2.932 (2H, m, C$\underline{H}_2^f$), 2.838 (4H, m, C$\underline{H}_2^b$ and C$\underline{H}_2^c$), 2.518 (2H, m, C$\underline{H}_2^e$), 1.439 (18H, s, Boc, -COO-C-(C$\underline{H}_3$)$_3$). ^{13}C-NMR [$CDCl_3$, 500 MHz, δ(ppm)]: 170.81 (-$\underline{C}$OOH), 156.25 (Boc, -$\underline{C}$OO-C-(CH$_3$)$_3$), 79.76 (Boc, -COO-$\underline{C}$-(CH$_3$)$_3$), 53.22 (-$\underline{C}H_2^f$), 50.52 (-$\underline{C}H_2^a$ and -$\underline{C}H_2^d$), 37.44 (-$\underline{C}H_2^b$ and -$\underline{C}H_2^c$), 30.54 (-$\underline{C}H_2^e$), 28.37 (Boc, -COO-C-($\underline{C}$H$_3$)). For NMR assignment identification system is depicted in supplemental scheme 2. Retention time (analytical HPLC, Method 3): 6.2 min. LC-ESI-MS: theoretical MW for $C_{17}H_{33}N_3O_6 = 375.4$; observed 376.2 $[M + H]^+$.



SUPPLEMENTAL SCHEME 1. Synthesis of Boc-DETA-PA. (i) Boc_2O, THF 0 °C, 4 h; (ii) $BrCH_2CH_2COOC_2H_5$, $(Et)_3N$, KI, CH_3CN, Δ, 48 h; (ii) MeOH, 5% NaOH, RT, 3 h. Identification system for NMR assignments is displayed in Boc-DETA-PA (*2*).

Boc-DETA-PA (10 mg, 26.6 μmol) was activated in 600 μL of DMF with tetrafluorphenol (6.6 mg, 39.9 μmol, 1.5 eq.), DCC (8.2 mg, 39.9 μmol, 1.5 eq.) and DIPEA (6 μL). After reacting for 5 hours, the activated ester was slowly added (330 μL,

14.8 μmol, 4.5 eq., 4 additions) to L-Cys-Pep (5 mg, 3.28 μmol). After overnight reaction the Boc-protecting groups were removed by reaction with a TFA cocktail for 3 hours. After precipitation with cold diethylether, $(DETA\text{-}PA)_2$-L-Cys-Pep was isolated by prep-HPLC using Method 1. Retention time (analytical HPLC, Method 3): 5.8 min. LC-ESI-MS: theoretical MW for $C_{85}H_{126}ClN_{23}O_{17}S_2$ = 1841.6; observed 921.9 $[M + 2H]^{2+}$.

Preparation of the Non-Radioactive fibrin binding probes

^{nat}Cu-D-Cys-FBP8. $(NODAGA)_2$-D-Cys-Pep ligand (0.4 mg, 0.178 μmol) was dissolved in 335 μL of a 1 mM solution of $CuSO_4$ in NaOAc 10 mM pH 5.5 (0.335 μmol, 2.5 eq.). The mixture was heated at 60 °C for 60 min. EDTA (1 mL of 10 mM solution) was added to scavenge any excess Cu^{2+}. The compound was purified by Sep-Pak. ^{nat}Cu-EDTA was washed with 4 mL of H_2O and the bis-copper complex eluted in 3 mL of MeOH (0.4 mg, 0.169 μmol). Retention time (analytical HPLC, Method 3): 6.0 min. LC-ESI-MS: theoretical MW for $C_{101}H_{136}ClCu_2N_{23}O_{29}S_2$ = 2362.9; observed 1182.4 $[M + 2H]^{2+}$.

^{nat}Ga-FBP14. $(NODAGA)_2$-L-Cys-Pep ligand (0.8 mg, 0.357 μmol) was dissolved in 890 μL of a 1 mM solution of $Ga(NO_3)_3$ in NaOAc 10 mM pH 5.5 (0.890 μmol, 2.5 eq.). The mixture was heated at 60 °C for 90 min. The compound was purified by Sep-Pak to isolate the bis-gallium complex (0.7 mg, 0.294 μmol). Retention time (analytical HPLC, Method 3): 6.2 min. LC-ESI-MS: theoretical MW for $C_{101}H_{136}ClGa_2N_{23}O_{29}S_2$ = 2375.3; observed 1188.4 $[M + 2H]^{2+}$.

^{nat}In-FBP15. $(DOTA)_2$-L-Cys-Pep ligand (0.5 mg, 0.217 μmol) was dissolved in 625 μL of a 1 mM solution of $InCl_3$ in NaOAc 10 mM pH 5.5 (0.625 μmol, 3 eq.). The mixture was heated at 85 °C for 90 min. The compound was purified by Sep-Pak to isolate the bis-indium complex (0.5 mg, 0.203 μmol). Retention time (analytical HPLC, Method 3): 6.5 min. LC-ESI-MS: theoretical MW for $C_{103}H_{142}ClIn_2N_{25}O_{29}S_2$ = 2523.6; observed 842.1 $[M + 3H]^{3+}$.

^{nat}Re-FBP16. An excess of $[Re(CO)_3(H_2O)_3]Br$ (163 μL of 10 mM solution, 1.63 μmol), synthesized from $Re(CO)_5Br$ as described in (*3*), was added to 1 mg of $(DETA\text{-}PA)_2$-L-Cys-Pep (1 mg, 0.54 μmol) dissolved in NaOAc 10 mM pH 7. The solution was heated at 85 °C for 5 h with stirring. Quality control of the reaction mixture was done by RP-HPLC. After completion 20 μL of diethylenetriamine (185 μmol) was added and allowed

to stand for 20 minutes at room temperature. The desired product was purified by HPLC (Method 1) and lyophilized. Retention time (analytical HPLC, Method 3): 6.9 min. LC-ESI-MS: theoretical MW for $C_{91}H_{126}ClN_{23}O_{23}Re_2S_2$ = 2382.1; observed 1192.2 $[M + 2H]^{2+}$.

Synthesis of ^{64}Cu, ^{68}Ga, ^{111}In and ^{99m}Tc labeled probes

^{64}Cu-labeling. A 25 μL aliquot of a 1 mg/mL solution of $(NODAGA)_2$Pep (11.15 nmol, Pep = L-Cys-Pep or D-Cys-Pep) was combined with a solution of $^{64}CuCl_2$ (4 - 8 mCi) in NaOAc (40 mM, pH 5.5, total volume 200 μL). After reaction at 60 °C for 30 min the reaction was analyzed by analytical radio-HPLC (Method 4, Rt 8.8 min) and instant thin layer chromatography (iTLC). Under these conditions, FBP8 (L-Cys-Pep) and D-Cys-FBP8 (D-Cys-Pep) were obtained in quantitative yields, at a ligand concentration of 56 μM and with specific activities of 0.35 - 0.71 mCi/nmol.

^{68}Ga-labeling. The $^{68}Ga/^{68}Ge$ generator (iThemba/IDB Holland BV, 50 mCi) was eluted with 6 mL of 0.6 M HCl. The third mL of the eluate was mixed with 1 mL of 3 M sodium acetate buffer pH 5.5, and 500 μL of this solution (2 – 3 mCi) was added to 25 μL of a 1 mg/mL solution of $(NODAGA)_2$-L-Cys-Pep. After heating for 15 - 20 min at 60 °C FBP14 was obtained in quantitative yields as ascertained by radio-HPLC (Method 4; Rt 8.6 min), at a ligand concentration of 22.3 μM and with specific activities of 0.26 - 0.35 mCi/nmol. ^{68}Ge breakthrough was removed by Sep-Pak C18 purification. Free ^{68}Ge was removed by washing the cartridge with 4 mL of water and FBP14 was collected in 3 mL ethanol. The purified probe was diluted with NaCl 0.9% and the organic solvent evaporated on the rotavap.

^{111}In-labeling. 50 μL of the $(DOTA)_2$-L-Cys-Pep conjugate solution (1 mg/mL, 21.73 nmol) was diluted with 150 μL of NaOAc buffer 40 mM pH = 5.5 and 20 μL of $^{111}InCl_3$ 0.05 M HCl (4 - 5 mCi). The solution was heated at 85 °C for 45 min. After cooling to room temperature, the labeling mixture was challenged with ethylenediaminetetraacetic acid (EDTA) to coordinate any excess of ^{111}In. After 10 min at room temperature a sample of the resulting solution was analyzed by radio-HPLC (Method 4, Rt 8.3 min). The radiochemical yield ranged from 92 – 95% (specific activities of 0.18 - 0.23 mCi/nmol). Pure radiolabeled peptide was obtained after purification using a C18 Sep-

Pak column.

^{99m}Tc-labeling. Na[$^{99m}TcO_4$] was eluted from a ^{99m}Tc/^{99}Mo generator (Mallinckrodt Ultra-TechneKow DTE) using 0.9% saline. $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared using a home made kit composed by 4.5 mg of $K_2[BH_3CO_2]$, 8.5 mg of sodium tartrate, 2.5 mg of sodium tetraborate and 7.15 mg of sodium carbonate(*4*). After addition of 2 mL of $^{99m}TcO_4$ (10 – 12 mCi) to the kit, the reaction mixture was incubated at 100 °C for 20 min. The radiochemical purity of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was checked by RP-HPLC (Method 4, Rt of "$^{99m}TcO_4$" = 5.4 min and "$^{99m}Tc(CO)_3$" = 11.1 min). After neutralization with 120 μL of 1 M HCl, 950 μL of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was added to 50 μL of peptide conjugate (1 x 10^{-3} M, 50 nmol) and heated at 100 °C for 25 min. FBP16 was obtained in 99.5% yield as determined by radio-HPLC (Method 4, Rt of 10 min), at a ligand concentration of 50 μM and with specific activities of 0.10 - 0.12 mCi/nmol. Radiochemical yield was also determined by iTLC using saline as eluent (Rf of FBP16 = 0 and Rf of "$Tc(CO)_3$" = 0.9). The presence of colloids was evaluated by iTLC using MeOH/ 1% HCl 6M as eluent. Using this method Tc colloidal stays at the baseline of the TLC plate (Rf = 0) and FBP16 moves with the solvent (Rf = 0.9). No colloids were present (*5*).

Labeling of ^{125}I-Fibrinogen

Fibrinogen (Calbiochem) was labeled with Na[^{125}I] (Perkin-Elmer) using the Iodogen technique (Pierce Chemical Corp.) (*6, 7*). Briefly, 200 μL of fibrinogen (5 mg/ml) suspended in 0.05 M phosphate buffer pH 7.4 were placed in the tube and 3.5 mCi of carrier-free ^{125}I was added. The iodination was allowed to proceed for 45 min at 0 °C under agitation using a magnetic stirrer. The labeling efficiency of the ^{125}I-fibrinogen was 94.3 ± 3.8% (two independent labeling reactions) as determined by thin layer radiochromatography. ITLCs (0.5 x 7 cm strips, ITLC-SG, GelmanSciences) were eluted using NaCl 0.9% as mobile phase. The radioactivity of the strips was analyzed using a BioScan (AR-2000) scanner. Free iodine was removed by size exclusion chromatography using a Sephadex G-25 (PD-10) column (Amersham Pharmacia Biotech) and PBS as elution buffer. Specific activity of ^{125}I-fibrinogen was 0.487 mCi/mmol. Labeled fibrinogen was stored in PBS buffer at -80 °C. Under these conditions the product is

stable for at least 6 months. The degree of functional damage caused by the labeling was assessed by determining the % of clottability of the fibrinogen. Briefly, in eppendorf tubes ~20 μCi of ^{125}I-fibrinogen was mixed with 100 μL of fibrinogen (5 mg/mL in TBS) and 100 μL of human thrombin (2 U/mL in TBS) containing 7 mM of $CaCl_2$. The solution was incubated at 37 °C for 60 min. The clottability (90.7 ± 2.8%,n=2) was determined by first measuring the total activity of the fibrinogen solutions using a gamma counter ($^{125}I_{Total}$). After incubation the solutions were centrifuged to separate the clot. The clot was washed with PBS and the activity of the supernatant ($^{125}I_{supernatant}$) was measured. The clottability is given by $\%C = ((1 - {}^{125}I_{supernatant})/{}^{125}I_{total}) \times 100$.

In vivo Stability

Blood samples collected at 2, 15, 120 and 240 minutes into EDTA tubes were immediately centrifuged for 10 minutes at 5000 rpm and the plasma passed through a 0.22-μm Millipore filter and injected onto an analytical HPLC column (Method 5). The eluent was collected every 0.5 minutes, and the activity of each fraction was measured by the gamma counter. Data obtained from the gamma counter were plotted to reconstruct the HPLC chromatograms (supplemental figure 3).

Determination of K_i

The affinity of the probes was assessed using a DD(E) fluorescence polarization displacement assay that was described previously *(8)*. The displacement of a tetramethylrhodamine labeled peptide (TRITC-Tn6) from DD(E) was detected by observing the corresponding change in fluorescence anisotropy. The K_d of the TRITC-Tn6 probe was determined by titrating it with the DD(E) protein and fitting the resultant fluorescence data as described previously *(8)*. This experiment was performed at room temperature using a concentration of TRITC-Tn6 of 0.1 μM in the following assay buffer: Tris base (50 mM), NaCl (100 mM), $CaCl_2$ (2 mM), Triton X- 100 (0.01%), pH = 7.8. The anisotropy measurements were made using a TECAN Infinity F200 Pro plate reader equipped with the appropriate filter set for tetramethylrhodamine (excitation 535 nm, emission 590 nm). A series of solutions of the probes (^{nat}Cu-D-Cys-FBP8, ^{nat}Ga-FBP14, ^{nat}In-FBP15 and ^{nat}Re-FBP16) were prepared through serial dilutions (25 to 0.1

μM). These solutions were then added to a mixture of the DD(E) protein and TRITC-Tn6 peptide. The final concentrations of protein and fluorescent probe used in these experiments were 2 μM and 0.1 μM, respectively. All measurements were performed at room temperature in a 384-well plate from Greiner Bio One. The inhibition constants, K_i of the probes were then calculated using least-squares regression and the known K_d of the fluorescent probe (supplemental table 1), as described previously (*3*). Concentrations were determined by analyzing for ^{nat}Cu, ^{nat}Ga, ^{nat}In, and ^{nat}Re by inductively coupled plasma-mass spectrometry.

Functional Fibrin Binding Assay

Human fibrinogen (American Diagnostica) was dialyzed against 50 mM Tris, pH 7.4, 150 mM sodium chloride, 5 mM sodium citrate (TBS·citrate) prior to use. The fibrinogen concentration was adjusted to 5 mg/mL (based on the absorbance at 280 nm and ε_{280} = 1.512 L•g^{-1}cm^{-1}), and $CaCl_2$ was added (7 mM). The fibrinogen solution (50 μL) was dispensed into the wells of a 96-well polystyrene microplate (Immulon-II). A solution (50 μL) of human thrombin (2 U/mL) in TBS was added to each well to clot the fibrinogen and to yield a final fibrin concentration close to 2.5 mg/mL (7.3 μM based on fibrinogen MW = 340 kDa). The plates were incubated at 37 °C and evaporated to dryness overnight. Blood tubes were centrifuged (2000 rpm for 20 minutes at 4 °C), and then plasma incubated in fibrin immobilized wells as well as in empty wells, and the sealed plate incubated for 2 h on a shaker at 300 rpm and room temperature. The plate was sealed with tape to prevent evaporation and agitated at 300 rpm on a shaker. After incubation, the counts in the supernatant in both the fibrin-containing and empty wells were measured on a gamma counter and divided by the weight of plasma to determine the concentration of unbound probe, [unbound], and total probe, [total], respectively. The amount of radioactive species bound to fibrin, [bound], was calculated from [bound] = [total] − [unbound]. As a positive control, an aliquot of the injected dose was spiked into blood plasma and used to estimate the total possible fibrin binding by each FBPs in the assay (% bound at t = 0). The amount of functional probe in the blood at time t was determined by taking the ratio of the % bound to fibrin at time t compared to the % bound at t = 0, and multiplying this ratio by the measured total % ID/g in the blood.

SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1. K_i values (μM) determined for fibrin binding peptides through the competitive displacement of the peptide TRITC-Tn6 from DD(E). EP-2104R was used as positive control *(9)*.

EP-2104R	D-Cys-FBP8	FBP14	FBP15	FBP16
0.31	>1000	-	-	0.83
0.35	-	0.53	0.60	-

SUPPLEMENTAL TABLE 2. Biodistribution data (% ID/g ± SEM) at 120 minutes post injection in rats with crush injury.

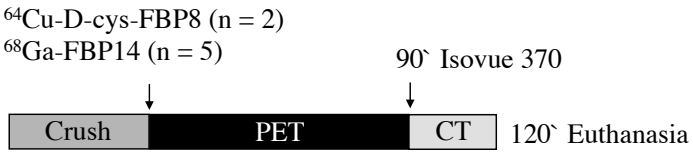
Tissue	**FBP14**	**FBP15**	**FBP16**
Thrombus	0.977 ± 0.182	1.043 ± 0.137	0.730 ± 0.070
Left Carotid	0.480 ± 0.085	0.277 ± 0.041	0.767 ± 0.103
Liver	0.302 ± 0.022	0.216 ± 0.041	3.240 ± 0.545
Kidney	4.070 ± 0.321	2.015 ± 0.293	4.928 ± 0.546
Heart	0.038 ± 0.008	0.060 ± 0.006	0.078 ± 0.005
Lungs	0.1056 ± 0.007	0.124 ± 0.020	1.468 ± 0.343
Spleen	0.189 ± 0.028	0.183 ± 0.036	1.862 ± 0.615
Intestine	0.222 ± 0.047	0.153 ± 0.017	0.536 ± 0.162
Muscle	0.084 ± 0.026	0.034 ± 0.006	0.036 ± 0.003
Bone	0.037 ± 0.006	0.080 ± 0.010	0.188 ± 0.009
Brain	0.004 ± 0.001	0.007 ± 0.002	0.010 ± 0.001
Blood	0.061 ± 0.014	0.100 ± 0.013	0.171 ± 0.022

n	5	8	7

SUPPLEMENTAL FIGURES

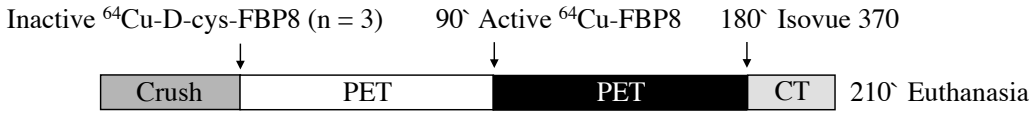
SPECT/PET–CT imaging protocols

A) Carotid crush injury to evaluate and directly compare ^{64}Cu-D-Cys-FBP8 and ^{68}Ga-FBP14

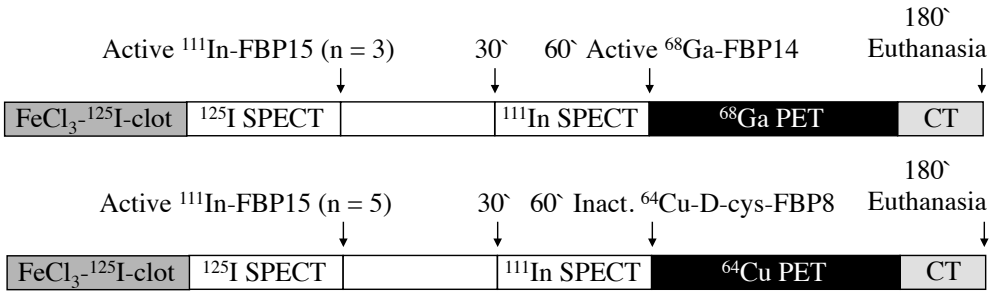


B) Carotid crush injury to evaluate and directly compare ^{111}In-FBP15 and ^{99m}Tc-FBP16

C) Carotid crush injury to evaluate nonbinding probe ^{64}Cu-D-cys-FBP8

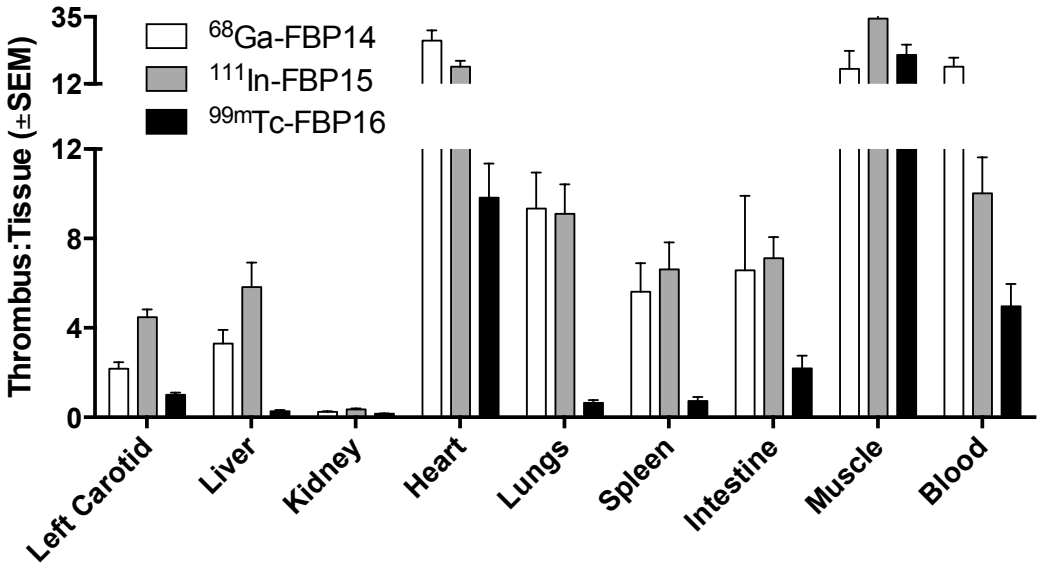


D) Evaluation of target specificity using multimodal approach



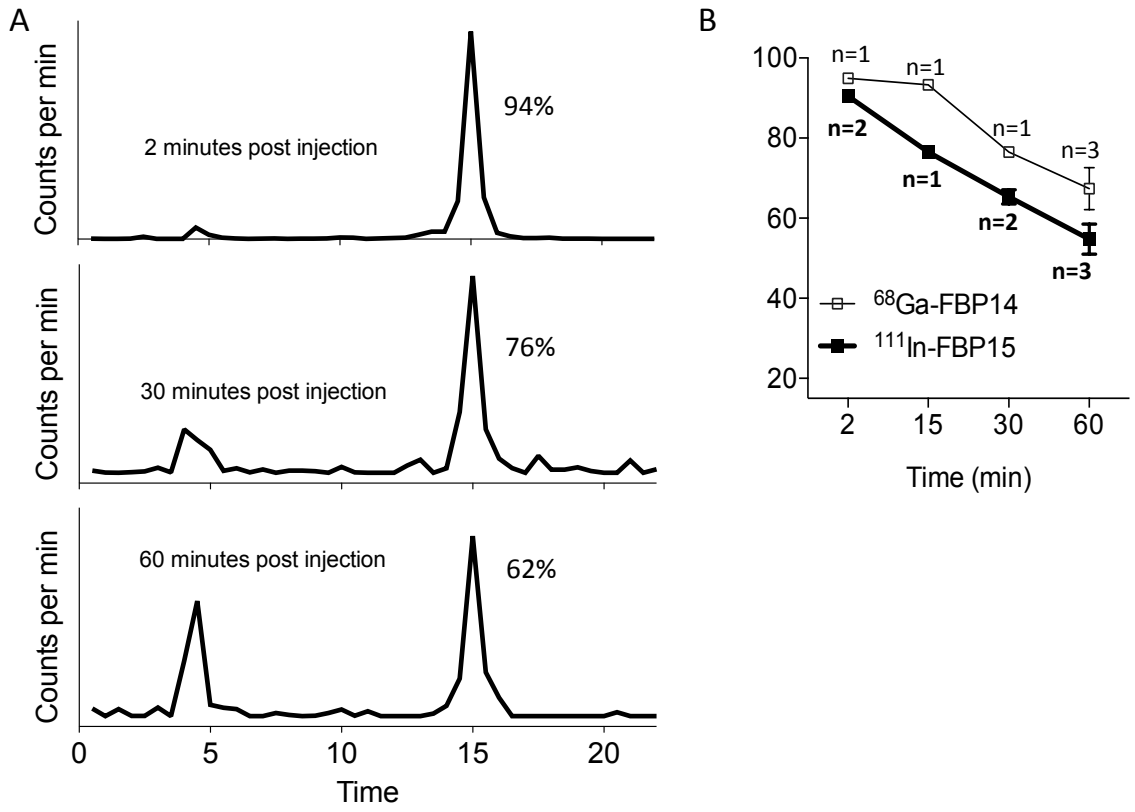
SUPPLEMENTAL FIGURE 1. Timeline of the carotid crush injury (crush) and $FeCl_3$-induced ^{125}I-clot experiments. A) To directly compare the new probes, rats with carotid crush injury were imaged dynamically for 90 minutes immediately after the injection of

the PET probes ^{64}Cu-D-Cys-FBP8 or ^{68}Ga-FBP14 or B) statically for 60 minutes beginning 30 minutes after injection of the SPECT probes ^{111}In-FBP15 or ^{99m}Tc-FBP16. Blood was drawn before and at several time points after injection of the probe. C) To characterize the negative control probe ^{64}Cu-D-Cys-FBP8, rats with crush injury were injected with ^{64}Cu-D-Cys-FBP8 and imaged with dynamic PET for 90 minutes; active probe ^{64}Cu-FBP8 was then injected and the animal PET imaged for a further 90 minutes followed by CT angiography and euthanasia. D) Target specificity was evaluated using a multimodal triple-isotope approach in rats with a ferric chloride induced thrombus that was labeled with ^{125}I-fibrin. SPECT imaging was performed for 10 minutes to image the ^{125}I-labeled thrombus. Rats were administered ^{111}In-FBP15 and static SPECT imaging was performed for 30 minutes beginning 30 minutes after injection. Then, either inactive probe ^{64}Cu-D-cys-FBP8 or active probe ^{68}Ga-FBP14 was injected and dynamic PET was performed for 60 minutes. CT angiography followed the PET scan and then sacrifice.

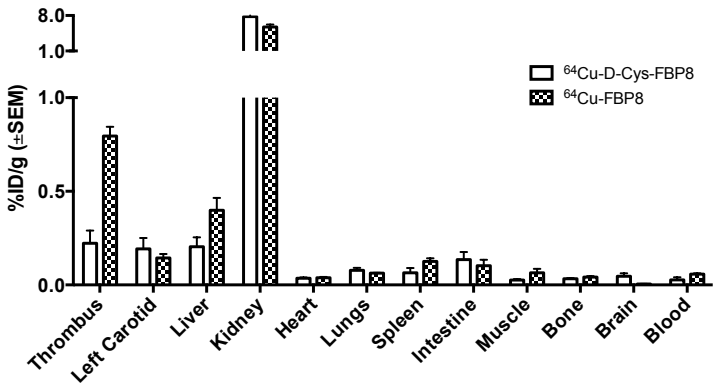


Thrombus:tissue ratios (± SEM)	^{68}Ga-FBP14	^{111}In-FBP15	^{99m}Tc-FBP16
Left Carotid	2.171 ± 0.297	4.062 ± 0.515	1.000 ± 0.099
Liver	3.303 ± 0.611	5.826 ± 1.105	0.273 ± 0.051
Kidney	0.237 ± 0.030	0.634 ± 0.037	0.156 ± 0.016
Heart	26.866 ± 3.475	17.878 ± 2.018	9.832 ± 1.520
Lungs	9.340 ± 1.616	9.110 ± 1.311	0.649 ± 0.126
Spleen	5.623 ± 1.276	6.614 ± 1.210	0.729 ± 0.184
Intestine	6.582 ± 3.324	7.124 ± 0.932	2.193 ± 0.560
Muscle	17.130 ± 6.169	34.429 ± 6.214	21.988 ± 3.519
Blood	17.925 ± 3.032	10.427 ± 1.853	4.960 ± 1.011
n	5	8	7

SUPPLEMENTAL FIGURE 2. Thrombus:tissue ratios from biodistribution data for ^{68}Ga-FBP14, ^{111}In-FBP15 and ^{99m}Tc-FBP16 at 120 min (crush model).

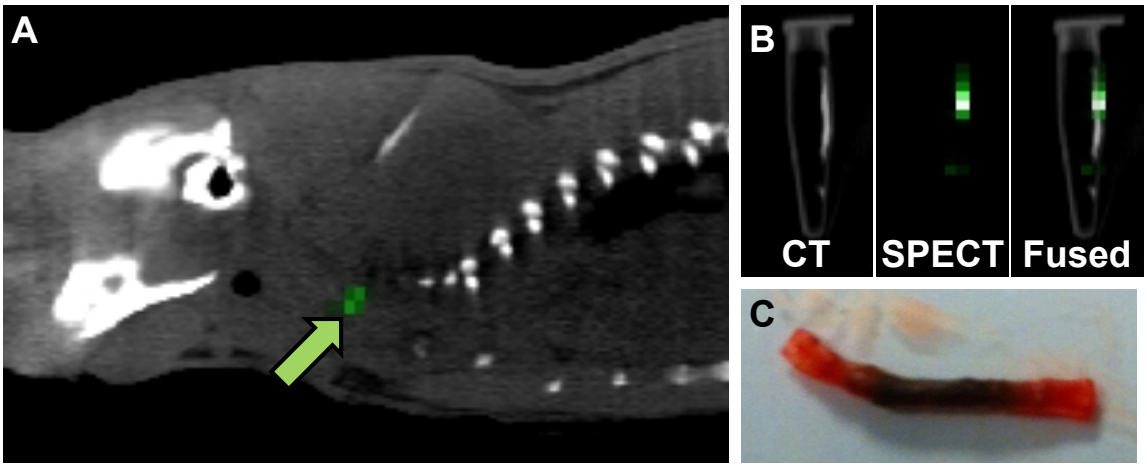


SUPPLEMENTAL FIGURE 3. (A) Typical chromatograms of ^{68}Ga-FBP14 prior to injection. Rat plasma from blood draws at different time points (2, 15, 30 and 60 min) was injected onto an analytical HPLC and the eluent was collected every 30 seconds. HPLC traces were reconstructed by measurement of the activity contained in the individually collected fractions using a gamma counter (Packard, CobraII Auto gamma). (B) % of intact ^{68}Ga-FBP14 and ^{111}In-FBP15 relative to total activity in rat blood at time points 2, 15, 30 and 60 minutes.

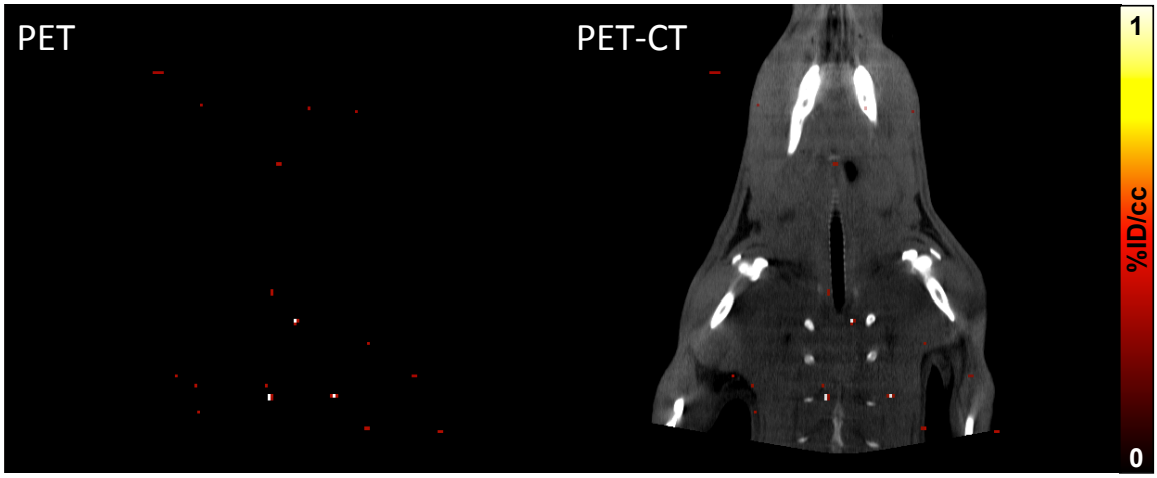


Tissue	^{64}Cu-D-Cys-FBP8	^{64}Cu-FBP8
Thrombus	0.222 ± 0.068	0.795 ± 0.050
Left Carotid	0.192 ± 0.058	0.144 ± 0.021
Liver	0.204 ± 0.050	0.399 ± 0.066
Kidney	7.781 ± 0.987	5.732 ± 0.482
Heart	0.036 ± 0.004	0.039 ± 0.004
Lungs	0.078 ± 0.013	0.063 ± 0.002
Spleen	0.065 ± 0.026	0.126 ± 0.016
Intestine	0.135 ± 0.040	0.102 ± 0.032
Muscle	0.026 ± 0.056	0.065 ± 0.021
Bone	0.033 ± 0.003	0.042 ± 0.005
Brain	0.046 ± 0.016	0.005 ± 0.001
Blood	0.027 ± 0.013	0.058 ± 0.004
n	5	7

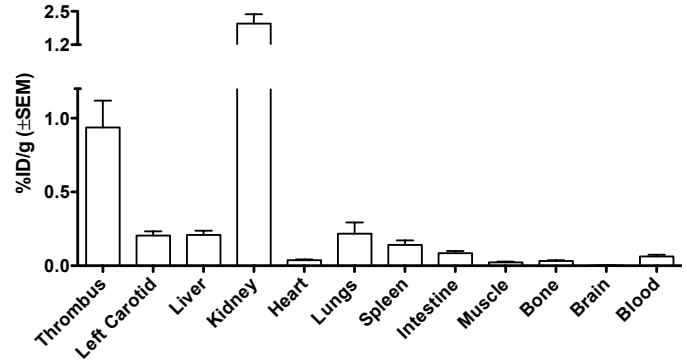
SUPPLEMENTAL FIGURE 4. Ex vivo biodistribution data (% ID/g ± SEM) for the nonbinding probe ^{64}Cu-D-Cys-FBP8 at 120 minutes after tracer injection in rats with crush injury (n = 5). Biodistribution data for the active analog ^{64}Cu-FBP8 (n = 7) in the same model and at same time point is also depicted (*10*).



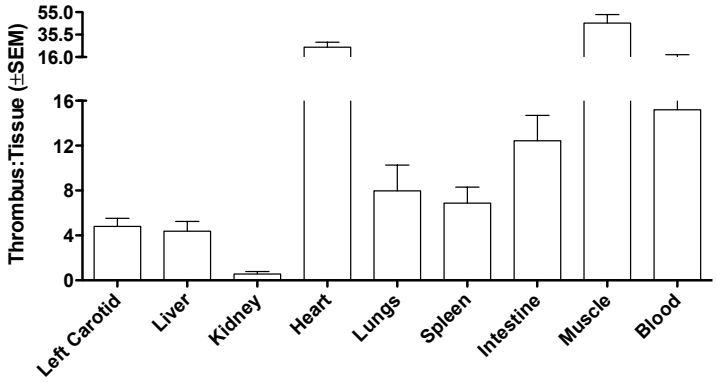
SUPPLEMENTAL FIGURE 5. (A) SPECT-CT after formation of a "hot clot" by injecting ^{125}I-labeled fibrinogen in the common carotid artery followed by application of $FeCl_3$ to initiate thrombosis. (B) Ex vivo SPECT-CT imaging performed on dissected thrombosed artery (C) confirms the presence of "hot clot" consistent with incorporation of ^{125}I-fibrinogen in the thrombus.



SUPPLEMENTAL FIGURE 6. PET scan (10 min) acquired before injection of the PET probe ^{68}Ga-FBP14 in an animal previously imaged by ^{111}In-FBP15-SPECT demonstrating that the presence of ^{111}In does not contribute to the PET image.



Tissue (± SEM)	FBP15
Thrombus	0.938 ± 0.182
Left Carotid	0.205 ± 0.028
Liver	0.226 ± 0.026
Kidney	2.022 ± 0.597
Heart	0.038 ± 0.005
Lungs	0.218 ± 0.085
Spleen	0.141 ± 0.032
Intestine	0.086 ± 0.017
Muscle	0.022 ± 0.007
Bone	0.032 ± 0.005
Brain	0.010 ± 0.000
Blood	0.063 ± 0.012
n	9



Thrombus:tissue ratios (± SEM)	FBP15
Left Carotid	4.801 ± 0.732
Liver	4.371 ± 0.873
Kidney	0.566 ± 0.015
Heart	24.576 ± 4.260
Lungs	7.696 ± 2.302
Spleen	6.885 ± 1.420
Intestine	12.442 ± 2.251
Muscle	93.750 ± 18.183
Blood	15.193 ± 3.292
n	9

SUPPLEMENTAL FIGURE 7. Whole-body biodistribution for ^{111}In-FBP15 (n = 9) at 180 minutes after ferric chloride thrombosis. Error bars are SEM.

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