SUPPLEMENTAL MATERIAL

In vivo molecular imaging of thrombosis and thrombolysis using a fibrin-binding PET probe

Ilknur Ay*, MD PhD; Francesco Blasi*, PharmD PhD; Tyson A. Rietz, BS; Nicholas J. Rotile, BA; Sreekanth Kura, MS, Anna Liisa Brownell, PhD, Helen Day, MS; Bruno L. Oliveira, PhD; Richard J. Looby, BS; Peter Caravan, PhD

Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital East, 149 13th Street, Charlestown, Massachusetts 02129, USA

SUPPLEMENTAL METHODS

Reagents

CB-TE2A [C16H30N4O4 (2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)diacetic acid)] was a gift of Prof. Carolyn Anderson, University of Pittsburgh Medical Center. $^{64}CuCl_2$ was obtained from the University of Wisconsin, Medical Physics Department. All other chemicals were purchased commercially and used without further purification.

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 mm × 2 mm, 5 μm). This system is 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 column (150 x 4.6 mm, 5 μm; to check radiochemical yields and purity) or a MetaChem Polaris C18 (150 x 4.6 mm, 5 μm; for analysis of urine and blood samples). Four different HPLC methods were used depending on whether HPLC was being used for preparative purification (Method 1), to assess purity (Method 2), to check radiochemical yields and purity (Method 3) or to analyze urine and blood metabolites (Method 4). For all the methods the mobile phase A was H_2O with 0.1% trifluoroacetic acid (TFA) and mobile phase B was CH_3CN with 0.1% TFA. Method 1 (flow rate of 15 mL/min): 0 – 3 min, 5% B; 3 – 15 min, 5 to

95% B; 15 – 18 min, 95% B; 18 – 19 min, 95 to 5% B; 19 – 22 min, 5% B. Method 2 (flow rate of 0.8 mL/min): 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 3 (flow rate of 1.0 mL/min): 0 – 12 min, 5 to 80% B; 12 - 12.1 min, 80 to 5% B; 12.1 – 13 min, 5% B. Method 4 (flow rate of 1 mL/min): 0 – 17 min, 0 to 100% B; 17 – 17.1 min, 100 to 0% B; 17.1 – 18 min, 0% B.

Probe Synthesis

The cyclic disulfide peptide precursor F.H.C.Hyp.Y(3-CI).D.L.C.H.I.L.PXD (Hyp=L-4-hydroxyproline, Y(3-Cl)=L-3-chlorotyrosine, PXD=paraxylenediamine) was prepared with the use of a rink amide MBHA resin, Fmoc coupling strategy, and aqueous DMSO cyclization. This peptide (24 mg), CB-TE2A (17.2 mg, 3eq), and Pybop (3eq) were added to 5 ml DMF while the pH of the reaction (monitored by spotting the reaction on a wet pH stick) was adjusted to ca. 6 with diisopropylethylamine. The reaction was stirred at RT for 16 h. The reaction was concentrated to an oil and purified using Method 1. Lyophilization of fractions containing the desired product provided 10 mg of white powder (theoretical MW for $[M + 2H]^{2+}$ = 1089.3, observed 1089.0).[1]

The peptide-$(CB2)_2$ ligand (25 μL, 1.0 mM in 10mM in NH_4OAc) was diluted in 400 μL of 40 mM NH_4OAc, pH 4.5. $^{64}CuCl_2$ was added (0.5 - 5 mCi) and heated at 95 °C for 60 minutes. The reaction was monitored by radio-HPLC, which showed 90 - 95% conversion to the desired product. EDTA (1 mL of 10 mM solution) was added and the mixture stirred an additional 20 min to scavenge any unchelated copper ion. The compound was purified using Method 3, concentrated via rotary evaporation, and reconstituted with 10 mM NH_4OAc and saline (1:1). This purified material was again checked by HPLC (Method 3) for final radiochemical purity.

In vitro stability assay

FBP7 was incubated with citrated rat plasma (Innovative Research Inc) at 37°C. Aliquots (100 µL) were collected after 0, 2 and 16 hours of incubation and mixed with 100 µL ice-cold methanol to precipitate plasma protein. Proteins were then sedimented by centrifugation (10,000 g x 5 min) and the supernatant was injected into HPLC (Method 4).[1]

DD(E) Binding Assay

The affinity of FBP7 to the soluble fibrin fragment DD(E) was assessed in the presence and absence of heparin using a fluorescence polarization assay as previously described.[1, 2] Here we used FBP7 labeled with natural abundance ^{63,65}Cu. This assay measures the displacement of a tetramethylrhodamine-labeled peptide (TRITC-Tn6) from DD(E) upon addition of FBP7 by observing the corresponding change in fluorescence anisotropy. The K_d of the TRITC-Tn6 probe binding to DD(E) is 0.92 µM[2], and based on this Kd and the observed displacement as a function of FBP7 concentration, an inhibition constant K_i for FBP7 can be determined.[2] The experiment was performed at room temperature using a probe concentration 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. For the heparin competition, the assay buffer was mixed in 33 IU sodium heparin blood collection tubes to allow for a 5U/ml final concentration. 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 FBP7 were prepared through serial dilutions from a stock solution. These solutions were then added to a mixture of the DD(E) protein and TRITC-Tn6 peptide in assay buffer with or without heparin. The final concentrations of DD(E) and fluorescent probe used in these experiments were 2 µM

and 0.1 μM, respectively. All measurements were performed in a 384-well plate. The inhibition constant, K_i of FBP7, were then calculated using least-squares regression and the known Kd of the fluorescent probe.

Biodistribution analysis

Serial blood samples were collected from the femoral artery at 0, 2, 5, 10, 15, 30, 60, 120, 240, and 300 min post-injection into EDTA blood tubes. Blood was weighed and radioactivity in the blood was measured on a gamma counter (Packard, CobraII Auto gamma) to assess clearance of total activity.

To quantify organ and body fluid distribution of copper-64, blood, chest and abdominal organs, brain, left rectus femoris muscle, and left femur were collected from all animals after euthanasia. Additionally, the right common carotid artery containing the intramural thrombus and the contralateral common carotid artery were collected from animals that had carotid crush injury. The tissues were weighed and radioactivity in each tissue was measured using a gamma counter, along with an aliquot of the injected FBP7 solution.[1]

The radioactivity was reported as percent injected dose per gram (%ID/g) which was calculated by dividing the counts of copper-64 per gram of tissue by the total counts of the injected dose, all decay corrected to the time of injection.

Autoradiography

Autoradiography of the common carotid arteries and the brain sections was performed using a multipurpose film and a Perkin-Elmer Cyclone Plus Phosphor Imager and PerkinElmer OptiQuant 5.0 software. Exposure time was between 1-10 min.[1]

Metabolic Stability Assay

Blood samples collected at 2, 15, 120 and 240 min were immediately centrifuged for 10 min at 2000 rpm and the supernatant passed through a 0.22-μm Millipore filter and injected onto an analytical HPLC column at a flow rate of 1 mL/min using Method 4. The eluent was collected every 0.5 min and the activity of each fraction was measured by the gamma counter. The HPLC analysis was performed in duplicate or triplicate. Data obtained from the gamma counter were plotted to reconstruct the HPLC chromatograms.[1]

Functional Fibrin Binding Assay

Human fibrinogen (American Diagnostica) was dialyzed against Tris (50 mM, pH 7.4), sodium chloride (150 mM), and sodium citrate (TBS·citrate; 5 mM), and the fibrinogen concentration was adjusted to 5 mg/mL (based on the absorbance at 280 nm and ε_{280} = 1.512 L/g/cm). After adding $CaCl_2$ (7 mM), 50 μL of this fibrinogen solution was dispensed into a 96-well polystyrene microplate (Immulon-II). Human thrombin solution (50 μL; 2 U/mL in TBS) was added into each well to clot the fibrinogen yielding 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 overnight and evaporated to dryness. Blood plasma that was obtained after centrifugation (2000 rpm for 20 min at 4°C) diluted in TBS (1:1) was incubated in fibrin immobilized wells as well as in empty

wells for 2h on a shaker at 300 rpm and room temperature. 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 and total probe, respectively. The amount of copper-64 containing species bound to fibrin was calculated from [bound] = [total] − [unbound]. 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 copper-64 %ID/g in the blood.[1]

Histopathology

Ipsilateral (right) and contralateral (left) common carotid arteries were collected two hours after crush injury (n=2); brains were harvested one hour after embolic stroke induction (n=2). Samples were carefully rinsed in Phosphate Buffer, embedded in OCT mounting media (Tissue-Tek), and snap-frozen in -45°C isopentane. The brains and the arteries were cryosectioned (20 μm thickness) and processed for Hematoxylin and Eosin staining according to the standard protocol. For fibrin immunostaining, adjacent slices were incubated with a mouse anti-fibrin alpha-chain antibody (Abcam, U45). Griffonia Simplicifolia Lectin 1 (Vector Laboratories) was used to stain the vascular endothelium; Hoechst 33342 (Sigma) was used as nuclear marker. Negative control experiments were performed by omitting the primary antibody anti-fibrin U45, resulting in the absence of specific fibrin immunostaining. Images were acquired using a Nikon TE-2000 microscope equipped with epifluorescence illumination.

Supplemental Figures and Figure Legends

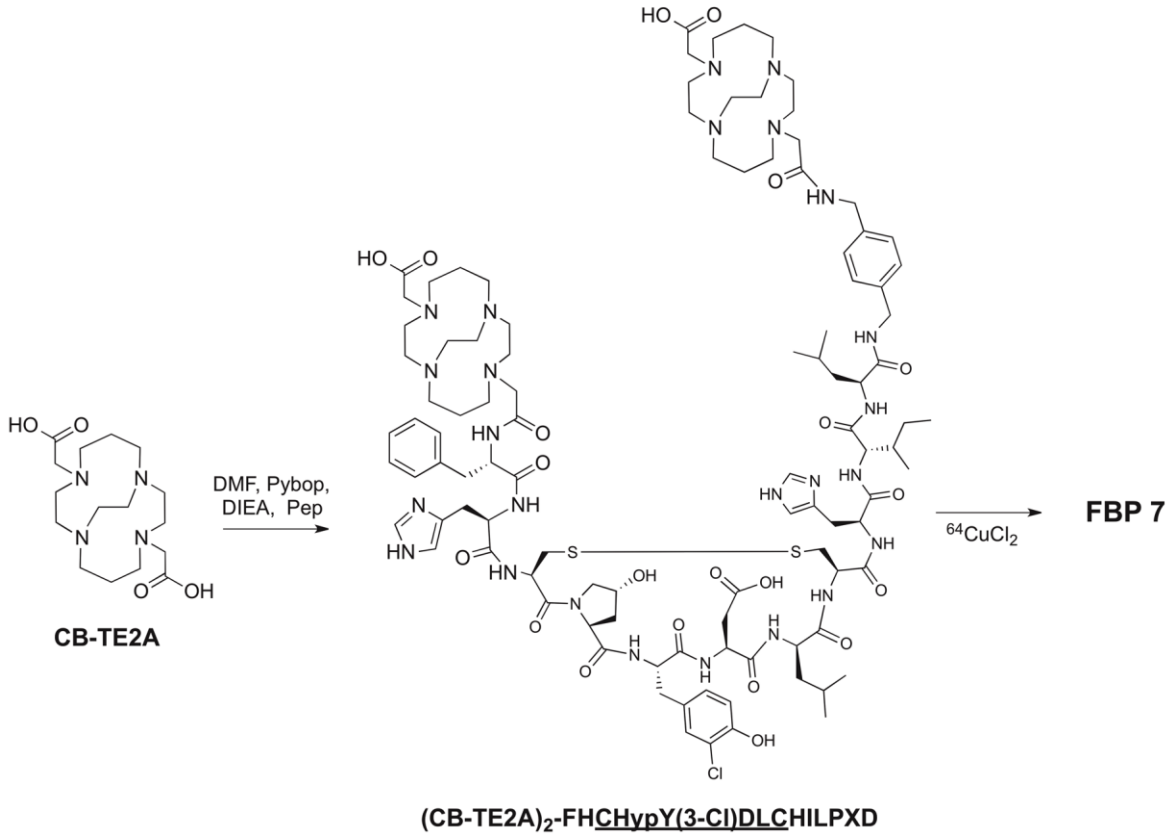

Figure I. Synthesis and chemical structure of FBP7.

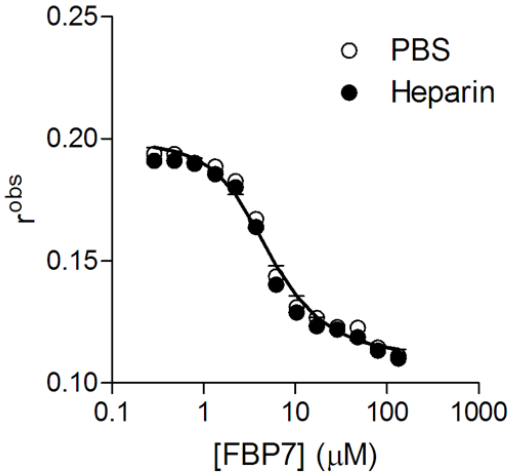

Figure II. Displacement of TRITC-Tn6 peptide (0.2 μM) from DD(E) by FBP7 in absence (clear symbols) and presence of heparin (5U/mL, solid symbols). The reduction in fluorescence anisotropy is used to calculate the inhibition constant (Ki). The binding of FBP7 to DD(E) in presence (Ki = 620 nM) or absence (Ki = 660 nM) of heparin was comparable.

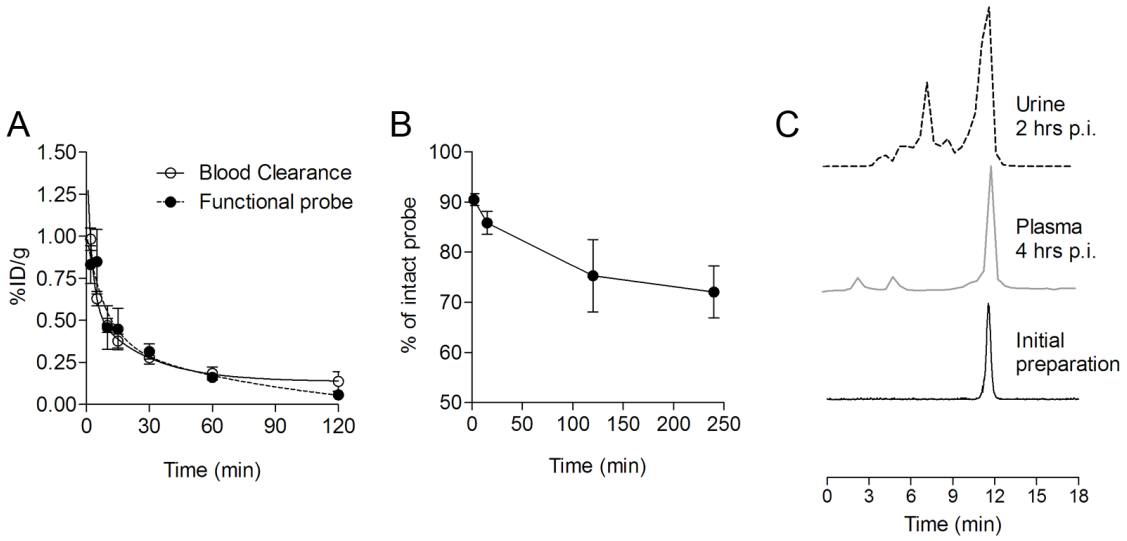

Figure III. (A) Data showing blood clearance (clear symbols) of FBP7 and result of fibrin-binding assay (solid symbols). (B) Radio-HPLC analysis from serial blood draws showing the percentage of intact probe at 2, 15, 120 and 240 min post-injection. (C) Representative radio-HPLC tracing of plasma and urine showing high levels of probe and just few metabolites up to 4 hours post-injection.

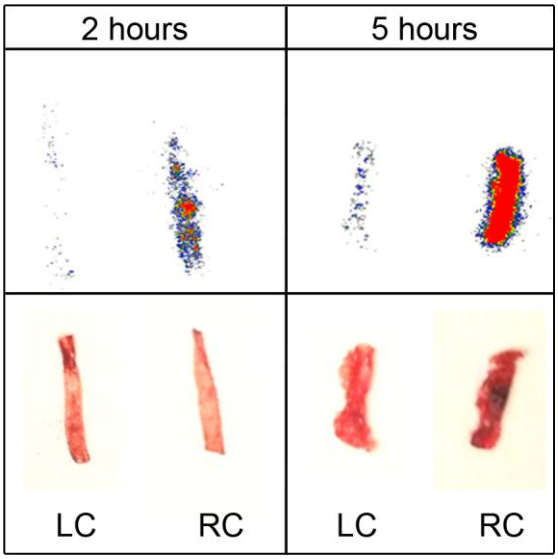

Figure IV. *Ex vivo* autoradiography (upper panels) of crushed (RC) and contralateral (LC) common carotid arteries at 2 hours and 5 hours post-injection confirmed high thrombus uptake of FBP7. Lower panels show the photographs of arteries taken before autoradiography.

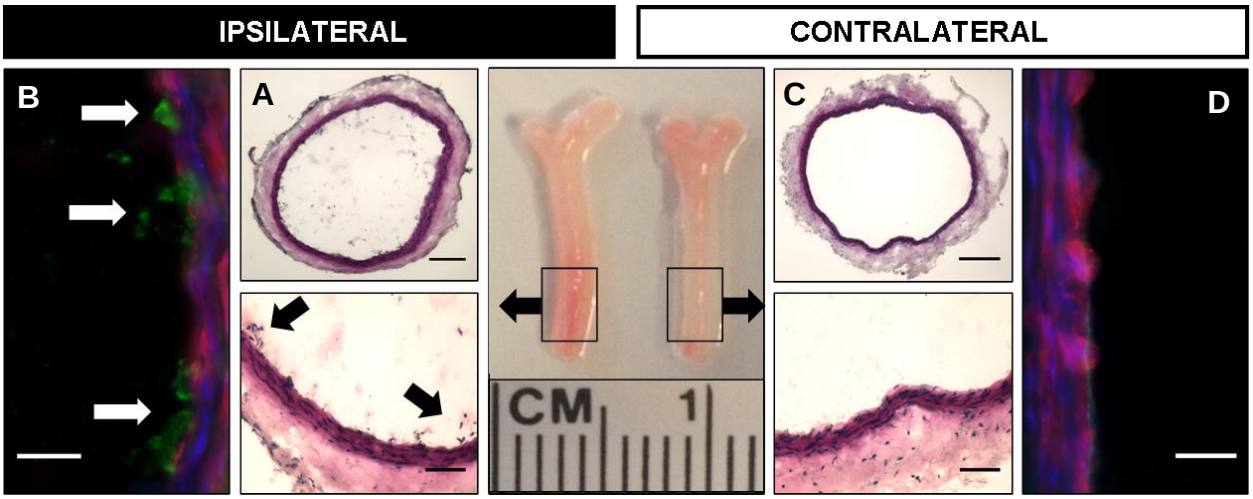

Figure V. Macroscopical inspection of the carotid arteries suggests thrombosis at the level of the ipsilateral common carotid artery, but not contralaterally (center panel). Hematoxylin and Eosin staining reveals the presence of arterial thrombosis after carotid crush injury (A, top). High magnification microphotograph shows that mural thrombi are mainly localized at the level of the endothelial cells layer (A, arrows, bottom); this finding is consistent with previously reported models of mural thrombosis[3]. Fibrin immunostaining performed on adjacent histological sections reveals an immunopositive signal (green) in proximity of the endothelial cells layer (B, arrows), consistent with fibrin deposition.

Histopathology did not reveal the presence of mural thrombi (C) or fibrin (D) in the contralateral common carotid artery.

Green, fibrin; red, endothelial cells layer; blue, nuclei. Arrows in A, mural thrombi; arrows in B, fibrin deposits. Scale bars: 0.2 (A and C, top), 0.05 (A and C, bottom), and 0.025 mm (B and D).

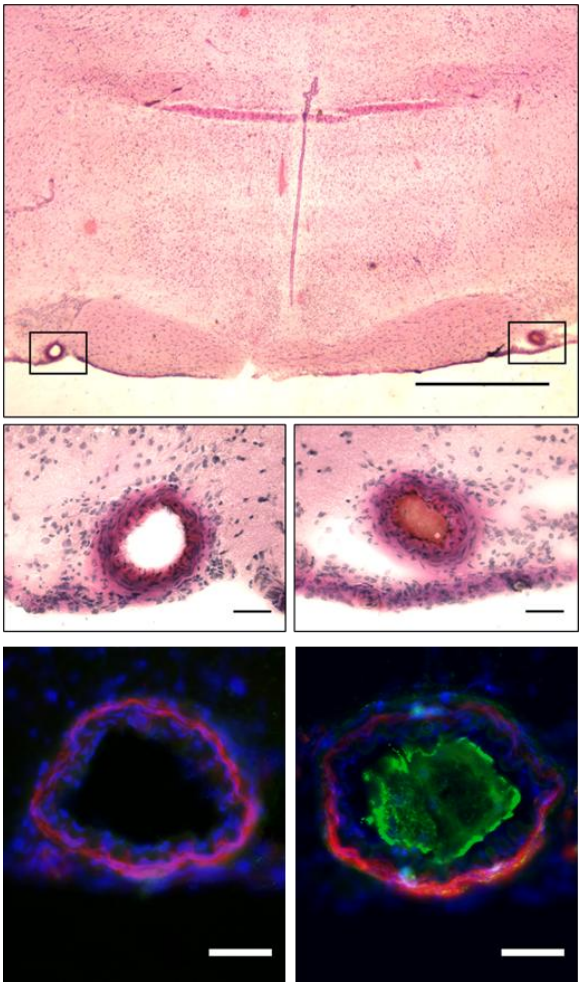

Figure VI. Hematoxylin and Eosin staining reveals an occlusive thrombus in the right middle cerebral artery of rats subjected to embolic stroke (top panel). Insets (middle panels) show high magnification (40X) images of the contralateral (left) and ipsilateral (right) cerebral arteries. The entire lumen of the right middle cerebral artery is occluded by the embolus. Adjacent sections were immunostained for fibrin (bottom panels). An immunopositive signal (green) was present in the lumen of the ipsilateral middle cerebral artery (right), but not contralaterally (left). Green, fibrin; red, endothelial cells layer; blue, nuclei. Scale bars: 0.2 (top) and 0.05 mm (middle and bottom).

SUPPLEMENTAL VIDEO LEGENDS

Video I. 3D reconstruction of a representative rat following carotid crush injury and FBP7 injection. Carotid thrombus is clearly visible and distinguishable from background organs and tissues. A superficial hyperintense area is also present at the level of the surgical incision.

SUPPLEMENTAL REFERENCES

1. Ciesienski KL, Yang Y, Ay I, Chonde DB, Loving GS, Rietz TA, Catana C and Caravan P. Fibrin-targeted PET probes for the detection of thrombi. *Mol Pharmaceutics*. 2013;10:1100-10.

2. Kolodziej AF, Nair SA, Graham P, McMurry TJ, Ladner RC, Wescott C, Sexton DJ and Caravan P. Fibrin specific peptides derived by phage display: characterization of peptides and conjugates for imaging. *Bioconjug Chem*. 2012;23:548-56.

3. Yamashita A, Furukoji E, Marutsuka K, Hatakeyama K, Yamamoto H, Tamura S, Ikeda Y, Sumiyoshi A and Asada Y. Increased vascular wall thrombogenicity combined with reduced blood flow promotes occlusive thrombus formation in rabbit femoral artery. *Arterioscler Thromb Vasc Biol*. 2004;24:2420-2424.