Material and Methods

-Material and Methods are available in the online-only Data Supplement-

Non-human primate DVT model and experimental design

This model uses a 6 hour temporary balloon occlusion in juvenile baboons. On day 0, standard contrast venography and ultrasound were performed in order to acquire baseline information regarding the vein to be thrombosed. First, a retroperitoneal incision was made and the hypogastric vein and other large draining side-branches to the iliac vein were divided and ligated. Then, a thrombus was created in the iliac vein by threading one balloon catheter via the internal jugular vein caudally down the inferior vena cava by fluoroscopy, to just below the iliac bifurcation. Another catheter is moved cranially up the femoral vein to near the pelvic crest. This isolates a vein segment of approximately 3.5 cm in length. During the 6 hours of venous occlusion, the area between balloons was monitored hourly for absence of flow and increasing echogenicity representative of thrombus formation, using ultrasonography. After 6 hours, the balloon catheters were removed, incisions closed, an ultrasound performed to confirm the presence of a thrombus within the iliac vein and the animal monitored for post-operative recovery. Animals were evaluated on days 2, 6, 14, and 21 with venous duplex ultrasound imaging, standard contrast venography, and magnetic resonance venography (MRV), Blood analysis was also performed on these days. On day 21, the animals were humanely euthanized and vein samples were harvested from the thrombosed iliac (experimental samples) and from the non-thrombosed iliac (control samples) for histology. A total of nineteen baboons were divided into 6 experimental groups [**Figure 1**].

<u>Groups included</u>:

Controls that did not receive any drug treatment (n=3); Animals receiving P-selectin inhibitor in a treatment arm (n=3, 2mg/kg IV on day 2, then 1mg/kg subcutaneous (SQ) followed by 1mg/kg SQ twice a day (BID) until euthanasia on day 21 after thrombosis); or prophylactic arm (n=4): Begun the day of surgery 3mg/kg (2mg/kg IV and 1mg/kg SQ) and continued 1mg/kg SQ for 6 days; Euthanized on day 21 after thrombosis). Animals receiving von Willebrand Factor (VWF) inhibitor in a treatment arm (n=3, Started on Day 2. 250µg/kg IV, on day 2. Repeat single dose of 250µg/kg IV on Days 6, 10, and 14 after testing platelet aggregates with PFA-100 test. Euthanasia occurred on day 21 after thrombosis); or prophylactic arm (n=3, Dosed pre-surgery. 250µg/kg IV, once. Dose repeated on day 2, after testing platelet aggregates with PFA-100 test, and the same was done on day 6; Euthanized on day 21 after thrombosis). Animals receiving enoxaparin (LMWH) in a treatment arm (n=3, 1.5mg/kg SQ once on day 2, then 1.5mg/kg SQ once daily until euthanasia on day 21 after thrombosis). Aptamer doses were determined by preliminary drug level studies in anesthetized baboons (see below).

ARC5692 and ARC15150 aptamer levels

Prior to experiments, a dosing study was conducted to determine the effective plasma concentration for the ARC5692, an anti-P-selectin aptamer and ARC15105, an anti-von Willebrand Factor (VWF) aptamer. Blood samples (~200 µL) were collected via femoral venipuncture into tubes containing K_2EDTA as an anticoagulant, placed on ice and centrifuged within 30 minutes of collection at approximately 4°C to obtain plasma. The plasma samples were stored frozen at approximately ≤ -80°C until analysis for anti-P-selectin aptamer and anti-VWF aptamer concentrations. Prior to analysis, to each aliquot (50 µl) of plasma containing the test article was added 25 µL of digestion buffer (60 mM Tris-HCL, pH 8.0, 100mM EDTA and

0.5% SDS) and 75 µl of proteinase solution (1.0 mg/mL proteinase K in 10 mM Tris HCl, pH 7.5, 20 mM $CaCl_2$, 10% glycerol v/v). Samples were then incubated at 55°C overnight with shaking. Following the incubation, samples were centrifuged (14,000 rpm; 4°C; 15 minutes) and 100 µL of the supernatant withdrawn and transferred to HPLC injection vials. The HPLC system was equipped with a column - temperature-controller, UV detector and a Dionex DNA PAK PA-100 (4 x 250 mm) column. The method used a mobile phase elution gradient made from phase A (75% 25 mM sodium phosphate dibasic buffer (pH 7.0) and 25% Acetonitrile) and B (75% 25 mM sodium phosphate dibasic in water (pH 7.0) and 25% Acetonitrile containing 400 mM $NaClO_4$). Flow rate was 0.5 mL/min with column oven temperature set at 80 ^oC. The assay injection volume was approximately 25 µL. The lower limit of quantitation (LLOQ) was 0.2 µg/mL with a linear concentration range of 0.2 to 500 µg/mL. The HPLC method was calibrated relative to concentration reference standards of anti-P-selectin aptamer and anti-VWF aptamer prepared in blank non-human primate plasma (K_2EDTA) and extracted by the same proteinase method used to prepare *in vivo* samples. All reported concentrations of anti-P-selectin aptamer and anti-VWF aptamer were based on the mass of aptamer, excluding the mass of PEG [1-5]. The mean circulating levels of anti-P-selectin aptamer were 24.59±6.2 µg/mL 4 hours post SQ administration. Longer acting anti-VWF aptamer had a mean concentration of 4.54±0.24 µg/mL 4 days post administration in this study. Based on these data, appropriate dosing schedules were created for both aptamers.

Magnetic Resonance Venography - Time of flight and gadolinium enhanced

Magnetic resonance venography (MRV) was performed on a Phillips Achieva 3T imaging system (Phillips Medical Systems, Bothell, WA). Thrombus resolution and lumen size was quantified by time-of-flight (TOF) imaging. The percent vein lumen size was determined as previously described[6]. Statistical analysis was performed using values obtained from the combined proximal and distal iliac vein segments of each primate. Evaluation for PE was assessed in all cases using a Gd enhanced MRI technique**.**

Thrombus fibrin stain

Formalin fixed, paraffin embedded sections were deparaffinized and hydrated to DH20, then were mordant in pre-warmed 60°C Bouins for 1 hour. Following a 15 minute was in tap water; sections were oxidized in 0.25% potassium permanganate, rinsed in DH20 water, and then bleached in 5% oxalic acid. Slides were washed well in DH20 then placed in room temperature PTAH overnight (~18 hours). Finally, sections were rapidly dehydrated and mounted in a synthetic resin. Quantification of fibrin was calculated as percentage of fibrin-covered area in the thrombus.

Masson's trichrome analysis for percent vein wall collagen

The IVC samples were paraffin-embedded, cut into 4μm sections, processed as slides, and stained with Masson's trichrome. Vein wall fibrosis was quantified by computerized analysis using Image J software (Scanalytics, Fairfax, VA). Three individual samples were analyzed per group for each time point. The mean fibrotic area was calculated from 10 to 12 regions of interest per vein wall section analyzed at 400× magnification[7, 8].

Vein wall morphometrics

Standard methods for tissue fixation with paraffin-embedded sections were used; sections were stained with hematoxylin/eosin. Veins were examined under high power (1000) oil immersion microscopy. Five representative high power fields were examined around the vein wall and the cell count analyzed.

Duplex ultrasound analysis

The baboon has a mid-iliac valve which can be evaluated for competency during venous thrombosis. This iliac valve in the baboon can be used to determine the effectiveness of novel therapeutics in protecting valve integrity post-thrombosis. On days 0 (baseline and T+6 hours), 2, 6, 14, and 21 (euthanasia; end of study), duplex ultrasonography was performed to evaluate the presence of thrombus and iliac valve function. The criteria for a clinical human evaluation of VT was used, including: a lack of compression, lack of flow, presence of dilated vein void of echos (acute), and presence of a vein full of echoes (chronic). Iliac valve closure times were documented as previously described[6]. Normal valve closure is defined as ≤1000 milliseconds (ms) after proximal compression**.**

Contrast venography

On days 0 (baseline), 2, 6, 14, and 21 (euthanasia; end of study), bilateral intravenous catheters were placed in the saphenous veins for the administration of contrast agent injected into both veins simultaneously. Images were recorded with a BV29 C-arm fluoroscopy unit and archived via computer files (Phillips Medical System, Cincinnati, OH).

Gross anatomy and venography

Non-perfusion fixed vein wall and thrombus segments from 3 areas (proximal, mid and distal thrombus area) were acquired at harvest and photographed. Then, they were paraffin-embedded, sectioned onto slides, and Masson's trichrome stain performed for quantification of vein wall fibrosis.

Anti-Xa monitoring

For all animals receiving enoxaparin, a chromogenic assay, Berichrome Heparin kit (Siemens Healthcare Diagnostics, Deerfield, IL), was performed. A LMWH calibrator was used to construct a LMWH standard curve which then was used to determine the plasma level of LMWH. Briefly, 50 µl of plasma from citrated blood was mixed with 50 µl antithrombin III and 500 µl factor Xa reagents. The chromogenic substrate was added to the reaction and measured at 405 nm on an automated coagulation analyzer (BCS, Siemens).per manufacturer's instructions to monitor enoxaparin levels drawn; 2 hours post administration on days 2, 6 and 14. The plasma level of administered LMWH was inversely proportional to the measured factor Xa activity. In patients, the recommended maintenance anti-Xa level of enoxaparin sodium is between 0.5 - 1.0 IU/mL 3-4 hours post administration [9].

Measurement of platelet function PFA-100

The effect of the anti-von Willebrand Factor aptamer on platelet function during venous thrombosis 5 minutes post administration in non-human primates was evaluated with the Platelet Function Analyzer (PFA-100; Siemens Healthcare Diagnostics, Deerfield, IL) in whole venous blood samples anti-coagulated with 3.2% sodium citrate. Anti-VWF aptamer was administered

IV post thrombosis on days 2, 6, 10, and 14 for treatment group and on days 2 and 6 in the prophylactic group; with the effect on platelet aggregation determined pre-administration and 5 minutes post on those days. Blood samples were placed in a test cartridge and aspirated through a capillary (200-mm diameter) under constant negative pressure (high shear stress) toward a membrane with a small aperture (150-mm diameter) coated with equine type I collagen to activate platelets and adenosine 50-diphosphate (CADP cartridges) to enhance platelet aggregation. The PFA-100 closure time (CT) represented the elapsed time in seconds (up to a maximum of 300 seconds) until aperture occlusion by formation of a platelet plug [5, 10, 11].

Coagulation tests and hematological analysis

Coagulation tests and hematological analysis were performed on days 0 (baseline and T+6 hours), 2, 6, 14, and 21. Activated partial thromboplastin time (aPTT), thrombin clotting time (TCT) (Dade Diagnostics, Miami, FL), semi-quantitative D-dimers (Dade Diagnostics, Miami, FL), fibrinogen (Siemens Healthcare, Malvern, PA), and template bleeding time (BT) (Allegiance Health Care Corp, McGraw Park, IL) were run according to the manufacturer's instructions. Blood cell counts were determined by an automated hematology system (HemaVET®, CDC Technologies Inc., Oxford, CT). All samples are run in duplicate within a 5% error margin**.**

Statistical evaluation and animal use

Statistical significance was calculated using an unpaired t-test with Welch's correction, Tukey's post hoc test after ANOVA and Krustal-Wallis test for nonparametric data (GraphPad Software, Inc., La Jolla, CA). Significance was defined as p=0.05. The health status of all animals was

monitored, and all animals were free of pathogens. All non-human primates were pair housed and cared for by the University of Michigan Unit for Laboratory Animal Medicine in accordance to Guide for the Care and Use of Laboratory Animals[12]. The University of Michigan is an AAALAC accredited facility and this research protocol was approved by the University's Committee on Use and Care of Animals (UCUCA).

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