SUPPLEMENTAL METHODS AND DATA

AB002 activity against thrombin substrates

To evaluate the activity of AB002 against select thrombin substrates, *in vitro*, AB002 was incubated with several physiological or synthetic thrombin substrates in 5 mM Tris HCl, 145 mM NaCl, 0.1% PEG8000, pH 7.4 at 37°C and substrate cleavage monitored over time as described in detail previously.[1] Briefly, generation of APC from human protein C (Haematologic Technologies, Inc.) in the presence of TM (rabbit; Haematologic Technologies, Inc.) was evaluated using a chromogenic substrate for APC (H-D-Asp-Arg-Arg-p-nitroanilide or DRR-pNA; American Peptide Company). Release of fibrinopeptide A (FpA) and B (FpB) from fibrinogen (Haematologic Technologies, Inc.) was measured by HPLC to determine specificity constants for fibrinogen and fibrin, respectively. Cleavage of the thrombin receptor PAR1 in the absence of TM was quantified by measuring the disappearance of the intact extracellular portion of PAR1 (residues 33-62; American Peptide Company) by HPLC. Antithrombin (ATIII; Haematologic Technologies, Inc.) inhibition of AB002 was evaluated by measuring cleavage of the thrombin substrate H-D-Phe-Pro-Arg-p-nitroanilide (FPR-pNA; American Peptide Company) in the presence of heparin.

Progress curves for p-nitroanilide (ε = 9920 $M^{-1}cm^{-1}$) substrate hydrolysis were collected using Cary UV-visible spectrophotometers equipped with WinUV Kinetics software. HPLC was performed using a Waters Alliance 2695 Separations Module for 1-mL injections into the HPLC system equipped with a Waters 4-µm C18 column. Empower software was used to integrate appropriate peaks of chromatograms. Progress curves of substrate cleavage were analyzed by established curve-fitting programs to determine kinetic constants.

For the thrombin time (TT) assay, platelet-poor plasma was used (SSC/ISTH Secondary Coagulation Standard Lot #4, NIBSC, London, United Kingdom). Human α-thrombin (WT) was

purchased from Haematologic Technologies, Inc. (Essex Junction, VT). All measurements were made using a KC-4 coagulometer (Tcoag, Ltd, Ireland). WT α-thrombin (7.9 mg/mL; Haematologic Technologies, Inc.) and AB002 (107-113 µg/mL) were diluted from their initial stock concentrations to 2.5 µg/mL using Tris-buffered saline (25 mM Tris-HCl pH 7.4, 130 mM NaCl, 2.7 mM KCl, 0.1% (w/v) bovine serum albumin (BSA)) to reach final concentrations of 2.5 µg/mL. For each experiment, 50 µL of platelet-poor plasma was added to the KC-4 cuvettes and incubated at 37°C for 120 s. After the incubation, fibrin formation was initiated by addition of 50 µL of WT thrombin or AB002 (n=8 replicates for WT thrombin; n=4 replicates each for 5 distinct manufacturing batches for AB002). Experiments were terminated if no clot formation was observed within 1000 s.

Pharmacodynamics of AB002 in baboons

Three baboons (Papio anubis, ~10 kg) received IV bolus doses of AB002 (from 1.25 to 10 µg/kg). Additionally, one baboon received 5 µg/kg of the catalytically inactive recombinant thrombin analog, S195A. All baboon experiments were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Blood samples were taken at each time point: pre-dose, 15, 30, and 60 min after AB002 administration. Blood was collected into heparin (10 U/mL) for the measurement of activated protein C-protein C inhibitor (APC-PCI) complex as a biomarker for AB002 activity. Heparinized plasma samples were stored at -80°C until tested.

APC-PCI ELISA for baboon samples

For measuring APC-PCI in heparinized baboon plasma, a matched-pair antibody set for ELISA (BioPorto Diagnostics, Denmark) was used per the manufacturer's instructions. APC-PCI standards were prepared as per the manufacturer's instructions in sample diluent supplemented with 1% pooled normal baboon plasma. Plasma samples were diluted 1:100 to 1:2,000 in

sample diluent with pooled baboon plasma to a final plasma level of 1% (pre-dose plasma was diluted 1:100 so as to be 1% plasma final). This ELISA utilizes a monoclonal anti-PCI antibody for capture and biotinylated monoclonal anti-protein C antibody for detection of captured APC-PCI complex. Coating, sample application, and detection were performed as described in the manufacturer's protocol and the standard curve was used to quantify APC-PCI in plasma samples which corresponds to levels of APC generated. The following reagents were used for detection: HRP-streptavidin (Pierce 21124) diluted to 100 ng/mL in sample diluent (SB001RA) and 1-Step Ultra TMB substrate (Life Technologies 34028). Following 30 min incubation with TMB substrate, 100 µl of 2 M H_2SO_4 was added per well to quench reactions.

AB002 pharmacology evaluation during cynomolgus monkey toxicology studies

Male and female cynomolgus monkeys (2 to 4 year-old *Macaca fascicularis*, n=40) were administered single IV bolus injections of AB002 (12.5, 25 or 50 µg/kg) or vehicle at Charles River Laboratories (CRL) in accordance with Good Laboratory Practices. Experiments were approved by the Institutional Animal Care and Use Committee of CRL. Blood samples were taken at pre-dose, 15, 30, 60, 240 min, and 24 h post-administration, processed to platelet-poor plasma and stored frozen until analysis. Blood was drawn into sodium citrate containing 400 µM biotinylated-PPACK (Bio-PPACK, Molecular Innovations) and AB002 plasma concentration was determined by ELISA as described below. For measurement of APC-PCI complexes, blood was drawn into heparin at a final concentration of 10 U/mL and assayed as described above using pooled cynomolgus monkey plasma from Bioreclamation/IVT. For aPTT measurements, blood was drawn into 3.2% sodium citrate and standard aPTT reactions were performed with HemosIL reagent.

AB002 ELISA

The concentration of Bio-PPACK-bound AB002 in cynomolgus monkey plasma samples was measured using an ELISA 96-well microtiter format. Plates were coated with a mouse monoclonal antibody to AB002 that cross-reacts with both WT and E-WE thrombin, and incubated at 4°C overnight. After removing the capture antibody and washing, plates were blocked with BSA buffer for 1 h at room temperature. Plates were then washed, samples added, followed by incubation for 1 h at room temperature. An AB002:Bio-PPACK calibration standard was used for this assay to generate a standard curve for each assay run. AB002:Bio-PPACK was generated by incubating AB002 with Bio-PPACK for 2 h, followed by dialysis to remove unbound Bio-PPACK, and was stored frozen until use. After washing, the detection molecule (peroxidase-conjugated streptavidin) was added and incubated for an additional 30 min at room temperature. The plates were washed again and o-phenylenediamine dihydrochloride (OPD) was added to the wells and color development stopped by the addition of 2 N sulfuric acid. The absorbance at 490 nm was measured and concentration of AB002:Bio-PPACK was calculated using a 4PL standard calibration curve. Plasma samples were tested at 25% dilution. Samples and controls were tested in duplicate and the mean absorbance values at 490 nm of the duplicate values were used to calculate the plasma concentration.

APC generation on platelet aggregates *in vitro*

Human venous blood was collected into sodium citrate (3.2% w/v) and acid/citrate/dextrose. Washed platelets were prepared in modified HEPES-Tyrodes (HT) buffer as described previously [2], and aliquots of 2.5×10^8 platelets were stimulated with 10 µg/mL collagen (Chrono-log) in the presence of 5 mM $CaCl_2$. Collagen-activated platelets were washed 3x with HT containing 2 mM EDTA, once with HT, and then resuspended in HT containing 5 mM $CaCl_2$. Aggregates were incubated with either 50 nM WT α-thrombin, AB002, or active site-mutated S195A-WE thrombin along with 100 nM human protein C for 1 h at 37°C. For receptor blocking

experiments, platelet aggregates were incubated for 1 h at room temperature with 40 µg/mL of anti-human CD31 (RayBiotech), anti-human TM (anti-TM; Sekisui), anti-human endothelial protein C receptor (anti-EPCR; Novus), or 20 µg/mL of recombinant human LDL-receptor associated protein (rhRAP; Enzo Life Sciences). The reactions were stopped with 100 nM hirudin, and APC was quantified using HAPC 1555 antibody capture ELISA as described previously.[3]

TAFI activation assays

Purified TAFI (500 nM; HTI) was incubated at room temperature with WT α-thrombin (150 nM) and/or AB002 (150 nM) with or without rabbit TM (15 nM) in HEPES-buffered saline (pH 7.4) for various times, after which 100 µM D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK, Molecular Innovations) was added. Samples were then prepared as per the manufacturer's instructions for reducing conditions with the Wes-Mouse (12-230 kDa) Master Kit. Mouse anti-human TAFI (AHTAFI-5024; HTI) was used as the primary antibody. Quantitative TAFI activation was determined as previously described using 300 nM TAFI, 5 nM TM, 5 nM α-thrombin, and increasing concentrations of AB002 (5 nM to 200 nM).[4]

GMP manufacturing

The AB002 drug substance clinical batch was produced from the MCB using a fed-batch process. The small-scale process was adapted for 30 and 100 L fermentation processes and subsequent purification. Briefly, cells from the MCB were cultured using LB broth (BD Biosciences) and ampicillin (Research Products International), induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; Calbiochem) to express E-WE prethrombin-2. Harvested inclusion bodies (IBs) were isolated from a cell paste by high pressure homogenization and repeated washes to remove soluble components. The IBs were solubilized and then reduced with a basic buffer solution containing the denaturant guanidine HCl (7 M) and the reductant dithiothreitol

(DTT; 5 mM). Refolding was performed at room temperature in tris buffer (pH 8.3) and the mixture was purified by heparin Sepharose chromatography, followed by zymogen activation using ecarin (Pentapharm). Further chromatography steps (heparin and butyl Sepharose) were performed to remove residual ecarin (limit < 8 mU/mL), endotoxin (max 10 EU/mL), and to purify activated E-WE thrombin, followed by concentration and diafiltration into formulation buffer solution for intravascular injection.

APC-PCI, protein C, and immunogenicity testing for human samples

APC-PCI concentrations were determined using a qualified ELISA method. Heparinized plasma samples were diluted up to 1000-fold and incubated at room temperature in a microtiter plate coated with a capture antibody that binds cleaved PCI (Bioporto Diagnostics, Denmark). Calibration standards and quality control (QC) samples were prepared from APC-PCI complex reference standard in sample diluent (Bioporto Diagnostics, Denmark). After 2 h, the plate was washed and a biotinylated secondary antibody against the APC-PCI complex (Bioporto Diagnostics, Denmark) was added to the wells and incubated at room temperature. After 1 h, the plate was washed and a streptavidin-HRP conjugate (Jackson Immunoresearch) was added to the wells and incubated at room temperature. After 1 h, the plate was washed and tetramethylbenzidine substrate added to the wells and the reaction quenched by the addition of 2 N sulfuric acid. Signal absorbance was read at 450 nm on a microplate reader. APC-PCI concentration was determined by 4PL fit of the standard curve.

Protein C levels were measured in plasma using a commercial competitive ELISA kit per the manufacturer's instructions (Innovative Research, Inc). Briefly, 25 µL of human protein C standard or sample (diluted 1:8 in ELISA kit diluent) were added to each well followed immediately by 25 µL of biotinylated human protein C. This mixture was incubated for 2 h at room temperature then rinsed 5X with the ELISA wash buffer. Detection of bound material was

performed by sequential addition of streptavidin-peroxidase conjugate, peroxidase chromogen substrate tetramethylbenzidine, and 0.5 N hydrochloric acid stop solution followed by an absorbance reading of 450 nm on a microplate reader. Protein C levels were determined by 4PL fit of the standard curve and are compared to pre-study plasma levels of protein C (100%).

D-dimer levels were measured in plasma using a commercial ELISA kit per the manufacturer's instructions (Sekisui Diagnostics, LLC). Briefly, 200 µL of human D-dimer standard, positive controls, or sample (diluted in ELISA kit diluent) were added to each well and incubated for 1 h at room temperature, rinsed 5X with the ELISA wash buffer, and then incubated with HRP-conjugated anti-human D-dimer antibody for 1 h at room temperature. After rinsing 5X with the ELISA wash buffer, detection of bound material was performed by sequential addition of tetramethylbenzidine and 0.45 M H_2SO_4 stop solution followed by an absorbance reading of 450 nm on a microplate reader. D-dimer levels were determined by curve fitting the standards and were calculated in ng/mL.

Prothrombin fragment 1.2 levels were measured in plasma using a commercial ELISA kit per the manufacturer's instructions (U.S. Biological). Briefly, 100 µL of human prothrombin fragment 1.2 standard, positive control plasma, or sample (diluted in ELISA kit diluent) were added to each well and incubated for 1 h at 37°C. Wells are then incubated with biotinylated anti-prothrombin fragment 1.2 antibody for 1 h at 37°C. After rinsing 3X with the ELISA wash buffer, detection of bound material was performed by sequential addition of HRP-conjugated avidin, tetramethylbenzidine, and sulfuric acid stop solution followed by an absorbance reading of 450 nm on a microplate reader. Prothrombin fragment 1.2 levels were determined by curve fitting the standards and were calculated in ng/mL.

Blood samples for immunogenicity testing were collected at pre-dose and at 14 and 28 days after administration of AB002 or placebo. The screening ELISAs were qualitative assays with microtiter plates coated with AB002 (1 µg/mL) or human thrombin (2 µg/mL), depending on the method. Samples were diluted with assay buffer (phosphate-buffered saline with 0.1% Tween-20 and 0.5% BSA) to a minimum required dilution and added to the ELISA plates for 90 min. Plates were washed with assay buffer and then 250 ng/mL T7-tagged AB002 or 100 ng/mL biotinylated thrombin in assay buffer was added for 90 min to form a complex with any antibody bound to the AB002- or thrombin-coated plates. Any bound antibodies were then detected with either anti-T7-tag antibody conjugated with HRP (Abcam ab19291, 1:20,000 in assay buffer) or streptavidin-HRP (Jackson Immunoresearch, 1:10,000 in assay buffer). After 90 min, plates were washed with assay buffer, TMB substrate was added for 10 min, and 4 N H_2SO_4 was used to quench reactions before reading the absorbance at 450 nm on a microplate reader (Molecular Devices). The data was analyzed with SoftMax Pro GxP software. All subject samples with a resulting signal less than the determined cut-point were considered negative.

Because signal generation could be due to non-specific interactions, all subject samples with a result greater than or equal to the cut-point required additional testing in confirmatory ELISAs to confirm the antibodies present are true positives, specific to either AB002 or human thrombin, depending on the method. The confirmatory ELISA methods share the same assay format as the screening ELISAs, except samples are diluted to the MRD in assay buffer with and without free AB002 or free thrombin (HCT-FPRCK, HTI). The responses from uninhibited samples (without free drug/thrombin) and inhibited samples (with free drug/thrombin) were used to determine a percent loss of signal that was compared to the cut-point. Samples with values less than the determined cut-point were considered negative. Samples with a percent loss of signal greater than or equal to the cut-point were confirmed positive for anti-AB002 or anti-thrombin antibodies and evaluated for antibody titers. The antibody titer ELISA methods share the same

assay format as the screening ELISAs. Each sample was serially diluted in negative control (pooled normal human plasma or serum) prior to dilution in assay buffer to satisfy the MRD. Titration curves were constructed by plotting the signal versus the log of the corresponding sample dilution factor. A 4PL model was used to calculate the antibody titer at which the signal was equal to the cut-point.

Supplemental Table 1. Specificity and activity of AB002

	AB002§ (E-WE thrombin)	BHK WE thrombin*	WT thrombin*
Protein C† k_{cat}/K_m ($mM^{-1}s^{-1}$)	**21 ± 2**	**33 ± 2**	**220 ± 10**
Fibrinogen k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	**0.00022 ± 0.00004**	**0.00089 ± 0.00007**	**17 ± 1**
Fibrin k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	**0.0008 ± 0.0001**	**0.0021 ± 0.0001**	**8.1 ± 0.5**
PAR1 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	**0.009 ± 0.001**	**0.026 ± 0.001**	**26 ± 1**
Antithrombin‡ k_{on} ($\mu M^{-1}s^{-1}$)	**0.020 ± 0.003**	**0.004 ± 0.003**	**13 ± 1**
Thrombin Time (s)	**>1000**	**>1000**	**12.8 ± 0.1**
RAP[II]	**7300 ± 400**	**2800 ± 300**	**1**

Values are ± SEM; Data for the different thrombins were collected in the same laboratory using the same reagents, protocols, and equipment, but were not assessed in parallel on the same day (except for thrombin times, which were tested in parallel).

* Values from Cantwell et al, except thrombin time. WT = wild-type and BHK WE = W215A/E217A mutant thrombin produced in baby hamster kidney (BHK) cells.

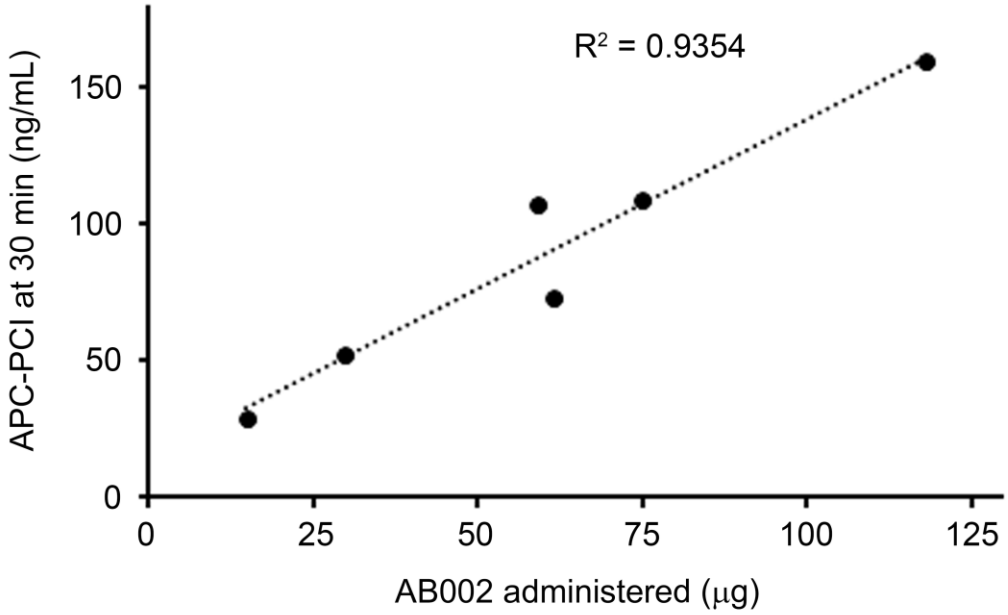
† In the presence of 50 nM TM, 5 mM $CaCl_2$. Performed in solution.

‡ In the presence of 0.5 U/mL heparin.

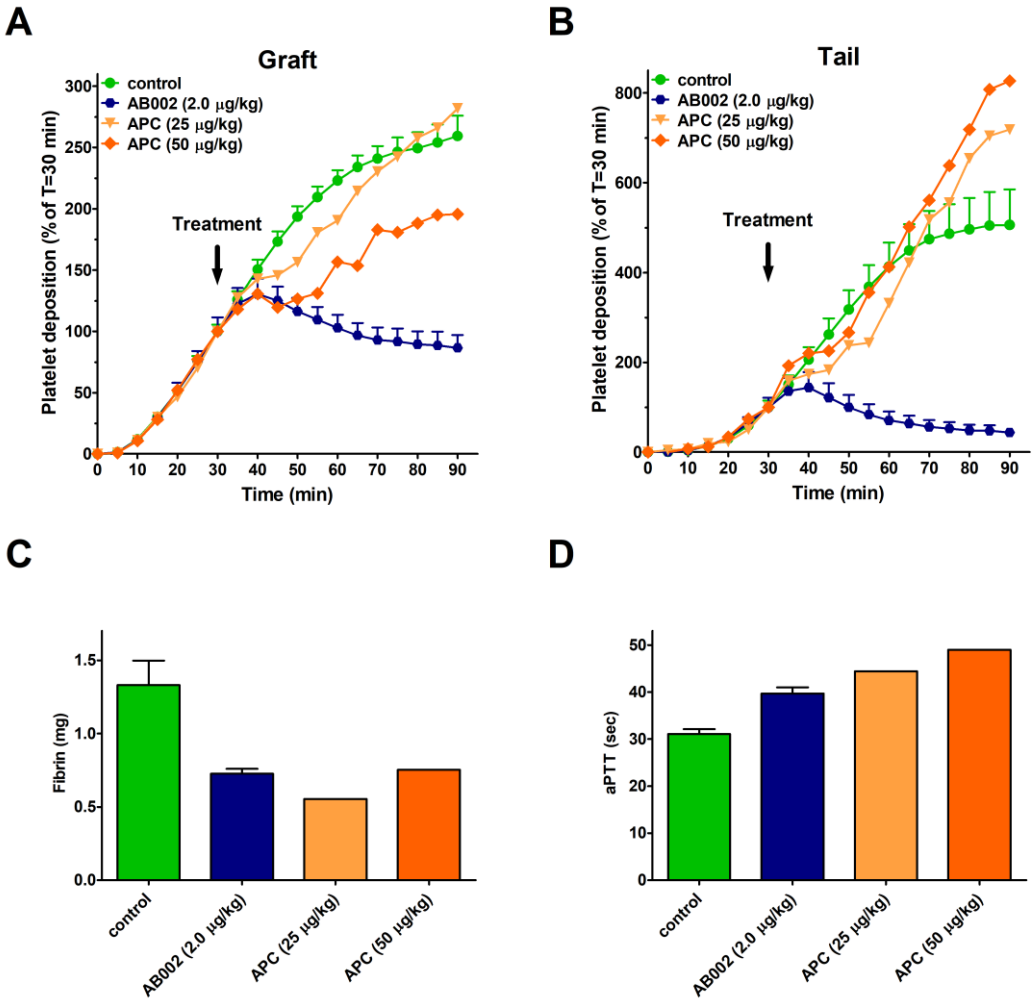
§ Average of three separate manufacturing lots of W215A/E217A mutant thrombin produced in *E. coli* (E-WE thrombin, AB002).

II The relative anticoagulant potency (RAP) was calculated as the ratio of the protein C activation rate over the rate for fibrinopeptide A release, relative to the same published ratio for WT thrombin.

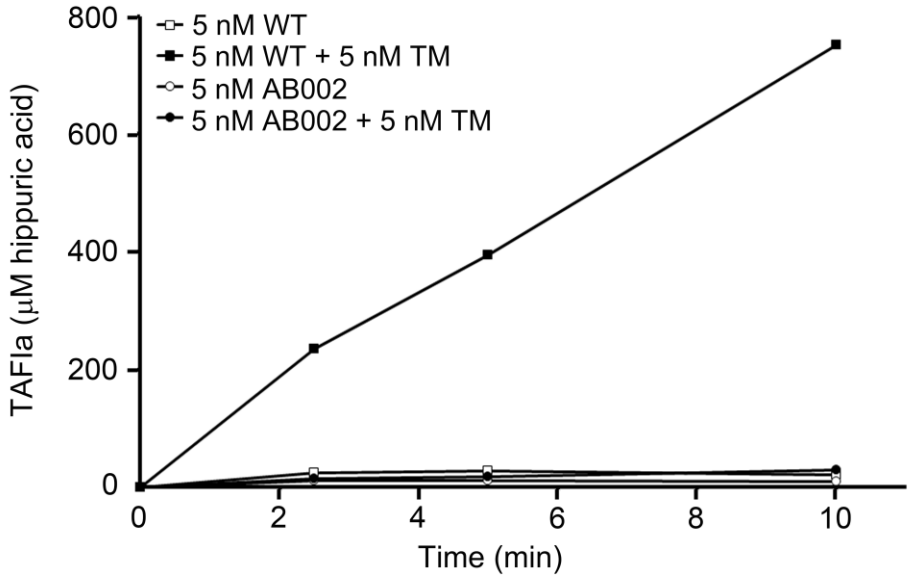
Supplemental Figure 1. AB002 is pharmacologically active in baboons and dose-dependently activates protein C. Three baboons were administered varying amounts of AB002 intravenously. Plasma was collected 30 minutes after AB002 injection and APC-PCI was measured by ELISA. Plasma APC-PCI concentration (ng/mL) is plotted against total AB002 administered (μg). Linear regression revealed that there is a statistically significant linear correlation between the amount of AB002 administered and APC-PCI plasma concentration measured after 30 min (*P* = 0.0016). No APC-PCI was detectable after administration of the catalytically inactive recombinant WE thrombin analog, S195A.



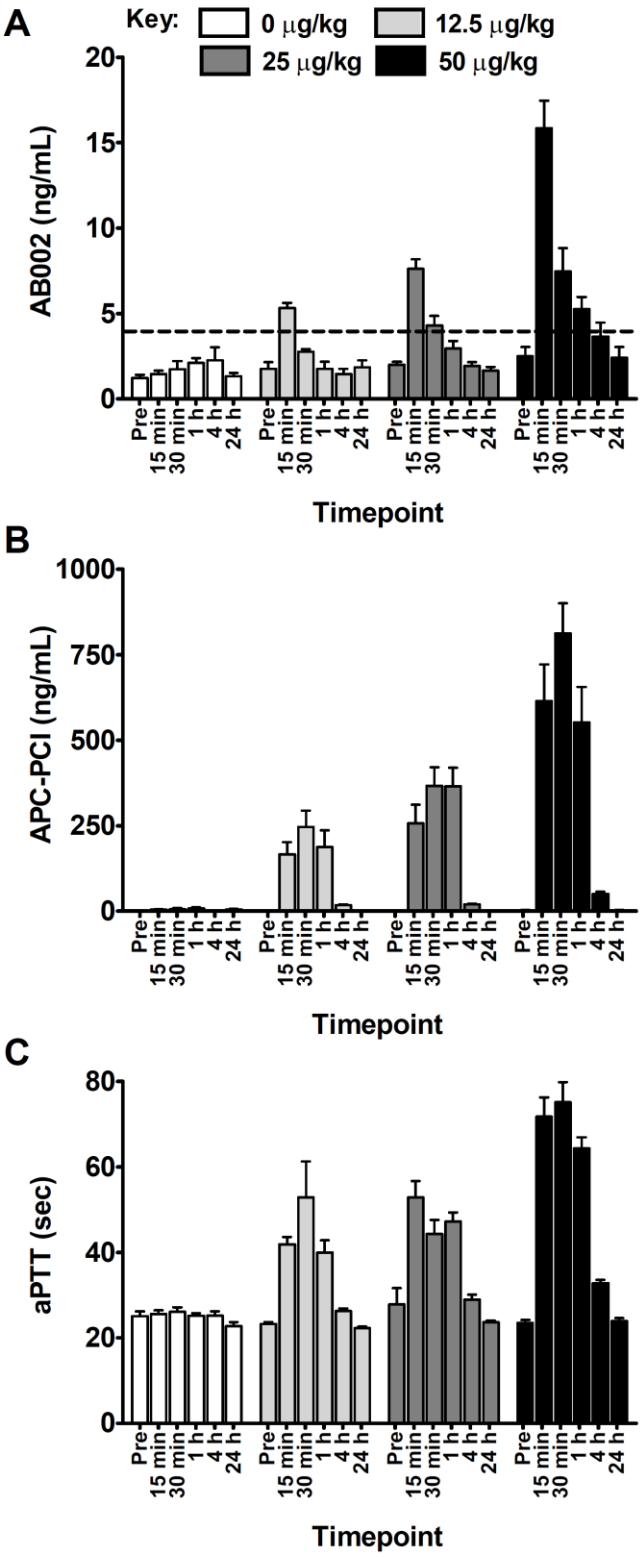
Supplemental Figure 2. AB002 appears superior to systemic APC at interrupting thrombus formation in a primate model. Effect of AB002 (2 μg/kg, IV) and activated protein C (APC, 25 μg/kg and 50 μg/kg, IV) on interrupting platelet accumulation (**A**-**B**) and fibrin deposition (**C**) in baboons. 4 mm diameter synthetic vascular grafts were coated with collagen and real-time platelet accumulation within graft (**A**) and the siliconized surgical tubing up to 10 cm downstream of the graft (**B**, "tail") were measured. Thrombi growth was interrupted after 30 min (black arrow) and post-treatment accumulation was observed for an additional 60 min. (**D**) 10 minutes after drug administration blood samples were taken and aPTT was measured in platelet-poor plasma within 6 ± 1 min of blood sampling. Control, n = 15; AB002, n = 7; APC, n = 1 each. All data are expressed as means ± SEM.



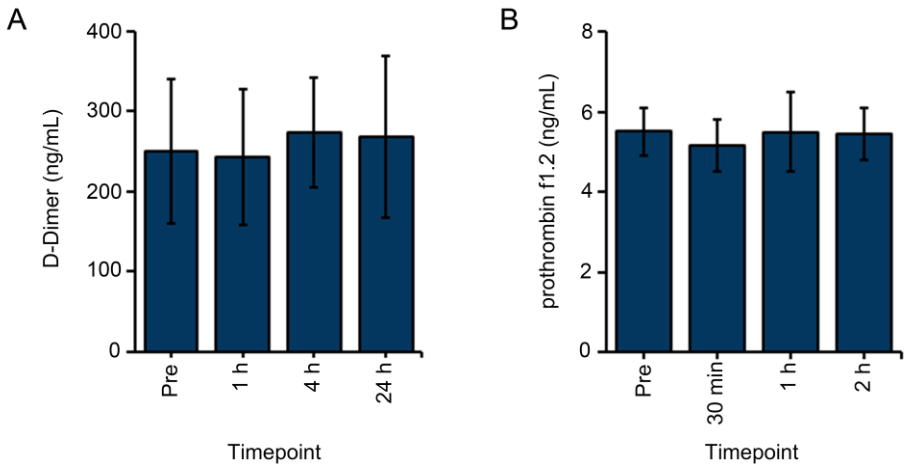
Supplemental Figure 3. AB002 is a poor activator of TAFI compared to WT thrombin when complexed with TM. To assess TAFI activation quantitatively, 300 nM of TAFI was incubated with 5 nM α-thrombin (WT) or 5 nM AB002 with and without 5 nM TM at room temperature, and reactions quenched at the indicated times by addition of 150 µM PPACK. Samples were then incubated with 20 mM hippuryl-arginine for 10 min at room temperature and the amount of hippuric acid generated was calculated by colorimetric analysis and used as a proxy for activated TAFI. The slope of hippuric acid generated over the course of the reaction was approximately 40-fold higher for WT thrombin in the presence of TM compared to AB002 in the presence of TM.



Supplemental Figure 4. Pharmacokinetic and pharmacodynamic profile of AB002 during toxicity studies in cynomolgus monkeys. Four groups of cynomolgus monkeys were administered a single intravenous bolus dose of AB002 (12.5 µg/kg, 25 µg/kg, 50 µg/kg) or vehicle (0 µg/kg). To assess the pharmacokinetic and pharmacodynamic effects of drug administration, plasma samples were collected before administration and at 15 min, 30 min, 1 h, 4 h, and 24 h post dose. Compared to vehicle treatment, administration of AB002 caused a dose-dependent, temporal elevation in (**A**) AB002 plasma concentration, (**B**) APC-PCI complex and (**C**) the activated partial thromboplastin time (aPTT). The highest concentration of AB002 was measured 15 min post administration, and peak concentration of APC-PCI complex was observed 30 min post administration. In (**A**), the dashed line indicates the limit of quantitation (LOQ) of the assay, as determined by the standard deviation of the blank. n = 10 monkeys per group (5 per sex) for AB002 plasma concentration and aPTT, and n = 6 monkeys per group (3 per sex) for APC-PCI concentration. Data are means ± SEM.



Supplemental Figure 5. Markers of procoagulant activity in subjects from the AB002 phase 1 clinical study. Plasma samples from subjects that received the highest dose of AB002 (cohort 4: 4.0 µg/kg, n = 4) were assayed at appropriate intervals to observe the effect of drug administration on (**A**) d-dimer levels and (**B**) prothrombin fragment 1.2 levels. The half-life of d-dimer is approximately 8 hours so in addition to the pre-dose sample, plasma was assayed for this marker at 1 h, 4 h, and 24 h. The half-life of prothrombin fragment 1.2 is approximately 90 min. Therefore, in addition to the pre-dose sample, plasma was assayed for this marker at 30 min, 1 h, and 2 h. All data are expressed as means ± SEM.



Supplemental References

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