Methods (Supplemental data)

Reagents

Human FXII, FXIIa, FXI, FXIa, FX, FXa, FIXa, pre-kallikrein, kallikrein, FVa, Protein S (PS), prothrombin, thrombin and mice FXa were from Enzyme Research Laboratories (South Bend, IN) or Hematologic Technologies (Essex Junction, VT). FIX (Benefix, recombinant FIX, protein-free) was from Wyeth-Pfizer (New York, NY). FVIIa (Novoseven, recombinant FVIIa, protein-free) was from NovoNordisk (Plainsboro, NJ). Lipidated tissue factor was from American Diagnostica/ Seikagaku (East Falmouth, MA). Control plasma (00676), APTT (STA-PTT Automate, 00606), and PT (Neoplastine CI Plus, 00595) reagents were from Diagnostica Stago (Asnières sur Seine, France). S2222 (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroaniline hydrochloride), S2238 (H-d-phenylalanyl-l-pipecolyl-l-arginine-*p*-nitroaniline dihydrochloride), S2366 (L-pyroglutamyl-L-propyl-L-arginine-*p*-nitroanilide), and S2302 (H-D-L-prolyl-L-phenylanyl-L-arginine-*p*-nitroanilide) were obtained from Chromogenix-Diapharma (West Chester, OH). Phosphatidylcholine, phosphatidylserine, heparin grade-I (H3149; 19 kDa), dextran sulphate (DS) 50K, DS500K, polyPs (commercialized as sodium phosphate glass type 45, S4379), kaolin, BK, and Evans blue were from Sigma Co. (Saint Louis, MO). Phosphatydilethanolamine was from Avanti Polar Lipids (Alabaster, AL). Corn Trypsin Inhibitor (CTI) was from Kerafast Inc. (Boston, MA). Human TFPI carrier free (2974-PI-010) was from R&D (Minneapolis, MN). Heparin-agarose columns, CM5 sensor chips, and all reagents for surface plasmon resonance (BIAcore) experiments were from GE-Healthcare-Pharmacia (Piscataway, NJ).

Desmolaris properties

Desmolaris molecular mass is 21555.44 Da (189 amino acids, mature form without His tag) with an estimated p*I* 8.57. Extinction coefficient at 280 nm is 19660; A280 nm/cm 0.1% (1 mg/mL), 0.92.

Cloning and protein expression

Genes for Desmolaris and mutated or truncated forms were synthesized by BioBasics (Ontario, Canada) and cloned in VR1020 vector, which contains the kanamycin-resistance gene and the cytomegalovirus promoter and the tissue plasminogen activator signal peptide.[1,2] The signal peptidase cleavage site is preserved in this plasmid, and exhibits a *BamH*I restriction site in the 3' end. Each synthetic sequence displays a *BamH*I restriction site (*Ggatcc*) at the 5' end. A sequence coding the C-terminal of the protein, 6xHis, a stop codon, and a *BamH*I restriction site comprised the 3' end. The following genes were synthesized based on the exon organization for TFPI-1.[3] Desmolaris (Asp^{1}-Iso^{189}), "R32L" (mutation at the P1 position, Arg for Leu), "K1K2" (Asp^{1}-Lys^{162}), "K1" (Asp^{1}-Leu^{103}), "K2Long" (Val^{104}-Iso^{189}), and "K2Short" (Val^{104}-Lys^{162}). VR1020 plasmid was digested with the *BamH*I site followed by ligation. All plasmids were sequenced and found to be in frame with the insert and used for transformation of TOP10 cells as described. [23,24] Plasmids were generated and used for transfection of human embryonic kidney 293-F cells at the Protein Expression Laboratory at NCI-Frederick (Frederick, MD) as described. [1,2] The supernatants were collected after 72 hours, centrifuged at 2000 rpm, and frozen.

Protein purification

Supernatants containing Desmolaris, mutant and variant forms were concentrated from 500 to 100 mL using an ultrafiltration cell unit (Millipore, Billerica, MA) under continuous stirring and 40 mPa pressure with 10-kDa ultrafiltration membranes (Millipore). The concentrate was centrifuged to remove particles. Then 400 mL of Tris-HCl, 0.5 M NaCl, pH 8.0 buffer (TBS-NaCl) was added to the supernatant. Samples were loaded in HiTrap® chelating high performance columns (GE Healthcare Biosciences, Pittsburgh, PA) previously loaded with nickel. Columns were washed with 100 mL of TBS-NaCl, followed by 50 mL each of 10 mM, 30 mM, 100 mM, and 300 mM imidazole diluted in TBS-NaCl. Fractions were concentrated in a centricon 10 kDa and analyzed by SDS-PAGE. Then 5% acetonitrile (ACN) was added to the samples, which were acidified with trifluoracetic acid (TFA) 0.1%. Samples were loaded in a reverse-phase HPLC (Vydac, Carpenteria, CA) previously equilibrated in ACN 5%/TFA 0.1%. Elution was carried out at 1 mL/ minute using a 0–100% ACN, TFA 0.1% in 1 hour. Samples were dialyzed extensively against PBS, and frozen.

SDS-PAGE

Samples were treated with 4× NuPAGE lithium dodecyl sulfate sample buffer and 10× sample reducing reagent, then loaded in NuPAGE-Bis-Tris 4% to 12% gels with 2-(N-morpholino)ethanesulfonic acid (MES) running buffer (Invitrogen). Gels were Coomassie blue stained.

aPTT and PT

aPTT and PT were evaluated on a STart® 4 stago coagulometer (Diagnostica Stago, Parsippany, NJ). Freeze-dried, citrated, normal human plasma was resuspended in ultra-pure water. For the aPTT, plasma (50 µL) was incubated with 7 µL Desmolaris or PBS (control) in appropriate cuvettes and placed in the coagulometer for 2 minutes at 37ºC. Then, 50 µL of pre-warmed aPTT reagent (STA® PTT; Diagnostica Stago, Asnières sur Seine, France) was added and incubated for 2 more minutes. $CaCl_2$ (50 µL at 25 mM) was added to start reactions. For the PT, plasma (50 µL) was incubated with Desmolaris or PBS (control) and placed in the coagulometer for 2 minutes at 37°C. Then, 100 µL of the PT reagent (NEOplastine CI plus; Diagnostica Stago) was added. Time for clot formation was recorded in duplicate. For PT and aPTT ex-vivo, Desmolaris (250 µg/kg) or phosphate-buffered saline (PBS) were administered i.v. to mice, and after 15 minutes blood was collected by cardiac puncture in 3.8% trisodium citrate in (9:1,v/v). Platelet-poor plasma was obtained by centrifugation at 2,000×g for 10 min. Plasma (50 µL) was incubated for 1 min at 37 ºC followed by addition of aPTT reagent (50 µL, 1 min) and then 25 mM $CaCl_2$ (100 µL) or PT reagent (100 µL). Time for clot formation was then recorded.

Protease inhibition assays

All assays were performed at 30°C in triplicate as described.[1] In 96-well fluorescent plates, Desmolaris (250 nM) was pre-incubated with each enzyme for 20 minutes before the addition of the corresponding substrate and appropriate buffer essentially as described.[1] The fluorogenic substrate hydrolysis rate was followed in kinetic mode in a Tecan Infinite® M200 96-well plate fluorescence reader (Tecan Group AG, Männedorf, Switzerland) using 365-nm excitation and 450-nm emission wavelengths with a cutoff at 435 nm. The observed substrate hydrolysis rate in

the absence of protein was considered as 100% and compared with the remaining enzymatic activity in the presence of the protein. For statistical analysis, we used the *t*-test, and a *P* value of ≤ 0.05 was considered statistically significant. Factor VIIa(1 nM)/lipidated TF(1 nM) activity was measured after incubation with PBS or Desmolaris for 30 minutes, followed by addition of S2236 (1 mM) as described.[4]

Kinetics studies

These were performed using chromogenic substrate hydrolysis (S2222 for FXa, S2366 for FXIa, and S2302 for kallikrein) using a Thermomax ELISA reader (Molecular Devices, Menlo Park, CA) as described.[1,4] All reagents were diluted in the reaction buffer, TBS-BSA (10 mM Tris, 0.15 M NaCl, 0.3% BSA, pH 7.4). Reactions were performed in 96-well flat-bottom plates (Corning 3596; Corning, NY) in a total volume of 200 μL. To characterize the interaction of Desmolaris and FXIa or FXa as tight (i.e. inhibition occurs at a concentration of the inhibitor that is near to that of the enzyme), reactions were started by addition of S2222 or S2366 (250 μM) to a mixture containing enzymes (at indicated concentrations) and inhibitor (0–200 nM) pre-incubated for 60 minutes at room temperature. Reactions were followed for 1 hour. Initial velocities (Vmax mode, mOD/minute) obtained were used to determine the ratio of substrate hydrolysis by the enzyme in the presence of the inhibitor [Vs(inhibited)/Vo(uninhibited)], which allows determination of the IC_{50} using the Morrison equation (see below).[1,5,6] For assays designed to determine whether Desmolaris is a slow (e.g. final degree of inhibition does not occur immediately) or fast inhibitor of FXI and FXa, and the type of inhibition (competitive or noncompetitive), the inhibitor (0–200 nM) was incubated with S2222 or S2366 (150, 300, 450, 600, 750, and 900 μM) at room temperature for 5 minutes followed by addition of FXa (0.5 nM)

or FXIa (0.5 nM). Reactions were followed for 2 hours. In all kinetic measurements, care was taken to ensure that substrate was less than 20% hydrolyzed. The linear part of the progress curves between 30–60 minutes was chosen to determine the steady-state kinetics (*Vmax* mode) of Desmolaris–FXIa or –FXa complex formation. These values were used to plot *Vs*/*Vo* versus Desmolaris for each S2222 or S2366 concentration, at a constant FXIa or FXa concentration. Data points were fitted with the Morrison equation[1,5,6] using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA), which allows for determination of IC_{50} for each substrate concentration. The plot IC_{50} (apparent K_i) vs. S2222 or S2366 was then used to calculate the type of inhibition and true Ki, as described in detail elsewhere.[1,5,6] Data points are the mean of 6 determinations, each performed in duplicate.

In some experiments to determine the effects of heparin in *Ki*, Desmolaris (at indicated concentrations), chromogenic substrate, and saturating concentrations of heparin (1 μg/mL) were incubated for 5 minutes, followed by addition of FXIa (0.5 nM) or FXa (0.5 nM). Reactions and data transformation to calculate *Ki* were performed as described above.

Prothrombinase assembly

Activation of prothrombin by human FXa was performed in Tris-buffered saline-Ca^{2+} (TBS-Ca^{2+}; 20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.3% BSA, pH 7.5), using a discontinuous assay,[7] with modifications. In a final total reaction volume of 200 μL, FXa (20 pM, final concentration), was incubated with Desmolaris (0–100 nM, final concentration) for 30 minutes at room temperature. Then, human FVa (1 nM, final concentration), and PC/PS vesicles (10 μM, final concentration) were added. After 15 minutes, reactions were initiated by addition of human prothrombin (1.4 μM, final concentration). Aliquots of 20 μL were removed every minute into

microplate wells containing 80 µL of TBS-EDTA (20 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA, 0.1% BSA, pH 7.5) to stop reactions. Then, 5 µL of S2238 (250 µM, final concentration) was added and absorbance at 405 nm was recorded at 37°C for 15 minutes at 11-second intervals using a Thermomax microplate reader (Molecular Devices).[1,4] Initial velocities (Vmax mode, mOD/minute) obtained were used to calculate the amount of thrombin formed using a standard curve. Alternatively, FVa, PC/PS, and FXa were previously incubated in TBS-Ca^{2+}; followed by addition of a mixture containing prothrombin/Desmolaris. Reactions proceeded as above to determine thrombin formation. PC/PS vesicles were prepared as described.[1] Absence of one of the components of the prothrombinase complex showed no thrombin formation.

Active site of Desmolaris

In a PCR tube, Desmolaris (7.5 µM) was incubated with human FXa and FXIa (1.7 µM) overnight at 37°C. All reactants were diluted in TBS. LDS loading buffer and DTT were added to the tubes, boiled for 10 minutes, and the mixture was loaded in a 4–12% NuPAGE gel (MES buffer) and Coomassie Blue stained.

Surface plasmon resonance

All surface plasmon resonance (SPR) experiments were carried out in a T100 instrument (Biacore Inc, Uppsala, Sweden) following the manufacturer's instructions. For immobilization using an amine-coupling kit (Biacore), carboxymethylated dextran chips were activated with 1-ethyl-3-(dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide before injection of Desmolaris (6.5 µg/mL) in acetate buffer, pH 5.5. Remaining activated groups were blocked with 1 mol/L ethanolamine, pH 8.5, resulting in a final immobilization of 871.3 RU. FXIa (10 µg/mL

in acetate buffer pH 5.0) was immobilized in CM5 sensor chips at 1617.4 RU. Kinetic experiments were carried out by injecting FXa, FXIa, or heparin for a contact time of 180 seconds at a flow rate 30 μL/minute at 25°C. For all runs, HBS-P buffer (10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20, pH 7.4) was used. FXIa- or FXa-Desmolaris complex dissociation was monitored for 900 seconds, and the sensor surface was regenerated by a pulse of 80 seconds of 1 mM HCl, 2 M NaCl (30 μL/minute). In some experiments, other coagulation factors (FVIIa, FIXa, FX, FXI, prothrombin, thrombin) were tested (64 nM) as analytes. Blank flow cells were used to subtract the buffer effect on sensorgrams. After subtraction of the contribution of bulk refractive index and nonspecific interactions with the CM5 chip surface, the individual association (*ka or kon*) and dissociation (*kd or koff*) rate constants were obtained by global fitting of data using the 1:1 model (Langmuir) interaction model using BIAevaluation™ (Biacore, Inc.). Values were then used to calculate the equilibrium constant (*KD*). In some experiments, association phase was allowed to proceed for 30 min to reach equilibrium binding (steady-state) and resonance values at binding stability (30 min) were used to calculate the *KD*. The values of average-squared residual obtained were not significantly improved by fitting data to models that assumed other interactions. Conditions were chosen so that the contribution of mass transport to the observed values of *KD* was negligible. Also, models in the T100 evaluation software fit for mass transfer coefficient to mathematically extrapolate the true *ka* and *kd*.

Isothermal titration calorimetry

Desmolaris binding to FXIa and FXa was performed using an isothermal titration calorimetry (ITC) microcalorimeter (Microcal, Northampton, MA) at 30°C. Before the run, the proteins were dialyzed against 20 mmol/L Tris-HCl, 0.15 M NaCl, pH 7.4, and concentrations carefully

checked and adjusted based on the extinction coefficient of the enzymes. Titration experiments were performed by making successive injections of 5 µL each of 10 µM FXa or FXIa into the 2-mL sample cell containing 1 µM Desmolaris until near-saturation. The calorimetric enthalpy (ΔHcal) for each injection was calculated after correction for the heat of Desmolaris dilution obtained in control experiments performed by titrating Desmolaris into buffer. The binding isotherms were fitted according to a model for a single set of identical binding sites by nonlinear square analysis using Origin® software (Microcal, Northampton, MA). The enthalpy change (ΔH) and stoichiometry (n) were determined as described.[1,2]

Heparin-agarose column

A HiTrap®-heparin HP column (5 mL; GE HealthCare Life Sciences, Piscataway, NJ) was connected to an AKTA purifier system (Amersham Biosciences, Piscataway, NJ). Before use, the column was extensively washed with 2 M NaCl. The column was then equilibrated with 10 mM Tris, 0.15 M NaCl (TBS) at a flow rate of 1 mL/minute. Desmolaris and truncated forms (24-90 µg, 200 µL in TBS) were loaded into the column, followed by washing for 20 minutes with TBS. Then, a gradient was applied (0–2 M NaCl in TBS) at 1 mL/minute for 40 minutes; elution conductivity for each protein was recorded.

FIX activation by FXIa

In all experiments, plasma- and albumin-free recombinant FIX was used (BeneFIX, 250 U diluted at 1 U/µL in distilled water). In a final volume of 25 µL, FXIa (3 nM) was incubated for 20 minutes with Desmolaris (300 nM) followed by addition of recombinant FIX (1 µM). After 60 minutes at 37°C, reactions were stopped by addition of Laemmli buffer and boiling with 2-

mercaptoethanol for 5 minutes. Proteins were separated by 4% to 12% NuPAGE (MES buffer). Coomassie Blue R-250 was used to stain the gel.

Reciprocal activation

Factor XII (0.2 nM final concentration) was preincubated with Desmolaris (0, 62, 125, 250, 500, and 1000 nM) in 20 mM Tris, 0.15 M NaCl, 0.3% BSA, pH 7.4, for 20 minutes at room temperature. Reciprocal activation was started by addition of prekallikrein (10 nM) and DS 500 (0.2 μg/mL). After 10 minutes' incubation, S-2302 (250 μM) was added, and the increase in absorbance at 405 nm was recorded at time intervals of 1 minute. Background hydrolysis of S-2302 by FXIIa was minimal and precluded the use of CTI to inhibit FXIIa.

FXI activation by FXIIa

In a final volume of 20 μL, FXIIa (25 nM) was incubated with Desmolaris (0, 50, and 250 nM) in TBS for 20 minutes followed by addition of FXI (100 μg/mL). After 8 hours at 37°C, reactions were stopped by addition of reducing SDS sample buffer. Samples were loaded on 4–12% NuPAGE gel, followed by staining with Coomassie Blue. CTI was used as control inhibitor.

Bradykinin (BK) generation

Ten μL of human plasma (Diagnostica Stago) was preincubated with (1 μL) Desmolaris (0–300 nM) or PBS. To begin contact activation, 2.5 μL of kaolin (4% in PBS, stock solution) was added and further incubated for 1 minute at 37^{o}C. The mixture was then transferred to isolated guinea pig ileum placed in a 1.5-mL glass chamber and bathed in modified Tyrode's solution

(with 10 mM HEPES buffer, pH 7.4). Generated BK was assayed by measuring the contraction response using a transducer from Harvard Apparatus Inc. (Holliston, MA). Known amounts of BK were used as standard, and the area under the contraction curve was used to estimate BK formation. During the course of the experiment, solutions were kept oxygenated by bubbling air into the bath.

Paw edema in mice

All in vivo experiments were approved by the National Institute of Allergy and Infectious Diseases. Female C57BL/6 mice, 6 to 8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at the Animal Care Facility. Posterior footpad thickness of each mouse was recorded using a caliper before each injection (Mitutoyo America Corp, Aurora, IL).[1] Subsequently, PBS, FXa alone, or FXa incubated with Desmolaris was injected intradermally into the paw using a 0.3-mL syringe. As an index of edema formation, paw thickness was then measured at 15, 30, 45, and 60 minutes. For each data point, 4 posterior paws were injected. Statistical analysis of variance (ANOVA) using Tukey as a multiple comparison post-test was used. $P \leq 0.05$ was considered statistically significant.

$FeCl_3$-induced artery thrombosis

BALB/c mice were anesthetized with intramuscular xylazine (16 mg/kg) followed by ketamine (100 mg/kg). The right common carotid artery was isolated through a midline cervical incision, and blood flow was continuously monitored using a 0.5-VB Doppler flow probe coupled to a TS420 flow meter (Transonic Systems, Ithaca, NY) as described.[1] Fifteen minutes before induction of thrombosis, animals were injected in the tail vein with 50 μL Desmolaris (0.01 or

0.1 mg/kg) or vehicle (PBS). Thrombus formation was induced by applying a piece of filter paper (1×2 mm) saturated with 7.5% $FeCl_3$ solution on the adventitial surface of the artery for 3 minutes. After exposure, the filter paper was removed, and the vessel was washed with sterile normal saline. Carotid blood flow was continuously monitored for 60 minutes or until complete occlusion (0 flow for at least 10 seconds) occurred. ANOVA using Tukey as a multiple comparison post-test was used. $P \leq 0.05$ was considered statistically significant.

Tail bleeding assay

Mice were anesthetized with intramuscular xylazin (16 mg/kg) followed by ketamine (100 mg/kg) and injected intravenously with phosphate-buffered saline (PBS) or Desmolaris (0.1, 0.25, 0.5 or 1.0 mg/kg) in a 100 μL volume. After 15 minutes, the distal 2 mm segment of the tail was removed and immediately immersed in 40 mL distilled water warmed to 37 ºC. The samples were properly homogenized and the absorbance determined at 540 nm to estimate hemoglobin content. No animal was allowed to bleed for more than 30 min.

Miles vascular permeability assay

For the Miles assay, Evans blue dye (100 μL of a 0.25% solution in 0.9% NaCl) premixed with Desmolaris (resulting in 500 μg/kg) or PBS was injected intravenously into anesthetized mice. After 5 minutes, 40 μL of polyP (0.2 mg/mL in PBS), or 40 μL PBS was injected intradermally into the skin, in the dorsal region of mice as described,[8] with modifications. After 20 minutes, the animals were euthanized with CO_2, and an area of skin that included the entire injection site was carefully removed and photographed. Evans blue dye was extracted from the skin by incubation

with 1500 μL of formamide for 24 hours at 55°C. After centrifugation at 4000 rpm for 20 minutes, absorbance of the supernatant was measured at 620 nm. ANOVA using Tukey as a multiple comparison post-test was used. $P \leq 0.05$ was considered statistically significant.

Pulmonary thromboembolism

Balb/c mice were anesthetized with intramuscular xylazin (16 mg/kg) followed by ketamine (100 mg/kg). Either Desmolaris (0.1, 0.5 and 1.0 mg/kg) or vehicle was slowly injected into the inferior vena cava 5 min prior to challenge. A mixture of 0.8 mg/kg collagen and 60 μg/kg epinephrine was then injected into the inferior vena cava. Animals still alive after 30 min were considered to be survivors. For histopathologic analyses mice were killed 5 minutes after challenge, and lungs were rapidly removed and fixed in buffered 4 % formalin, pH 7.4. Tissues were dehydrated and embedded in paraffin, cut into 8 μm sections, and stained with hematoxylin and eosin.

Statistical analysis

Results are expressed as means ± SE. Statistical differences among the groups were analyzed by *t*-test or ANOVA using a multiple comparison post-test. Significance was set at $P \leq 0.05$ (GraphPad Prism software, San Diego, CA).

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