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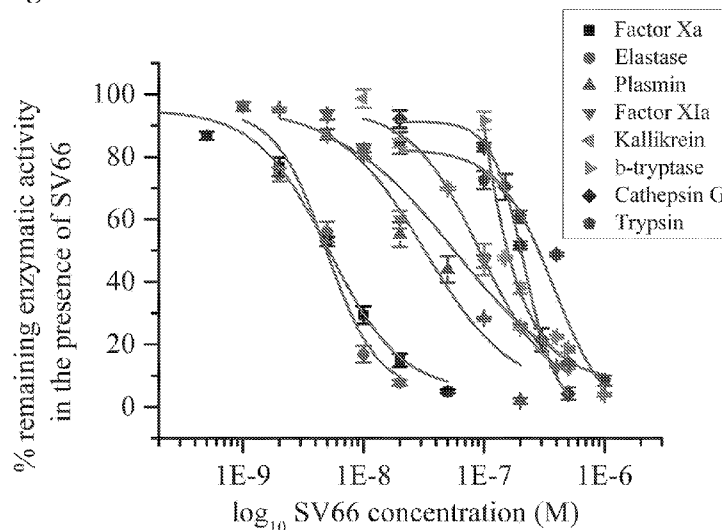
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[Continued on next page]

(54) Title: SIMUKUNIN

Figure 8



(57) Abstract: The present invention includes a novel protein, also referred to herein as simukunin, that inhibits the function of several physiologically important enzymes. Simukunin is a potent inhibitor of the blood coagulation cascade, inhibiting Factor Xa and functioning as an efficient anticoagulant. Simukunin also inhibits the serine proteases elastase and cathepsin and demonstrates anti-inflammatory properties. Also included are methods of making and using simukunin.



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SIMUKUNIN

CONTINUING APPLICATION DATA

10 This application claims the benefit of U.S. Provisional Application Serial No. 61/490,149, filed May 26, 2011, which is incorporated by reference herein.

GOVERNMENT FUNDING

15 This invention was made with government support under Grant No. 200704549, awarded by that United States Department of Agriculture. The Government has certain rights in the invention.

BACKGROUND

20 Certain medical conditions, such as strokes, cardiovascular blockages, deep vein thrombosis, and pulmonary embolism, are treated with the administration of an anticoagulant, to prevent the formation of blood clots and the extension of existing clots. A widely used anticoagulant is heparin. However, some patients develop allergic responses to heparin and a serious side-effect of heparin is heparin-induced thrombocytopenia (HIT). HIT is caused by an immunological reaction that makes
25 platelets a target of immunological response, resulting in the degradation of platelets and causing thrombocytopenia. These patients would benefit from the availability of alternative anticoagulants.

Heparin results in the inactivation of thrombin and other proteases involved in blood clotting (most notably factor Xa). The actions of heparin are limited to
30 anticoagulant activity and heparin does not demonstrate anti-inflammatory activity. Alternatives to heparin, possessing both anticoagulant activity and additional activities, such as anti-inflammatory activity, are needed. Such alternatives, demonstrating, for example, both anticoagulant and anti-inflammatory activities, could be administered in the early stages of a medical crisis to both antagonize clot formation and to limit tissue
35 damage caused by inflammatory responses.

SUMMARY OF THE INVENTION

The present invention includes an isolated simukunin polypeptide having an amino acid sequence at least about 80% identical to SEQ ID NO:3 and fragments thereof, wherein the simukunin polypeptide inhibits Factor Xa. In some aspects, the isolated simukunin polypeptide also inhibits elastase and/or cathepsin G.

The present invention includes an isolated simukunin polypeptide having an amino acid sequence at least about 95% identical to SEQ ID NO:3 and fragments thereof, wherein the simukunin polypeptide inhibits Factor Xa. In some aspects, the isolated simukunin polypeptide also inhibits elastase and/or cathepsin G.

The present invention includes an isolated simukunin polypeptide having an amino acid sequence at least about 95% identical to SEQ ID NO:3 and fragments thereof, wherein the isolated simukunin polypeptide inhibits Factor Xa, inhibits elastase, and inhibits cathepsin G.

In some aspects, an isolated simukunin polypeptide of the present invention also inhibits Factor IXa, Factor XIa, plasmin, kallikrein, trypsin, and/or β -tryptase.

The present invention includes compositions of an isolated simukunin polypeptide as described herein. In some aspects, the composition includes a pharmaceutically acceptable carrier. In some aspects, the pharmaceutically acceptable carrier is pyrogen free. In some aspects, a composition is pyrogen free.

In some aspects, the present invention includes a composition including an isolated simukunin polypeptide having an amino acid sequence at least about 95% identical to SEQ ID NO:3 and fragments thereof, wherein the isolated simukunin polypeptide inhibits Factor Xa, inhibits elastase, and inhibits cathepsin G, wherein the composition is pyrogen free.

In some aspects, a composition of the present invention is formulated for parenteral application.

In some aspects, a composition of the present invention is formulated for topical application.

The present invention includes an isolated polynucleotide sequence encoding a simukunin polypeptide as described herein. In some aspects, the present invention includes a vector or host cell including an isolated polynucleotide sequence.

The present invention includes a method of producing a polypeptide, the method including culturing a host cell as described herein and purifying an expression product from the cell mass and/or the culture medium.

The present invention includes a method of inhibiting plasma coagulation, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting Factor Xa, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting one or more serine proteases, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting elastase and/or cathepsin G, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting Factor IXa, Factor XIa, and/or plasmin, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting kallikrein, trypsin, and/or β -tryptase, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of modulating inflammation, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of modulating the function of neutrophils, macrophages, and/or mast cells, the method including providing an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated polypeptide is provided in vitro. In some aspects, the isolated polypeptide is provided in vivo to a subject. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting plasma coagulation in a subject, the method including administering to the subject an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated simukunin polypeptide includes an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting Factor Xa in a subject, the method including administering to the subject an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated simukunin polypeptide includes an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting elastase and/or cathepsin G in a subject, the method including administering to the subject an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated simukunin polypeptide includes an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting Factor Xa and elastase in a subject, the method including administering to the subject an isolated simukunin

polypeptide as described herein or a composition as described herein. In some aspects, the isolated simukunin polypeptide includes an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of modulating inflammation in a subject, the method including administering to the subject an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated simukunin polypeptide includes an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of modulating the function of neutrophils, macrophages, and/or mast cells in a subject, the method including administering to the subject an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated simukunin polypeptide includes an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G. In some aspect, administration is parenteral. In some aspects, administration is topical.

The present invention includes a method of inhibiting plasma coagulation and inhibiting elastase and/or cathepsin G in a subject, the method including administering to the subject an isolated simukunin polypeptide as described herein or a composition as described herein. In some aspects, the isolated simukunin polypeptide includes an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G. In some aspect, administration is parenteral. In some aspects, administration is topical.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B. SV-66 and SV-170 belong to the Kunitz family of protease inhibitors. Fig. 1A shows nucleotide sequences of SV-66 (SEQ ID NO:1) and SV-170 (SEQ ID NO:4) and the translated polypeptide sequences of SV-66 and SV-170. Start and stop codons are in white with black shading. Numbers below the amino acid residues are designated based on the putative mature protein. Signal sequences predicted by SignalP are underlined. SV-66 encodes a 102 amino-acid polypeptide (Simukunin) (SEQ ID NO:2), which includes a 19 amino-acid N-terminal signal sequence. Mature Simukunin is predicted to consist of 83 amino-acid residues (SEQ ID NO:3), with a theoretical mass of 9627.22 Da and pI of 9.93. SV-66 also contains a putative O-glycosylation site at position 81 (Ser). SV-170 encodes a 78 amino-acid polypeptide (SEQ ID NO:5), which includes an N-terminal 22 amino-acid signal sequence. Mature SV-170 is predicted to consist of 56 amino-acid residues (SEQ ID NO:6), and theoretical mass and pI are 6526.66 Da and 8.87, respectively. Fig. 1B shows alignment of representative Kunitz domain sequences with SV-66 (SEQ ID NO:7) and SV-170 (SEQ ID NO:8). Each Kunitz domain was separated from the original sequences for alignment (numbers denote amino-acid positions in the original mature peptides). All reference sequences were retrieved from GenBank. Accession numbers are as follows: Accession number P10646 for TFPI (human: three Kunitz domains - KU1 (SEQ ID NO:9), KU2 (SEQ ID NO:10), and KU3 (SEQ ID NO:11)); Accession number AAI49369 for BPTI (*Bos taurus*: one Kunitz domain (SEQ ID NO:12)); Accession number AAR97367 for Amblin (*Amblyomma hebraeum*: two Kunitz domains - KU1 (SEQ ID NO:13) and KU2 (SEQ ID NO:14)); and Accession number CAC82583 for Boophilin (*Rhipicephalus microplus*: two Kunitz domains - KU1 (SEQ ID NO:15) and KU2 SEQ ID NO:16). Strictly conserved cysteine residues are indicated with white arrows, and predicted conserved disulfide bonds are shown in solid lines. The reactive site loop (RSL) P₄-P₂' residues, conserved in canonical binding inhibitors, are indicated by asterisks. The P₁ residue is indicated with a black arrow. Highly conserved P₁-P₁' (Arg/Lys-Ala/Gly) residues are shaded and boxed. Other identical residues across the domain are shaded and indicated with a plus (+) and conserved or semi-conserved residues are shaded with grey.

Figures 2A and 2B. SV-66 is constitutively expressed in the salivary glands of adult female *S. vittatum*. Fig. 2A shows sex and tissue-specific expression of Simukunin. Transcript was detected in the adult female body (head and thorax without

abdomen), but not in adult female carcasses (bodies without salivary glands or heads). M: male; F: mature female; NTC: no-template control. Five individuals were pooled for each sample. Actin PCR products are shown as a positive control indicating equivalent concentrations of template among samples. Each panel is a composite of two rows (upper and lower) of wells, run in the same gel at the same time. Fig. 2B shows a time-course of expression of Simukunin. Transcript was detected before, and at selected time points up to 48 h post blood meal. Fresh: freshly eclosed non-blood-fed female adult; hpb: hours post blood feeding; NTC: no-template control. Two samples, each comprised of five pooled individuals, were analyzed for each time point.

Figure 3. rSV-66 delays clotting of human plasma. Indicated concentrations of rSimukunin (rSV-66) and rSV-170 were tested by the recalcification time assay. Citrated human plasma (50 μ l) was mixed with recombinant proteins (in 50 μ l 0.15 M NaCl, 10 mM HEPES pH7.4) and pre-warmed at 37°C for 15 min before clotting was initiated by the addition of 50 μ l prewarmed CaCl_2 (25 mM). Recalcification (clotting) time was determined by monitoring absorbance at 650 nm at 10-sec intervals in a SpectraMax 340 microtiter plate reader, with onset time (the time to a linear increase in the OD, which reflects the maximal rate of formation of insoluble fibrin) set at an OD of 0.04. Clotting times (mean $\pm$ SD) for rSimukunin and rSV-170 are shown in black bars and grey bars, respectively. The white bar is the Ca^{2+} -only control. One-way analysis of variance indicted a significant difference between treatments ($F_{6,21}=119.3$; $P<0.001$) for rSimukunin but not rSV-170. Subsequent multiple comparisons between various treatments and the positive control were performed using the Holm-Sidak method. Statistically significant increases in clotting time at $p<0.05$ and $p<0.01$ are indicated by * and ** respectively. Results shown are representative of three independent experiments.

Figure 4. Point mutations in the reactive site loop of rSimukunin disable anti-coagulation activity. Each recombinant protein was tested by adding 0.5 μ g (grey bars) or 1 μ g (black bars) to a fixed volume of plasma. Plasma was then pre-warmed at 37°C for 15 min before addition of 25 mM (8.3 mM final concentration) CaCl_2 (pre-warmed) to initiate clotting. White bar shows the Ca^{2+} -only plasma control. The graph shows mean $\pm$ SD from three independently conducted experiments. p-values for significant differences by one-tailed t-test are shown, where the alternative hypothesis is that sample recalcification time is greater than the Ca^{2+} -only control. Statistically significant increases in clotting time at $p<0.05$ and $p<0.01$ are indicated by * and ** respectively

Figure 5. rSimukunin inhibits several serine proteases. Enzyme activity in the presence of 500 nM of rSimukunin, as a percentage of the total activity in the absence of inhibitor, is shown. Results with statistically significant (t-test, $p < 0.01$) inhibition compared to the control are shown in white bars. The graph shows mean $\pm$ SEM. u-PA: urokinase-type plasminogen activator, t-PA: tissue plasminogen activator. Each inhibition assay was conducted in triplicate.

Figures 6A to 6C. rSimukunin displays high-affinity binding to FXa and elastase. Fig. 6A shows surface Plasmon Resonance (SPR) Sensorgrams show mouse, bovine, and human FXa, the FXa derivatives des-GLA-hFXa and DEGR-hFXa, and other coagulation factors (all tested at 200 nM) binding to immobilized rSimukunin. Fig. 6B shows sensorgrams for various concentrations of human FXa (50 nM, 25 nM, 12.5 nM, 6.25 nM, and 3.1 nM) binding to immobilized rSimukunin. Fig. 6C shows sensorgrams for various concentrations of elastase (3.75 nM, 1.8 nM, 0.9 nM, and 0.45 nM) binding to immobilized rSimukunin. Data were fitted using a 11 binding model (Langmuir). RU: resonance units.

Figure 7. Visualization of rSV-66 and rSV-170 following separation by SDS-PAGE (left panel) and immunoblotting (right panel). The SDS-PAGE gel was stained with Coomassie Brilliant Blue, while the immunoblot was probed with an anti-His primary antibody and visualized by chemiluminescence.

Figure 8. Determination of rSimukunin IC_{50} values for selected serine proteinases.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE PRESENT INVENTION

The present invention includes a novel protein, also referred to herein as simukunin, isolated from salivary glands of a blood-feeding fly (*Simulium vittatum*), that inhibits the function of several physiologically important enzymes. Simukunin is a potent inhibitor of the blood coagulation cascade, inhibiting Factor Xa, and to a lesser extent, inhibiting Factor XIa, and functioning as an efficient anticoagulant. Simukunin also inhibits elastase and cathepsin G, which play important roles in mast cell and neutrophil-driven inflammation. Accordingly, simukunin is a potent anticoagulant, with additional anti-inflammatory properties.

The present invention includes an isolated simukunin polypeptide, and fragments and derivatives thereof. Such a simukunin polypeptide, or fragment or derivative thereof, may exhibit one or more of the physical or functional characteristics described herein.

5 In some embodiments, a simukunin polypeptide may have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% amino acid identity to a simukunin amino acid sequence as described herein. For example, a
10 simukunin polypeptide may have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% amino acid identity to SEQ ID NO:2 or SEQ ID NO:3, or a fragment thereof.

15 In some embodiments, a simukunin polypeptide is encoded by a polynucleotide sequence with at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO:1.

20 In some embodiments, a simukunin polypeptide is encoded by a polynucleotide sequence that hybridizes under "stringent conditions," also referred to herein as "high stringency conditions," to a polynucleotide sequence described herein. For example, hybridizing under stringent conditions to SEQ ID NO:1.

Also included in the present invention are isolated *Simulium vittatum* SV-170
25 polypeptides, and fragments and derivatives thereof. Such a *Simulium vittatum* SV-170 polypeptide or fragment of derivative thereof may demonstrate one of more of the physical or functional characteristic of a *Simulium vittatum* SV-170 polypeptide, as described herein.

In some embodiments, a *Simulium vittatum* SV-170 polypeptide may have at
30 least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% amino acid identity to a *Simulium vittatum* SV-170 amino acid sequence as described herein. For example, a *Simulium vittatum* SV-170 polypeptide may have at

least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% amino acid identity to SEQ ID NO:5 or SEQ ID NO:6, or a fragment thereof.

5 In some embodiments, a *Simulium vittatum* SV-170 polypeptide is encoded by a polynucleotide sequence with at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO:4.

10 In some embodiments, a *Simulium vittatum* SV-170 polypeptide is encoded by a polynucleotide sequence that hybridizes under "stringent conditions," also referred to herein as "high stringency conditions," to a polynucleotide sequence described herein. For example, hybridizing under stringent conditions to SEQ ID NO:4.

 Also included in the present invention are fragments of a polypeptide described
15 herein. A fragment may be, for example, include about any 5, about any 10, about any 15, about any 20, about any 25, about any 30, about any 35, about any 40, about any 45, about any 50, about any 55, about any 60, about any 65, about any 70, about any 75, about any 80, about any 85, about any 90, about any 95, about any 95, or about any 100 consecutive amino acids of a polypeptide described herein, or any range thereof. For
20 example, a fragment may include about any 10, about any 15, about any 20, about any 25, about any 30, about any 35, about any 40, about any 45, about any 50, about any 55, about any 60, about any 65, about any 70, about any 75, about any 80, about any 85, about any 90, about any 95, about any 95, or about any 100 consecutive amino acids of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:6, or any range thereof.

25 Also included in the present invention are polypeptides, and derivatives, or fragments thereof, as described herein with additional amino acid residues not of simukunin, SV-170, and/or *Simulium vittatum*. Such heterologous sequences may, for example, encode additional enzymatic activities or other additional components, such as, for example, a linker, an immunogenic carrier protein, a detectable marker, or a fusion
30 protein.

 A polypeptide of the present invention may possess one or more of the physical characteristics described herein. For example, a polypeptide of the present invention may include one or more of the residues characteristic of a Kunitz domain. This includes, but is not limited to, one or more of the characteristic residues identified in Fig.

1B. For example, a polypeptide may include one, two, three, four, five, or six of the strictly conserved cysteine residues (for example, represented by amino acids 5, 14, 30, 38, 51, and 55 of SEQ ID NO:3, respectively); one, two, or three of the conserved disulfide bonds characteristic of a Kunitz domain (represented by disulfide bonds
 5 between cysteine residues C5 to C55, C14 to C38, and C30 to C51 of SEQ ID NO:3); one, two, three, four, five, or six of the conserved amino acid residues of the reactive site loop (RSL) P₄-P₂' residues (for example, represented by amino acid positions 12, 13, 14, 15, 16, and 17 of SEQ ID NO:3); a basic residue, such as, for example, Arg (R) or Lys (K), at site P₁ (represented by amino acid position 15 of SEQ ID NO:3); and/or an Ala
 10 (A) or Gly (G) at site P₁' (for example, represented by amino acid position 16 of SEQ ID NO:3).

A polypeptide of the present invention may possess one or more of the functional characteristics described herein. For example, a polypeptide described herein may demonstrate anticoagulant activity, anti-clotting activity, anti-platelet activity, anti-
 15 inflammatory activity, and/or immunomodulating activity. A polypeptide described herein may inhibit the function of one or more enzymes. A polypeptide as described herein may be a protease inhibitor, including but not limited to, a serine protease inhibitor. In some embodiments, a polypeptide of the present invention inhibits multiple proteases. Such an enzyme may be, for example, a mammalian enzyme, from, for
 20 example, human, mouse, rat, bovine, and/or non-human primates. Such an enzyme may be, for example, a bacterial or parasitic enzyme.

A polypeptide described herein may inhibit Factor Xa enzymatic activity. In some embodiments, such a polypeptide inhibits one or more additional serine proteases, for example, one or more of elastase, cathepsin G, plasmin, Factor IXa, Factor XIa,
 25 kallikrein, trypsin, β -tryptase, chymase, α -chymotrypsin, and/or enterokinase.

A polypeptide of the present invention may inhibit the enzymatic activity of one or more of Factor Xa, elastase, cathepsin G, plasmin, Factor IXa, Factor Xa, Factor XIa, kallikrein, trypsin, β -tryptase, chymase, α -chymotrypsin, and/or enterokinase.

A polypeptide of the present invention may bind to Factor Xa and/or elastase.
 30 In some embodiments, a polypeptide of the present invention does not inhibit, bind, and/or effect enzymatic activity or function of one or more of Factor XIIa, thrombin, matriptase, u-Pa and/or t-PA.

A polypeptide of the present invention may inhibit, reduce, or slow blood coagulation and/or may inhibit plasma coagulation. A polypeptide of the present

invention may inhibit an inflammatory response. A polypeptide of the present invention may inhibit or reduce mast and or neutrophil driven inflammatory processes, for example, by inhibiting the enzymatic activity of an enzyme contained in or released from a neutrophil, a mast cell, or other immune cell.

5 The functional characterization of a polypeptide of the present invention may be by any of a variety of available methods, including, but not limited to, any of those described in the examples section included herewith. In some embodiments, a measure of the enzymatic inhibition and/or binding of a polypeptide may be about any or more of those measurements (such as concentrations, ratios, IC₅₀, K_i, K_m, K_d) described in the
10 examples section included herewith.

 As used herein, the term "polypeptide" refers broadly to a polymer of two or more amino acids joined together by peptide bonds, regardless of length or post-translational modification. It should be understood that these terms do not connote a specific length of a polymer of amino acids, nor are they intended to imply or distinguish
15 whether the polypeptide is produced using recombinant techniques, chemical or enzymatic synthesis, or is naturally occurring.

 As used herein, "identity" refers to sequence similarity between two polypeptides or two polynucleotides. The sequence similarity between two polypeptides is determined by aligning the residues of the two polypeptides (e.g., a candidate amino acid
20 sequence and a reference amino acid sequence, such as SEQ ID NO:2, 3, 5, or 6, or a portion thereof) to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of shared amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. The sequence similarity is
25 typically at least 80% identity, at least 81% identity, at least 82% identity, at least 83% identity, at least 84% identity, at least 85% identity, at least 86% identity, at least 87% identity, at least 88% identity, at least 89% identity, at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity, or at least 99%
30 identity. Sequence similarity may be determined, for example, using sequence techniques such as the BESTFIT algorithm in the GCG package (Madison WI), or the Blastp program of the BLAST 2 search algorithm, as described by Tatusova, et al. (*FEMS Microbiol Lett* 1999, 174:247-250), and available through the World Wide Web, for instance at the internet site maintained by the National Center for Biotechnology

Information, National Institutes of Health. Preferably, sequence similarity between two amino acid sequences is determined using the Blastp program of the BLAST 2 search algorithm. Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap
 5 x_dropoff = 50, expect = 10, wordsize = 3, and optionally, filter on. In the comparison of two amino acid sequences using the BLAST search algorithm, structural similarity is referred to as "identities."

The sequence similarity between two polynucleotides is determined by aligning the residues of the two polynucleotides (e.g., a candidate nucleotide sequence and a
 10 reference nucleotide sequence, such as SEQ ID NO:1, 3, 5, 7, or a portion thereof) to optimize the number of identical nucleotides along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of shared nucleotides, although the nucleotides in each sequence must nonetheless remain in their proper order. The sequence similarity is typically at least
 15 80% identity, at least 81% identity, at least 82% identity, at least 83% identity, at least 84% identity, at least 85% identity, at least 86% identity, at least 87% identity, at least 88% identity, at least 89% identity, at least 90% identity, at least 91% identity, at least 92% identity, at least 93% identity, at least 94% identity, at least 95% identity, at least 96% identity, at least 97% identity, at least 98% identity, or at least 99% identity.

20 Sequence similarity may be determined, for example, using sequence techniques such as GCG FastA (Genetics Computer Group, Madison, Wisconsin), MacVector 4.5 (Kodak/IBI software package) or other suitable sequencing programs or methods known in the art. Preferably, sequence similarity between two nucleotide sequences is determined using the Blastn program of the BLAST 2 search algorithm, as described by
 25 Tatusova, et al. (1999, *FEMS Microbiol Lett.*, 174:247-250), and available through the World Wide Web, for instance at the internet site maintained by the National Center for Biotechnology Information, National Institutes of Health. Preferably, the default values for all BLAST 2 search parameters are used, including reward for match = 1, penalty for mismatch = -2, open gap penalty = 5, extension gap penalty = 2, gap x_dropoff = 50,
 30 expect = 10, wordsize = 11, and optionally, filter on. In the comparison of two nucleotide sequences using the BLAST search algorithm, sequence similarity is referred to as "identities."

Conditions that "allow" an event to occur or conditions that are "suitable" for an event to occur, such as an enzymatic reaction, or "suitable" conditions are conditions that

do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event.

The present invention includes an isolated polynucleotide sequence encoding a simukunin polypeptide as described herein, and fragments and derivatives thereof. Such a simukunin polypeptide, or fragment or derivative thereof, may have one or more of the functional characteristics described herein. Also included are isolated polynucleotide sequences encoding a *Simulium vittatum* SV-170 polypeptide as described herein, and fragments and derivatives thereof. Such a *Simulium vittatum* SV-170 polypeptide or fragment of derivative thereof may demonstrate one or more of the functional characteristic of a *Simulium vittatum* SV-170 polypeptide, as described herein.

In some embodiments, a polynucleotide sequence of the present invention may have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to a polynucleotide sequence described herein. For example, a derivative thereof may have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO:1 or SEQ ID NO:4, or a fragment thereof.

In some embodiments, a polynucleotide sequence of the present invention may encode a polypeptide as described herein. For example, a polynucleotide sequence of the present invention may encode an amino acid sequence with at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity SEQ ID NO:3 or SEQ ID NO:6.

Two polynucleotide sequences may be compared using the Blastn program of the BLAST 2 search algorithm, as described by Tatusova and Madden, 1999, FEMS Microbiol Lett; 174:247-250), and available on the world wide web at ncbi.nlm.nih.gov/gorf/bl2.html. Preferably, the default values for all BLAST 2 search parameters are used, including reward for match = 1, penalty for mismatch = -2, open gap penalty = 5, extension gap penalty = 2, gap x_dropoff = 50, expect = 10, wordsize = 11, and filter on.

In some embodiments, a polynucleotide sequence of the present invention is a polynucleotide sequence that hybridizes under "stringent conditions," also referred to herein as "high stringency conditions," to a polynucleotide sequence described herein. For example, hybridizing under stringent conditions to SEQ ID NO:1 or SEQ ID NO:4.

5 The polypeptide encoded by such a polynucleotide sequence may exhibit one or more of the various physical and/or functional characteristics described herein. Stringency of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper

10 annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures

15 would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995). "Stringent conditions" or "high stringency conditions," as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for

20 washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5X

25 SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2X SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1X SSC containing EDTA at 55°C.

30 An isolated polynucleotide of the present invention may include additional sequences not of simukunin origin. Such heterologous sequences may, for example, encode additional enzymatic activities or other additional components, such as promoter, transcription initiation, and/or and termination sequences.

The present invention also includes recombinant vectors including an isolated polynucleotide as described herein. A wide variety of plasmid and viral vectors are known in the art. The appropriate DNA sequence can be inserted into a vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) in a vector by procedures known in the art. Such procedures are deemed to be within the scope of those skilled in the art. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial vectors include, for example, pQE70, pQE60, pQE-9, pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, and pRIT5. Eukaryotic vectors include, for example, pWLNEO, pSV2CAT, pOG44, pXT1, pSG, pSVK3, pBPV, pMSG, and pSVL. However, any other plasmid or vector can be used. A large number of such vectors have been described in various publications and many such vectors are commercially available.

Included with the present invention are host cells that incorporate an isolated polynucleotide sequence encoding a simukunin polypeptide, or derivative or fragment thereof, and host cells that incorporate a vector incorporating an isolated polynucleotide sequence encoding a simukunin polypeptide, or derivative or fragment thereof. A host cell can be a higher eukaryotic cell, such as a mammalian or insect cell, or a lower eukaryotic cell, such as a yeast cell. Or, the host cell can be a prokaryotic cell, such as a bacterial cell, or a plant cell. Introduction of a vector construct into the host cell can be effected by any suitable techniques, such as, for example, calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation.

As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxynucleotides, and includes both double- and single-stranded RNA and DNA. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical techniques. A polynucleotide can be linear or circular in topology. A polynucleotide may be, for example, a portion of a vector, such as an expression or cloning vector, or a fragment. A polynucleotide may include nucleotide sequences having different functions, including, for instance, coding regions, and non-coding regions such as regulatory regions.

As used herein, the terms "coding region" and "coding sequence" are used interchangeably and refer to a nucleotide sequence that encodes a polypeptide and, when placed under the control of appropriate regulatory sequences, expresses the encoded polypeptide. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. A "regulatory sequence" is a nucleotide sequence that regulates expression of a coding sequence to which it is operably linked. Non-limiting examples of regulatory sequences include promoters, enhancers, transcription initiation sites, translation start sites, translation stop sites, and transcription terminators. The term "operably linked" refers to a juxtaposition of components such that they are in a relationship permitting them to function in their intended manner. A regulatory sequence is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

A polynucleotide that includes a coding region may include heterologous nucleotides that flank one or both sides of the coding region. As used herein, "heterologous nucleotides" refer to nucleotides that are not normally present flanking a coding region that is present in a wild-type cell. For instance, a coding region present in a wild-type cell and encoding an HCN polypeptide is flanked by homologous sequences, and any other nucleotide sequence flanking the coding region is considered to be heterologous. Examples of heterologous nucleotides include, but are not limited to regulatory sequences. Typically, heterologous nucleotides are present in a polynucleotide of the present invention through the use of standard genetic and/or recombinant methodologies well known to one skilled in the art. A polynucleotide of the present invention may be included in a suitable vector.

As used herein, an "exogenous polynucleotide" refers to a polynucleotide that has been introduced into a cell by artificial means. As used herein, the term "endogenous polynucleotide" refers to a polynucleotide that is normally or naturally found in a cell. An "endogenous polynucleotide" is also referred to as a "native polynucleotide."

The terms "complement" and "complementary" as used herein, refer to the ability of two single stranded polynucleotides to base pair with each other, where an adenine on one strand of a polynucleotide will base pair to a thymine or uracil on a strand of a second polynucleotide, and a cytosine on one strand of a polynucleotide will base pair to a guanine on a strand of a second polynucleotide. Two polynucleotides are complementary to each other when a nucleotide sequence in one polynucleotide can base

pair with a nucleotide sequence in a second polynucleotide. For instance, 5'-ATGC and 5'-GCAT are complementary. The term "substantial complement" and cognates thereof as used herein, refer to a polynucleotide that is capable of selectively hybridizing to a specified polynucleotide under stringent hybridization conditions.

- 5 The present invention includes compositions including one or more isolated polypeptides or polynucleotides as described herein. In a preferred form, an isolated polypeptide is purified and substantially free of other agents. As used herein, an "isolated" substance is one that has been removed from its natural environment, produced using recombinant techniques, or chemically or enzymatically synthesized.
- 10 For instance, a polypeptide, a polynucleotide, or a cell can be isolated. Preferably, a substance is purified, i.e., is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components with which they are naturally associated.

- Compositions may be administered in any of the methods described herein and
- 15 may be formulated in a variety of forms adapted to the chosen route of administration. The formulations may be conveniently presented in unit dosage form and may be prepared by methods well known in the art of pharmacy. A composition may include a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable," as used herein, means that the compositions or components thereof so described are suitable for
- 20 physiological contact with a subject without undue toxicity, incompatibility, instability, allergic response, and the like. A composition may be a pharmaceutical composition.

- The preparation of such compositions is well understood in the art. The formulations of this invention may include one or more accessory ingredients including, but not limited to, diluents, buffers, binders, disintegrants, surface active agents,
- 25 thickeners, lubricants, preservatives, including, for example, antioxidants, and the like. Pharmaceutically acceptable includes salts, amides and esters that are well known in the art. Representative acid addition salts include, for example, hydrochloride, hydrobromide, sulfate, bisulfate, acetate, oxalate, valerate, oleate, palmitate, stearate, laurate, borate, benzoate, lactate, phosphate, toluenesulfonate, methanesulfonate, citrate,
- 30 maleate, fumarate, succinate, tartrate, ascorbate, glucoheptonate, lactobionate, lauryl sulfate salts, and the like. Representative alkali or alkaline earth metal salts include, for example, aluminum, calcium, lithium, magnesium, potassium, sodium, or zinc salt, an ammonium salt such as a tertiary amine or quaternary ammonium salt, and an acid salt such as a succinate, tartarate, bitartarate, dihydrochloride, salicylate, hemisuccinate,

citrate, isocitrate, malate, maleate, mesylate, hydrochloride, hydrobromide, phosphate, acetate, carbamate, sulfate, nitrate, formate, lactate, gluconate, glucuronate, pyruvate, oxalacetate, fumarate, propionate, aspartate, glutamate, or benzoate salt, and the like. Pharmaceutically acceptable carriers includes, for example, non-toxic, inert solid, semi-
5 solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Examples of materials that may serve as pharmaceutically acceptable carriers include, but are not limited to, sugars, such as, for example, lactose, glucose and sucrose, starches such as, for example, corn starch and potato starch, cellulose and its derivatives such as, for example, sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate,
10 powdered tragacanth, malt, gelatin, talc, excipients such as, for example, cocoa butter and suppository waxes, oils such as, for example, peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil, glycols, such as, for example, propylene glycol, polyols such as, for example, glycerin, sorbitol, mannitol and polyethylene glycol, esters such as, for example, ethyl oleate and ethyl laurate, agar,
15 buffering agents such as, for example, magnesium hydroxide and aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants such as, for example, sodium lauryl sulfate and magnesium stearate, as well as coloring agents,
20 releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants may also be present in the composition, according to the judgment of the formulator.

For parenteral administration in an aqueous solution, the solution may be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline
25 or glucose. For enteral administration, the inhibitor may be administered in a tablet or capsule, which may be enteric coated, or in a formulation for controlled or sustained release. Many suitable formulations are known, including polymeric or microparticles or nanoparticles encapsulating drug to be released, ointments, gels, or solutions which can be used topically or locally to administer drug, and even patches, which provide
30 controlled release over a prolonged period of time. These can also take the form of implants. Compositions for nasal administration may be formulated for aerosol or inhalation administration. Such compositions may include solutions in saline which may also contain, for example, benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, and/or other solubilizing or dispersing agents such

as those known in the art. Compositions for rectal administration include, for example, suppositories which may contain a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ordinary temperatures, but liquify and/or dissolve in the rectal cavity to release the drug.

5 For human and veterinary administration, compositions of the present invention may meet sterility, pyrogenicity, and general safety and purity standards as required by federal regulatory agencies, such as the FDA. Such compositions are considered suitable for parenteral or enteral administration to a mammal. Such compositions may be pyrogen-free.

10 Certain medical conditions, such as strokes and some cardiovascular blockages, involve both clot formation and inflammation. A simukunin polypeptide as described herein may be used in the treatment for these conditions. For example, administered in the early stages of a medical crisis, the protein may limit damage by antagonizing clot formation and inflammatory responses in the affected tissue.

15 A polypeptide as described herein may be used to inhibit the function of one or more enzymes. For example, a polypeptide as described herein may be used to inhibit the activity of one or more serine proteases. For example, a polypeptide as described herein may be used in methods to inhibit Factor Xa enzymatic activity. A polypeptide as described herein may be used in methods to inhibit elastase and/or cathepsin G. A
20 polypeptide as described herein may be used in methods to inhibit Factor Xa, elastase, and cathepsin G. A polypeptide as described herein may be used in methods to inhibit one or more additional serine proteases, such as for example, one or more of plasmin, Factor IXa, Factor XIa, kallikrein, trypsin, β -tryptase, chymase, α -chymotrypsin, and/or enterokinase.

25 A polypeptide as described herein may be used in methods to inhibit the enzymatic activity of one or more of Factor Xa, elastase, cathepsin G, plasmin, Factor IXa, Factor XIa, kallikrein, trypsin, β -tryptase, chymase, α -chymotrypsin, and/or enterokinase.

 As used herein, the term "inhibit" means prevent, decrease, or reverse. Such
30 contact may be *in vitro*, *ex vivo*, and/or *in vivo*. As used herein *in vitro* is in cell culture, *ex vivo* is a cell that has been removed from the body of a subject, and *in vivo* is within the body of a subject.

 The present invention provides for the use of a polypeptide as described herein as an anticoagulant and/or anti-inflammatory agent. Such an agent may demonstrate

anticoagulant activity, anti-clotting activity, anti-platelet activity, anti-inflammatory activity, and/or immunomodulating activity. As used herein, an anticoagulant agent prevents the coagulation (clotting) of blood or plasma.

A polypeptide as described herein may be used in the treatment of various
5 medical conditions that are treated with the administration of an anticoagulant, to prevent the formation of blood clots and the extension of existing clots. Examples include, but are not limited to, strokes, cardiovascular blockages, deep vein thrombosis, myocardial infarction, pulmonary embolism, venous thromboembolism, congestive heart failure, atrial fibrillation, and genetic or acquired hypercoagulability. A polypeptide as described
10 herein may be administered to a subject to prevent, discourage, minimize, or slow the rate of formation of blood clots. A polypeptide as described herein may be administered to a subject after a blood clot has formed and the anti-coagulant activity of the polypeptide destroying or dissolving the clot.

In some applications, a polypeptide as described herein may be used to coat or
15 treat various laboratory instruments or devices, including, but not limited to, blood transfusion bags, test tubes used for laboratory blood tests, and medical and surgical equipment, to prevent the formation of blood clots on the surface or within.

The present invention includes methods of modulating inflammation. Modulation may include inhibiting or reducing inflammation and/or reducing or
20 inhibiting tissue damage from inflammatory processes. The serine proteases elastase and cathepsin G are implicated in the host defense against invading bacterial and fungal pathogens, participating in the killing and digestion of engulfed pathogens. Both elastase and cathepsin G are secreted by activated neutrophils and play important roles in the inflammatory response. Elastase and cathepsin G regulate the ability of immune cells
25 known as neutrophils to secrete chemicals that attract other immune cells and start the local inflammatory process. Over time, the excessive accumulation of immune cells can lead to tissue and cartilage damage in joints, causing pain and limiting mobility. See for example, the review by Pham, 2006, *Nat Rev Immunol*; 6(7):541-50 ("Neutrophil serine proteases: specific regulators of inflammation"). A polypeptide as described herein may
30 be used in methods to inhibit the enzymatic activity of one or more serine proteases, such as elastase and/or cathepsin G. Such inhibition may reduce the tissue damage associated with elastase and/or cathepsin G release. A polypeptide as described herein may be used in methods to modulate the function of immune cells such as neutrophils, macrophages, monocytes, and/or mast cells. A polypeptide as described herein may be used in the

treatment of conditions in which the pathology is associated with elastase and/or cathepsin G enzymatic activity, such as for example, in the treatment of pulmonary emphysema, α_1 -antitrypsin deficiency, cystic fibrosis, rheumatoid arthritis, keratoconus, asthma, allergies, eczema, and cancer. In some applications, a simukunin polypeptide as
5 described herein may be administered the form of a topical treatment to manage inflammatory conditions of the skin.

A polypeptide as described herein may be used in methods of modulating both coagulation and inflammation, including, but not limited to modulation of any two, three, four, five, six, or more activities described herein.

10 A polypeptide as described herein may be used in methods of inhibiting the enzymatic activity of one or more of plasmin, Factor IXa, Factor XIa, kallikrein, trypsin, β -tryptase, chymase, α -chymotrypsin, and/or enterokinase and methods of inhibiting a pathology associated with the expression of such an enzyme in the tissues of a subject.

With the methods of the present invention, any of a variety of modes of
15 administration may be used. For example, administration may be intravenous, topical, oral, rectal, intranasal, subcutaneous, intraperitoneal, intramuscular, intracardiac, intraosseous, intracerebral, intrathecal, epidural, transdermal, subcutaneous, intracavernous, intravitreal, intra-articular, intrasynovial, transscleral, or intratumor. In some aspects, administration is parenteral, that is, administration into the body is via a
20 route other than the gastro-intestinal tract,

An agent of the present disclosure may be administered at once, or may be divided into a number of multiple doses to be administered at intervals of time. For example, agents of the invention may be administered repeatedly, e.g., at least 2, 3, 4, 5, 6, 7, 8, or more times, or may be administered by continuous infusion. It is understood
25 that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted
30 over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that any concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions and methods.

By a "therapeutically effective amount" is meant a sufficient amount of the compound to treat the subject at a reasonable benefit/risk ratio applicable to obtain a desired therapeutic response. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including, for example, the disorder being treated and the severity of the disorder, activity of the specific compound employed, the specific composition employed, the age, body weight, general health, sex and diet of the patient, the time of administration, route of administration, and rate of excretion of the specific compound employed, the duration of the treatment, drugs used in combination or coincidentally with the specific compound employed, and like factors well known in the medical arts.

In some therapeutic embodiments, an "effective amount" of an agent is an amount that results in a reduction of at least one pathological parameter. Thus, for example, in some aspects of the present disclosure, an effective amount is an amount that is effective to achieve a reduction of at least about 10%, at least about 15%, at least about 20%, or at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%, compared to the expected reduction in the parameter in an individual not treated with the agent.

In accordance with the present invention, a simukunin polypeptide may be administered in combination with the administration of one or more previously known treatment modalities. As used herein, the term "additional therapeutic agent" represents one or more agents previously known to be effective in the treatment of conditions for which the administration of an anti-coagulant or an anti-inflammatory agent is appropriate. In some embodiments of the present invention, the administration of a simukunin polypeptide in combination with additional therapeutic agents may demonstrate therapeutic synergy. As used herein, a combination may demonstrate therapeutic synergy if it is therapeutically superior to one or other of the constituents used at its optimum dose. In some embodiments, a combination demonstrates therapeutic synergy if the efficacy of a combination is characterized as more than additive actions of each constituent. The administration of a simukunin polypeptide may take place before, during, and/or after the administration of the other mode of therapy.

The present invention includes methods of administering one or more simukunin polypeptides in combination with the administration of one or more previously known treatment modalities. The present invention also includes compositions of one or more simukunin polypeptides and one or more previously known treatment modalities.

5 As used herein, the term "subject" includes, but is not limited to, humans and non-human vertebrates. In preferred embodiments, a subject is a mammal, particularly a human. A subject may be an individual. A subject may be an "individual," "patient," or "host." Non-human vertebrates include livestock animals, companion animals, and laboratory animals. Non-human subjects also include non-human primates as well as
10 rodents, such as, but not limited to, a rat or a mouse. Non-human subjects also include, without limitation, chickens, horses, cows, pigs, goats, dogs, cats, guinea pigs, hamsters, ferrets, mink, and rabbits.

 As used herein "in vitro" is in cell culture and "in vivo" is within the body of a subject. As used herein, "isolated" refers to material that has been either removed from
15 its natural environment (e.g., the natural environment if it is naturally occurring), produced using recombinant techniques, or chemically or enzymatically synthesized, and thus is altered "by the hand of man" from its natural state.

 The present invention also includes methods of making and using the polypeptides, polynucleotides, and compositions described herein.

20 The term "and/or" means one or all of the listed elements or a combination of any two, three, four, five, or more of the listed elements.

 The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances.

25 Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

 The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

30 Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

 Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

Unless otherwise indicated, all numbers expressing quantities of components,
5 molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the
10 doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible.
15 All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

The description exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves
20 only as a representative group and should not be interpreted as an exclusive list.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be
25 interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

- 5 Simukunin from the Salivary Glands of the Black Fly *Simulium vittatum*
 Inhibits Enzymes That Regulate Clotting and Inflammatory Responses

Black flies (Diptera: Simuliidae) feed on blood, and are important vectors of
Onchocerca volvulus, the etiolytic agent of River Blindness. Blood feeding depends on
10 pharmacological properties of saliva, including anticoagulation, but the molecules
responsible for this activity have not been well characterized. Two Kunitz family
proteins, SV-66 and SV-170, were identified in the sialome of the black fly *Simulium*
vittatum. As Kunitz proteins are inhibitors of serine proteases, SV-66 and SV-170 are
candidate proteins responsible for the anticoagulant activity of black fly saliva. This
15 example demonstrates that only recombinant (r) SV-66, but not rSV-170, inhibited
plasma coagulation. Mutational analysis suggested that SV-66 is a canonical BPTI-like
inhibitor. Functional assays indicated that rSV66 reduced the activity of ten serine
proteases, including several involved in mammalian coagulation. rSV-66 most strongly
inhibited the activity of Factor Xa, elastase, and cathepsin G, exhibited lesser inhibitory
20 activity against Factor IXa, Factor XIa, and plasmin, and exhibited no activity against
Factor XIIa and thrombin. Surface plasmon resonance studies indicated that rSV-66
bound with highest affinity to elastase ($K_D=0.4$ nM) and to the active site of FXa
($K_D=3.07$ nM). This novel protein has been named "Simukunin." This Example
demonstrates that Simukunin preferentially inhibits Factor Xa. The inhibition of elastase
25 and cathepsin G indicate that this protein likely modulates inflammation, which could
potentially affect pathogen transmission.

Both eukaryotes and prokaryotes produce Kunitz family protease inhibitors,
indicating an ancient origin for Kunitz family encoding genes (Ascenzi et al., 2003, *Curr*
Protein Pept Sci; 4:231–251; and Liener, 1986, *J Nutr*; 116:920–923). The most
30 conserved function of Kunitz family proteins is the reversible competitive inhibition of
serine proteases (Ascenzi et al., 2003, *Curr Protein Pept Sci*; 4:231–251). A single
Kunitz domain is small (about 60 amino acids) and forms a compact globular fold
typically containing three disulfide bonds. Based on the structure of bovine pancreatic
trypsin inhibitor (BPTI), a typical Kunitz domain contains cysteine residues at positions

5, 14, 30, 38, 51 and 55 in the mature peptide, which form three disulfide bonds C5–C55, C14–C38 and C30–C51 (Ascenzi et al., 2003, *Curr Protein Pept Sci*; 4:231–251). The Kunitz domain may exist singly, or as multiple domains within a single polypeptide (Corral-Rodriguez et al., 2009, *Insect Biochem Mol Biol*; 39:579–595). Many Kunitz domains act as protease inhibitors through their scissile bond at positions 15 (P1) and 16 (P1'). The P1 residue is often a basic amino acid (K or R) while the P1' position is an A or G, which together interact with the active site of one or more proteases (Ascenzi et al., 2003, *Curr Protein Pept Sci*; 4:231–251). Alternative modes of action have also been characterized. For example, snake venoms contain Kunitz family proteins named dendrotoxins that exhibit weak anti-protease activity but strongly block neuronal K⁺ channels (Harvey, 2001, *Toxicon*; 39:15–26).

Blood coagulation in mammals is a physiological response that is activated by a complex enzymatic cascade, consisting primarily of serine proteases, and which terminates with formation of a fibrin clot. Negative regulators of coagulation are primarily protease inhibitors, which include one Kunitz family protein named tissue factor pathway inhibitor (TFPI) (Crawley and Lane, 2008, *Arterioscler Thromb Vasc Biol*; 28:233–242) that inhibits formation of FXa by binding to the FVIIa-Tissue factor-FXa complex (Corral-Rodriguez et al., 2009, *Insect Biochem Mol Biol*; 39:579–595; and Crawley and Lane, 2008, *Arterioscler Thromb Vasc Biol*; 28:233–242). Blood-feeding arthropods also produce anti-hemostatic factors in their saliva, which facilitate blood feeding by interfering with host hemostatic responses (Ribeiro and Francischetti, 2003, *Annu Rev Entomol*; 48:73–88). A variety of anti-coagulation factors have been identified from arthropods including several Kunitz family proteins in the saliva of ticks (Corral-Rodriguez et al., 2009, *Insect Biochem Mol Biol*; 39:579–595; and Francischetti et al., 2009, *Front Biosci*; 14:2051–2088). For example, ixolaris from the deer tick, *Ixodes scapularis*, contains two kunitz domains. The N-terminal Kunitz displays a glutamic acid residue in the P1 position, while the C-terminal Kunitz atypically has only four cysteines (Francischetti et al., 2002, *Blood*; 99:3602–3612). Ixolaris binds to the heparin-binding exosite of coagulation Factor X (FX) and FXa through the C-terminal domain, and this complex forms a tight-binding inhibitor of the FVIIa/Tissue Factor complex. The saliva of *I. scapularis* also contains penthalaris, which has five Kunitz domains and inhibits the tissue factor pathway in a manner similar to ixolaris (Francischetti et al., 2004, *Thromb Haemost*; 91:886–898). Other Kunitz family proteins from tick saliva exhibit functions that range from anti-thrombin and anti-FXa activity to

anti-kallikrein and anti-platelet aggregation (Corral-Rodriguez et al., 2009, *Insect Biochem Mol Biol*; 39:579–595); and Maritz-Olivier et al., 2007, *Trends Parasitol*; 23:397–407.

Black flies (Diptera: Simuliidae) like *Simulium vittatum* are small, stout-bodied insects. Females of *S. vittatum* and most other species must feed on blood from a vertebrate host to produce multiple clutches of eggs. Black flies are not only a nuisance for humans and livestock but vector several pathogens including *Onchocerca volvulus* that causes onchocerciasis, (river blindness) in humans, and vesicular stomatitis virus that causes disease in livestock. The bites of *S. vittatum* induce a pronounced and persistent erythema (Cupp et al., 1994, *Am J Trop Med Hyg*; 50:241–246) due to the presence of a salivary protein named *S. vittatum* erythema protein (SVEP) (Cupp et al., 1998, *J Exp Biol*; 201:1553–1561). *S. vittatum* saliva also contains at least three anti-coagulation factors, which exhibit activity against thrombin, FXa, or FV (Jacobs et al., 1990, *Thromb Haemost*; 64:235–238; Abebe et al., 1994, *J Med Entomol*; 31:908–911; Abebe et al., 1995, *J Insect Physiol*; 41:1001–1006; and Abebe et al., 1996, *J Med Entomol*; 33:173–176). The identity of these anti-hemostatic factors, however, remains unknown.

A recent publication on the combined transcriptome and proteome (collectively called the “sialome”) of *S. vittatum* salivary glands detected many transcripts and corresponding tryptic peptide fragments including two Kunitz family proteins, named SV-66 and SV-170 (Andersen et al., 2009, *J Proteome Res*; 8(3):1474–1488). In this study, SV-66 and SV-170 were expressed and assessed their anti-coagulant activity. This example demonstrates that SV-66 is an anti-coagulant with anti-FXa activity that also inhibits several other serine proteases.

25

Results

SV-66 and SV-170 encode conserved Kunitz proteins. SV-66 and SV-170 consist of 309 (SEQ ID NO:1) and 237 (SEQ ID NO:4) nucleotides respectively that encode predicted proteins of 102 (SEQ ID NO:2) and 78 (SEQ ID NO:5) amino acids. Fig. 1A shows nucleotide and translated polypeptide sequences of SV-66 and SV-170. Start and stop codons are in white with black shading. Numbers below the amino acid residues are designated based on the putative mature protein. Signal sequences predicted by SignalP are underlined. SV-66 encodes a 102 amino-acid polypeptide (SEQ ID NO:2) (Simukunin), which includes a 19 amino-acid N-terminal signal sequence.

Mature Simukunin is predicted to consist of 83 amino-acid residues (SEQ ID NO:3), with a theoretical mass of 9627.22 Da and pI of 9.93. SV-66 also contains a putative O-glycosylation site at position 81 (Ser). SV-170 encodes a 78 amino-acid polypeptide (SEQ ID NO:5), which includes an N-terminal 22 amino-acid signal sequence. Mature SV-170 is predicted to consist of 56 amino-acid residues (SEQ ID NO:6), and theoretical mass and pI are 6526.66 Da and 8.87, respectively.

Fig. 1B shows alignment of representative Kunitz domain sequences with SV-66 and SV-170. Each Kunitz domain was separated from the original sequences for alignment (numbers denote amino-acid positions in the original mature peptides). All reference sequences were retrieved from GenBank. Accession numbers are: TFPI (human: 3 Kunitz domains), P10646; BPTI (*Bos taurus*: 1 Kunitz domain), AAI49369; Amblin (*Amblyomma hebraeum*: 2 Kunitz domains), AAR97367; Boophilin (*Rhipicephalus microplus*: 2 Kunitz domains), CAC82583. Strictly conserved cysteine residues are indicated with white arrows, and predicted conserved disulfide bonds are shown in solid lines. The reactive site loop (RSL) P4-P2' residues, conserved in canonical binding inhibitors, are indicated by asterisks. The P1 residue is indicated with a black arrow. Highly conserved P1-P1' (Arg/Lys-Ala/Gly) residues are shaded and boxed. Other identical residues across the domain are shaded and indicated with a plus (+) and conserved or semi-conserved residues are shaded with grey.

SignalP identified signal sequences for SV-66 and SV-170 of 19 and 22 amino acids respectively. Residue numbers were assigned based on the predicted mature proteins and indicated signal sequence residues as negative numbers (Fig. 1A).

Alignment with selected other Kunitz-domain containing proteins indicated that SV-66 and SV-170 possess six conserved cysteine residues and other conserved residues characteristic of Kunitz family members (Fig. 1B). SV-66 exhibited a basic arginine residue at position 15, which was the predicted P1 residue. This finding suggested that SV-66 may be an active protease inhibitor. In contrast, SV-170 had a threonine at the predicted P1 position, which suggested a lack of a canonical inhibitory activity against trypsin-like serine proteases, but which was similar to the C-terminal Kunitz domain of boophilin (Macedo-Ribeiro et al., 2008, *PLoS One*; 3:e1624).

SV-66 is expressed in female salivary glands. RT-PCR assays were performed to qualitatively assess SV-66 expression in adult *S. vittatum*. Amplicons of expected size were detected in cDNA samples prepared from the female body (head and thorax) but not in female carcasses (female body minus salivary glands and head) or adult males

(Fig. 2A). Time course studies indicated that SV-66 was expressed by adult females prior to blood feeding as well as at all time points sampled after blood feeding (Fig. 2B). Taken together, these results suggested that SV-66 was constitutively expressed in the salivary glands of adult females but was not expressed in males.

5 rSV-66 inhibits plasma clotting, while rSV-170 does not. SV-66 and SV-170 cDNAs were cloned into the pET-30 Ek/LIC vector (Novagen) and expressed each in *Escherichia coli* as recombinant (r) proteins with C-terminal double His tags. Two-step purification using Ni^{2+} resin and RP-HPLC yielded fractions highly enriched for rSV-66 or rSV-170 (Fig. 7).

10 The effect of rSV-66 and rSV-170 on the time needed for fibrin deposition from Ca^{2+} -stimulated normal human plasma (clotting time) was determined by measuring the increase in OD at 650 nm. rSV-66 prolonged coagulation time in a dose-dependent manner, beginning at concentrations as low as 12.5 nM, whereas rSV-170 exhibited no anti-coagulation activity up to a concentration of 400 nM (Fig. 3). Since rSV-170 did
15 not inhibit coagulation, the remainder of this example focused on rSV-66. Given its anticoagulant activity, rSV-66 was named “Simukunin”, after a contraction of *Simulium kunitz* inhibitor. Recombinant Simukunin is also referred to herein as rSimukunin.

Residues in the reactive site loop are important for rSimukunin anti-coagulation activity. Since canonical Kunitz inhibitors interact with target proteases through their
20 reactive site loop (RSL), whether alanine replacement of residues in or adjacent to the RSL affected Simukunin activity was addressed by producing three alanine replacement mutants, SV66V13A, SV66C14A, and SV66R15A. A fourth mutant, SV66K19A, was also produced as a control protein with an alteration outside of the RSL domain that should not affect anticoagulation activity. Following purification by Ni^{2+} affinity resin
25 and reversed-phase HPLC, it was observed that each recombinant protein ran as a doublet on SDS-PAGE gels. Tryptic digestion and mass spectrometry analysis, however, indicated that both bands consisted solely of Simukunin that were identical to full length native Simukunin. This analysis also indicated that the low molecular-weight band corresponded to loss of one of the epitope tags, possibly due to cleavage by an *E.*
30 *coli* protease during the lysis step. Thus, it was concluded that the presence of this lower band should not affect the RSL or activity. However, its presence necessitated the use of μg rather than molar concentrations in coagulation assays since precise calculation of molar concentrations was impossible. Therefore anticoagulation assays were conducted by adding 0.5 or 1.0 μg of WT or mutant rSimukunin to plasma and the rapidity of

clotting compared to plasma without rSimukunin by pairwise t-test. The presence of WT rSimukunin significantly increased clotting time as did addition of SV66K19A. In contrast, the addition of SV66C14A and SV66R15A to plasma had no significant effect on clotting activity. SV66V13A delayed coagulation, but this effect was reduced compared to the delay produced by WT rSimukunin (Fig. 4).

rSimukunin inhibits activity of multiple proteases including coagulation factors. Preliminary assays indicated that rSimukunin also inhibited the enzymatic activity of enterokinase, which suggested rSimukunin could inhibit other serine proteases besides those with roles in host coagulation. The inhibitory activity of rSimukunin against 15 different serine proteases, including the coagulation factors FXa, FXIa, FXIIa, and thrombin, was characterized. Results indicated that rSimukunin significantly reduced the activity of ten of these proteases (Fig. 5). In the coagulation cascade, rSimukunin significantly inhibited FXa and FXIa, but not thrombin or FXIIa. Other enzymes strongly inhibited by rSimukunin were elastase, plasmin, kallikrein, trypsin, β -tryptase, and cathepsin G (Fig. 5).

The IC_{50} of rSimukunin to the most strongly inhibited proteases was determined in order to assess the relative affinity of this inhibitor for each target (Table 1). To obtain linear reaction rates within the time frame of the experiment, each target enzyme was assayed at different molar concentrations. The molar ratio of rSimukunin to the enzyme at the IC_{50} (Table 1) was also calculated. Collectively, the results indicated that rSimukunin most strongly inhibited elastase with an IC_{50} of 4.9 nM and a ratio of inhibitor to enzyme of 27.22. Factor Xa was also strongly inhibited, with an IC_{50} of 5.2 nM and a molar ratio of 52.00, as was cathepsin G with a molar ratio of 32.45. The other enzymes tested, including FXIa, required molar excesses of hundreds- to thousands-fold for 50% inhibition of proteolytic activity.

Table 1. IC₅₀ values for rSimukunin against various serine proteases.

Enzyme	Concentration (nM)	IC ₅₀ (mean ± SEM (nM)	Ratio
Elastase	0.18	4.9±0.6	27.22
Cathepsin G	6.7	217.4±5.7	32.45
Factor Xa	0.1	5.2±0.3	52.00
Plasmin	0.2	32.2±5.2	161.00
Factor XIa	0.06	56.7±13.1	945.00
Kallikrein	0.08	91.8±6.3	1147.50
Trypsin	0.1	379.3±30.2	3793.00
β-Tryptase	0.01	66.8±14.6	6680.00

5 Titrated concentrations of rSimukunin were tested with constant concentrations of enzymes (in Concentration column) to determine the concentrations of rSimukunin that gave a 50% inhibition of the enzyme activity. Ratios of IC₅₀ to enzyme concentration are also shown as different concentrations of enzymes were necessary to obtain linear reaction rates. Titration curves are shown in Fig. 8.

10 As elastase, FXa, and cathepsin G were the most strongly inhibited, based on the ratio of inhibitor to enzyme (Table 1), the K_m for these three enzymes was determined, and then used the approach of Cheng and Prusoff (Cheng and Prusoff, 1973, *Biochem Pharmacol*; 22:3099–3108) to calculate the K_i from the IC₅₀, with a final substrate concentration of 250 μM. The K_m for the elastase substrate was 38.5±3.3 μM, and the estimated K_i was~0.65 nM. For FXa the substrate K_m was 161.6±17.4 μM, and the
15 estimated K_i was~2.1 nM. For Cathepsin G the K_m was 188±20 μM, and the K_i was 100 nM. A general chymotrypsin substrate was used to assess Cathepsin G activity; as this substrate is not optimized for Cathepsin G it is likely that our measurement of the K_i underestimates the effect of rSimukunin. Further studies of the effect of rSimukunin on Cathepsin G will be performed.

20 rSimukunin displays high affinity binding to FXa and elastase. The preceding results provided measures of affinity for elastase, FXa, and cathepsin G by rSimukunin,

but were insufficient for calculation of binding and dissociation kinetics. Therefore, Surface Plasmon Resonance (SPR) studies were conducted using rSimukunin immobilized on a sensor chip and used selected target enzymes as the analyte. These studies showed that rSimukunin bound FXa from several mammals (human, mouse and bovine) with high affinity (Fig. 6A). rSimukunin also bound DES-Gla-FXa, an FXa derivative lacking the Gla domain necessary for docking onto a negatively charged membrane surface. In contrast, rSimukunin did not bind DEGR-FXa, a derivative blocked at the active site, or FX, which is the zymogen precursor of FXa (Fig. 6A). rSimukunin also exhibited very weak binding responses to FIXa and FXIa, and no binding responses to FVIIa, FXIIIa or thrombin (Fig. 6A). Kinetic analysis determined that FXa bound rSimukunin with a K_D of 3.07 nM (Fig. 6B and Table 2), but assays with elastase revealed an even stronger affinity for rSimukunin with a $K_D \approx 0.4$ nM (Fig. 6C and Table 2). These K_D values are consistent with the K_i values we calculated based on the K_m and IC_{50} . The low K_D values for both FXa and elastase result from a fast association rate ($k_{a1} = 4.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for elastase) and a very slow off rate ($k_{d1} = 0.017 \text{ s}^{-1}$) indicative of rSimukunin functioning as a tight-binding inhibitor.

Table 2. Kinetics of FXa and Elastase interactions with rSimukunin.

Enzyme	$k_{a1}(\text{M}^{-1} \text{ s}^{-1})$	$k_{d1}(\text{s}^{-1})$	K_D (nM)
FXa	9.87×10^4	3.032×10^{-4}	3.071
Elastase	4.302×10^7	0.01727	0.40

Responses were obtained by injecting FXa or elastase over immobilized rSimukunin for 180 seconds at a flow rate of 30 $\mu\text{l}/\text{minute}$. Data were derived from K_{a1} and K_{d1} and fitted using the Langmuir (1:1 binding) equation. Kinetic values obtained from the sensorgrams presented in Figures 6B and 6C for FXa and elastase respectively.

Discussion

Salivary gland extracts from *S. vittatum* have long been known to have potent anticoagulant activity, but the identity of the molecules involved had heretofore remained uncharacterized. This Example demonstrates that of the two Kunitz family proteins expressed in the salivary glands of female *S. vittatum* (Andersen et al., 2009, *J*

Proteome Res; 8(3):1474-1488), rSV-66, which was named Simukunin, dose-dependently prolongs coagulation times of human plasma while rSV-170 lacks anticoagulant activity. Simukunin is constitutively expressed specifically in adult female salivary glands, which is consistent with a role in blood feeding.

5 SPR results strongly suggest that the anticoagulant activity of Simukunin is primarily due to high affinity binding to FXa. In contrast, rSimukunin does not bind to the zymogenic form of FX, which suggests this inhibitor only interacts with the activated enzyme. Results further show that rSimukunin exhibits binding to des-Gla-FXa but does not interact with DEGR-FXa, which is blocked specifically at the active site. These data
10 together with outcomes of the mutagenesis studies strongly suggest that rSimukunin interacts directly with the active site of FXa. That FXa is a primary target of rSimukunin is also supported by results showing that rSimukunin does not interact with FVIIa, FXIIa, FXIIIa, or thrombin, and only weakly interacts with FIXa and FXIa.

 The loss of anticoagulation activity by SV66R15A indicates that rSimukunin
15 interacts directly with the active site of FXa, and that Arg15 functions as the P1 residue. The reduced activity of SV66V13A could likewise reflect a less stable interaction with the catalytic active site of FXa, while the lack of activity of SV66C14A likely reflects an alteration in structure due to elimination of the Cys14–Cys38 disulfide bond. Taken together, the data indicate that Simukunin acts similarly to BPTI, whose basic P1
20 residue, Lys15, is critical for the inhibition of trypsin and chymotrypsin (Tschesche et al., 1987, *Biochim Biophys Acta*; 913:97–101) through stable docking into the S1 specificity subsite of these enzymes and formation of polar interactions with a negatively charged Asp189 side chain (Ascenzi et al., 2003, *Curr Protein Pept Sci*; 4:231–251).

 The anti-clotting activity of Simukunin may account for the anti-FXa activity of
25 black fly SGE described previously (Jacobs et al., 1990, *Thromb Haemost*; 64:235–238). However, the estimated size of a partially characterized FXa inhibitor was 18000 Da (Jacobs et al., 1990, *Thromb Haemost*; 64:235–238), which is much larger than the predicted size of Simukunin (9627.22 Da). Although Simukunin has a putative O-glycosylation site in the C-terminal region, the 1D SDS-PAGE gel band from which
30 Simukunin tryptic fragments were recovered migrated between the 14.1 and 6 kDa markers in Andersen et al. (Andersen et al., 2009, *J Proteome Res*; 8(3):1474-1488), which indicates that glycosylation does not significantly add to the native size of Simukunin. The relationship between Simukunin and the previously described anti-FXa

activity thus remains unclear but it is possible that other proteins contribute to the anti-FXa activity in black fly saliva.

Although rSimukunin inhibited kallikrein, the activity was modest, and further, it did not inhibit bradykinin production from kaolin-activated human plasma, indicating
5 that the FXII/prekallidrein/kallikrein pathway is not a target of this inhibitor.

Coagulation and inflammation are linked through a variety of cross-talk mechanisms (Levi and van der Poll, 2010, *Crit Care Med*; 38:S26–34). For example, proteinase-activated receptor 4, which is highly expressed on platelets, is activated following cleavage by FXa or thrombin and subsequently mediates inflammatory
10 responses (Levi and van der Poll, 2010, *Crit Care Med*; 38:S26–34; and McDougall et al., 2009, *Arthritis Rheum*; 60:728–737). As Simukunin inhibits FXa, which indirectly reduces subsequent thrombin activation, it is likely that Simukunin may also influence inflammatory responses at the bite site, though we have not yet examined this experimentally.

The comparative data of this example clearly show that rSimukunin binds to
15 and/or inhibits several other enzymes besides FXa including major components of neutrophil azurophil granules such as elastase, and mast cell proteases including cathepsin G, tryptase, and chymase. Indeed, the strongest binding affinity we detected was between rSimukunin and elastase. This finding together with inhibition of cathepsin
20 G is notable because both enzymes function in killing phagocytized microbes by neutrophils. Extracellular release of cathepsin G mediates platelet aggregation, which is known to provide a surface for assembly of the prothrombinase complex (Levi and van der Poll, 2010, *Crit Care Med*; 38:S26–34). Thus, inhibition of cathepsin G could indirectly antagonize coagulation. Cathepsin G, tryptase, and elastase also regulate the
25 function of several chemokines, cytokines, cell surface receptors and adhesion molecules (Pham, 2006, *Nat Rev Immunol*; 6:541–550; Pham, 2008, *Int J Biochem Cell Biol*; 40:1317–1333; Trivedi and Caughey, 2010, *Am J Respir Cell Mol Biol*; 42:257–267; and Zhao et al., 2005, *J Immunol*; 175:2635–2642), which leads to the possibility that Simukunin may affect inflammation or other responses in proximity to sites of black fly
30 feeding. Further experimental work will be necessary to examine the effect of Simukunin on macrophage, mast cell, and neutrophil function.

The inhibition of elastase by rSimukunin is somewhat surprising in light of prior studies showing that Kunitz domain inhibitors of elastase like Bikunin (formerly called acid stable trypsin inhibitor or ASTI, or urinary trypsin inhibitor) have an aliphatic

amino acid, such as methionine, leucine, or valine in the P1 position (Fries and Blom, 2000, *Int J Biochem Cell Biol*; 32:125–137; Albrecht et al., 1983, *Hoppe Seylers Z Physiol Chem*; 364:1697–1702; Albrecht et al., 1983, *Hoppe Seylers Z Physiol Chem*; 364:1703–1708; and Tschesche and Wenzel, 1983, *Adv Exp Med Biol*; 156:329–337).

- 5 Thus, it is likely that residues outside the RSL either orient the Simukunin/elastase interaction to permit tight binding despite the unfavorable P1 residue, or that binding and inhibition involves interactions with a domain distinct from the RSL. Further structure-function experiments will address how Simukunin interacts with this enzymatic target.

- Inhibition of multiple enzymes by rSimukunin, is also not unusual for Kunitz
 10 family inhibitors from blood-feeding arthropods and other organisms. For example, the archetypical Kunitz protein BPTI inhibits trypsin with a K_i of 0.06 pM, but it also inhibits chymotrypsin (K_i =9 nM), plasmin, and kallikrein (Vincent and Lazdunski, 1973, *Eur J Biochem*; 38:365–372). Boophilin, a tick salivary protein with two Kunitz domains, likewise exhibits significant inhibitory activity toward thrombin, trypsin,
 15 plasmin, and plasma kallikrein (Macedo-Ribeiro et al., 2008, *PLoS One*; 3:e1624), while the serpin IRS-2 has inhibitory activity against cathepsin G, chymase, and α -chymotrypsin (Chmelar et al., 2011, *Blood*; 117:736–744). On the other hand, Kunitz family thrombin inhibitors from soft ticks appear to specifically inhibit thrombin at pM concentrations (eg. ornithodorin, K_i =1 pM (van de Loch et al., 1996, *EMBO J*; 15:6011–
 20 6017); savignin, K_i =4.89 pM) (Nienaber et al., 1999, *Exp Parasitol*; 93:82–91). Lastly, it is noted that the binding affinities of Simukunin for FXa (K_D =3.071 nM) and elastase (K_D =0.4 nM) are broadly consistent with the binding affinities determined for other Kunitz inhibitors from blood feeding species. As examples, the tick salivary inhibitor ixolaris binds to FX and FXa with K_D s of 0.67 and 0.25 nM respectively (Monteiro et al.,
 25 2008, *Protein Sci*; 17:146–153), while boophilin binds thrombin with a K_i of 1.8 nM (Macedo-Ribeiro et al., 2008, *PLoS One*; 3:e1624), as does amblin, with a K_i of 20 nM (Lai et al., 2004, *Gene*; 342:243–249).

- Future functional studies will more fully characterize the role of Simukunin in blood feeding. Nonetheless, the results of this example collectively suggest Simukunin
 30 facilitates blood-feeding by disrupting coagulation and possibly by interfering with host inflammatory responses. Several examples from mosquitoes and sandflies also implicate saliva in potentiating pathogen transmission by modulating immune responses in the host skin (reviewed in Schneider and Higgs, 2008, *Trans R Soc Trop Med Hyg*; 102:400–408; Titus and Ribeiro, 1988, *Science*; 239:1306–1308; and Oliveira et al., 2008, *PLoS Negl*

Trop Dis; 2:e226). However, future studies will be required to determine whether Simukunin affects pathogen transmission by black flies. Lastly, recent studies of the sialome from another black fly, *Simulium nigritanum*, identified four Kunitz protein-encoding transcripts, which are divided into two subfamilies based on the presence or
 5 absence of a C-terminal extension of basic residues (Ribeiro et al., 2010, *Am J Trop Med Hyg*; 82:1060–1075). Therefore, *S. nigritanum* salivary glands may also produce multiple Kunitz anti-coagulation factors with potential roles in regulating coagulation and inflammation.

10 Materials and Methods

S. vittatum culture. *S. vittatum* was reared at the Department of Entomology, the University of Georgia, according to previously described conditions (Gray EW, Noblet R. Laboratory Rearing of Black Flies. In: Maramorosch K, Mahmood F, editors. Maintenance of Human, Animal and Plant Pathogen Vectors. New Delhi and Calcutta:
 15 Oxford & IBH Publishing Co. PVT. LTD; 1999; and Bernardo and Cupp, 1986, *J Med Entomol*; 23:666–679). Because *S. vittatum* is facultatively autogenous, no vertebrate animals were used in the study for rearing or maintenance of insects.

Salivary gland dissection, cDNA synthesis, and expression analyses. Adult female flies (collected 2–3 days post eclosion) were chilled on ice, and their salivary
 20 glands were collected by dissection under a stereomicroscope in sterile HEPES saline (HS) (10 mM HEPES pH 7.0, 150 mM NaCl). Fifty salivary gland pairs were collected in 20 µL HS, mixed with 20 µL RNeasy lysis buffer (Qiagen, Foster City, CA), and stored at –70°C until use. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Genomic DNA was removed using RNase-free DNase (Turbo DNase, Ambion, Foster
 25 City, CA). First strand cDNA was synthesized using 100 ng of total RNA, SuperScript III (Invitrogen, Carlsbad, CA) and an oligo d(T) primer (5'-(T)₂₅-VN-3') at 50°C for 60 min, followed by enzyme inactivation at 70°C for 15 min. Resultant cDNA was then stored at -20°C until use.

Total RNA was isolated and reverse transcribed as described above from female
 30 flies without salivary glands and head (termed “carcass”), intact female flies, and intact males flies. Since *S. vittatum* is autogenous for the first gonotrophic cycle, reverse transcribed RNA from female bodies without abdomens was also used (in order to avoid collecting vertebrate cells in the blood meal) collected after the first oviposition, and at 3, 6, 12, 24, and 48 hrs post blood-feeding (pbf). Blood-fed flies were kept at 27°C with

constant access to sugar water (10% sucrose). RT-PCRs were then run using an Eppendorf thermocycler in 25 µl reaction volumes containing 1 µl of cDNA and 2.5 µM of SV-66 UA and SV-66 DA primers. Primers are shown in Table 3. Cycling conditions were as follows: initial 2 min denaturation at 94°C, followed by 30 cycles at 30 sec at 5 94°C, 30 sec at 57°C 30 sec at 72°C, and a final 5 min extension at 72°C. Actin from *S. vittatum* (GenBank accession number AY083375.1) amplified using SVactinUA and SVactinDA primers was used as an endogenous control.

The PCR primers used in this example are shown in Table 3. For primers used for cloning in pET-30, direction-specific LIC sites are shown in italics. For primers used 10 for single His-tag constructs, bold letters indicate the stop codon (TAA) and the read-through Ala (GCA in reverse-complement orientation).

Table 3. Primers

Primer	Sequence (5' → 3')	SEQ ID NO
SvactinUA	TGTGTTACGTTGCCTTGGACTTTG	17
SvactinDA	TGATGGAGTTGTAGACGGTTTCGTG	18
SV66UA	TGAATTGGATCGAAATGAATATACTTCCA	19
SV66DA	TTAGTTTGAATGTCCTTTTTAGTCCAACGA	20
SV170UA	CACCTGAGAGAATCTTCTGCGTCAAA	21
SV170DA	CGGTCAATACATTTTTATCCTCTTGTGCT	22
SV66UB	GACGACGACAAGATGCAAGAGAACGTTTGCAATCTTC	23
SV66DB	GAGGAGAAGCCCGGTTAGTCCAACGAAATAATTGGTATC	24
SV66DC	GAGGAGAAGCCCGGTGCGTCCAACGAAATAATTGGTATCG	25
SV170UB	GACGACGACAAGATGAAGTCAGCTGACATCTGCAGA	26
SV170DB	GAGGAGAAGCCCGGTTACATACACTTGGCTTTACATTCT	27
SV170DC	GAGGAGAAGCCCGGTGCCATACACTTGGCTTTACATTCTTG	28
V13A-UA	CCGGTGGACGAAGGTGCATGTAGAGCGTTATTC	29
V13A-DA	GAATAACGCTCTACATGCACCTTCGTCCACCGG	30
C14A-UA	AATCTTCCGGTGGACGAAGGTGTAGCTAGAGCGTTATTCA	31
C14A-DA	TGAATAACGCTCTAGCTACACCTTCGTCCACCGGAAGATT	32
R15A-UA	CTTCCGGTGGACGAAGGTGTATGTGCAGCGTTATTCAAGC	33
R15A-DA	GCTTGAATAACGCTGCACATACACCTTCGTCCACCGGAAG	34
K19A-UA	GGTGTATGTAGAGCGTTATTCGCGCGTTTTTACTACGAACCC	35
K19A-DA	GGGTTCGTAGTAAAAACGCGCGAATAACGCTCTACATACACC	36

Cloning and sequence analysis of Kunitz family proteins. Full-length cDNAs for SV-66 and SV-170 (GenBank accession numbers EU930300 and EU930227, respectively) (Andersen et al., 2009, *J Proteome Res*; 8(3):1474-1488) were amplified from salivary gland cDNA using Platinum HIFI Taq DNA polymerase and gene specific primers (SV66 UA, SV66DA, SV170UA, SV170DA) in 25 µl reactions. PCR conditions were: initial 2 min denaturation at 94°C, followed by 35 cycles (30 sec at 94°C, 30 sec at 57°C 30 sec at 68°C), and a final 5 min extension at 68°C. The resulting amplicons were then TA cloned into pCR4-TOPO (Invitrogen), and transformed into *E. coli* TOP10 competent cells (Invitrogen). After overnight culture, the plasmids SV66/TOPO and SV170/TOPO were purified using the Wizard Plus miniprep DNA purification system (Promega, Madison, WI) and quantified using a NanoDrop

spectrophotometer (Thermo Scientific, Wilmington, DE). Inserts were sequenced (Macrogen, Rockville, MD) to confirm fidelity with the reference sequences. SignalP (Emanuelsson et al., 2007, *Nat Protoc*; 2:953–971) was used to predict signal peptide sequences, and NetOGlyc (Julenius et al., 2005, *Glycobiology*; 15:153–164) was used to
 5 predict potential glycosylation sites. Alignments were performed using Clustal W2 (Larkin et al., 2007, *Bioinformatics*; 23:2947–2948) against other Kunitz domains from the following Kunitz inhibitors: bovine pancreatic trypsin inhibitor (BPTI) (Genbank accession number AAI49369) from *Bos taurus* (a single Kunitz domain inhibitor); human tissue factor pathway inhibitor (TFPI) (P10646) (consisting of 3 Kunitz domains);
 10 a thrombin inhibitor, amblin, (AAR97367) from the tick *Amblyomma hebraeum* (2 Kunitz domains); and a prothrombinase inhibitor, boophilin, (CAC82583) from the tick *Rhipicephalus (Boophilus) microplus* (2 Kunitz domains).

Protein expression and purification. For bacterial expression of SV-66 and SV-170, full-length ORFs without signal peptides were PCR amplified using SV66/TOPO or
 15 SV170/TOPO as template, the primers SV66UB/SV66DB or SV170UB/SV170DB (Table 3), and Elongase polymerase enzyme mix (Invitrogen). The resulting products were then directly cloned in frame with the C-terminal His-tag of the vector pET-30 (Novagen) using T4 polymerase. Mutants SV66V13A, SV66C14A, SV66R15A, and SV66K19A were generated using the Quick Change site-directed mutagenesis kit
 20 (Stratagene) together with the primers V13A-UA/V13A-DA, C14A-UA/C14A-DA, R15A-UA/R15A-DA, and K19A-UA/K19A-DA respectively. Each of these constructs were confirmed by DNA sequencing, and then expressed by transforming into *E. coli* BL21 (DE3) cells cultured in SOC medium (0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM Glucose), supplemented
 25 with 10 ug/ml of kanamycin to an O.D. of 1.0 at 37°C. 0.1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) was then added to the cultures and grew an additional 17–24 h at 20°C. Bacterial cells were harvested by centrifugation at 4500 g for 10 min and used immediately or stored at -80°C.

Bacterial pellets from 0.8 L cultures were resuspended in 40 ml of lysis buffer
 30 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). After addition of lysozyme (1 mg/ml) in 50 mM Tris-HCl (pH 8.0), cells were incubated on ice for one hour followed by two freeze-thaw cycles and sonication with six, 10 sec bursts at 300 W using a Branson 450 Sonifier (VWR). The lysate was then centrifuged at 13,000 g for 10 min, followed by 3 h incubation of the supernatant with Ni-NTA Superflow beads (Qiagen)

pre-equilibrated with lysis buffer. After washing, attached proteins were eluted with three column volumes of elution buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 300 mM imidazole), followed by desalting and concentration using an Amicon Ultra-4 3000 MWCO spin column (Millipore, USA). Proteins were quantified using the Micro BCA Protein Assay Kit (Pierce) and visualized after SDS-polyacrylamide gel electrophoresis (PAGE) (4–20% precast gels (Lonza)) by staining with Coomassie Blue.

Recombinant proteins were further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Jupiter C4 column (5 µm particle size, 300 Å pore size, 250 mm length 2.00 mm ID) (Phenomenex, Torrance, CA) with a linear gradient between 95% H₂O/5% acetonitrile (ACN)/0.05% trifluoroacetic acid (TFA) and 95% ACN/5% H₂O/0.03% TFA) monitored at 220 nm. Fractions were collected every minute, lyophilized to remove ACN and TFA, resuspended in 20 mM Tris pH 8.0, and quantified by the BCA method described above. Purity of the proteins was assessed by SDS-PAGE, performed as described above, and immunoblotting. Separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio Rad, Hercules, CA), blocked in 1 PBS containing 0.05% Tween-20 and 2% non-fat skim milk for 1 hour at room temperature, and incubated with an anti-His primary antibody (His-probe (H-15); Santa Cruz Biotechnology) (15000). The membrane was washed three times with 1 PBS containing 0.05% Tween-20, incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Jackson Immuno Research) (110000), and visualized by chemiluminescence using the ECL Plus Western Blotting system (GE Healthcare, Piscataway, NJ).

Recalcification assay. Recalcification assays were performed as described by Valenzuela et al. (Valenzuela et al., 1996, *Exp Parasitol*; 83:184–190). Briefly, 10 µL of various concentrations of recombinant proteins, 40 µL of 0.15 M NaCl, 10 mM HEPES pH7.4 and 50 µL of citrated human plasma (TriniCHECK™ Level 1, Trinity Biotech, Co Wicklow, Ireland) were mixed in a flat-bottomed 96-well plate and prewarmed at 37°C for 15 min. To initiate plasma clotting, 50 µL of 25 mM CaCl₂ prewarmed to 37°C was added in the 96-well plate. Immediately after the addition of CaCl₂ absorbance was taken at 650 nm at 10-sec intervals by microtiter plate reader at 37°C (SpectraMax 340, Molecular Devices, Sunnyvale, CA). Clotting onset time (“Clotting Time”) was set at the time when the absorbance reached an optical density (OD) of 0.04, where the increase in OD is linear. One-way ANOVA, followed by pairwise comparisons of treatment values to the CaCl₂-only control using the Holm-Sidak method, was used for

the statistical analysis; when $p < 0.05$, the difference was considered as statistically significant.

Serine protease inhibition assays. rSimukunin (500 nM) was first pre-incubated with each enzyme for 10 min before the addition of the corresponding substrate. The amount of enzyme used in the assays was the lowest possible to give a linear substrate hydrolysis rate in the assays ($r^2 > 0.95$). After incubation for 5 min at 30°C, substrate hydrolysis rate was followed in a Tecan Infinite M200 96-well plate fluorescence reader (Tecan group Ltd, Switzerland) using 365 nm excitation and 450 nm emission wavelength with a cutoff at 435 nm for 20 min at 30°C. Wells without enzyme were used to monitor spontaneous substrate hydrolysis and protease contamination in the inhibitor preparation. A t-test was used for the statistical analysis of the observed inhibition in the presence of 500 nM rSimukunin and when $p < 0.05$, it was considered as statistically significant.

IC₅₀ estimates were determined as previously described (Kotsyfakis et al., 2007, *J Biol Chem*; 282:29256–29263), using decreasing concentrations of rSimukunin pre-incubated for 5 min with a given target enzyme, followed by addition of substrate. All experiments were performed in triplicate (for each enzyme and each concentration of the inhibitor). The mean percentage of enzymatic activity in the presence of various rSimukunin concentrations was then compared with enzymatic activity in the absence of rSimukunin. The sigmoidal fit of the data then yielded the estimate for the IC₅₀ of rSimukunin for the various enzymes. The resulting titration curves are provided in Fig. 8.

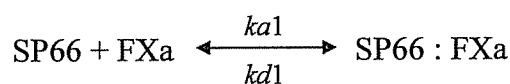
All enzymes used were of human origin and of the highest available purity. The source and concentration of the enzymes used in the serine protease screen assays follows: Thrombin (0.02 nM), α-chymotrypsin (0.025 nM), plasmin (0.2 nM) and chymase (1.8 nM) were purchased from Sigma (St. Louis, MO), skin β-tryptase (0.01 nM) was purchased from Promega (Madison, WI), activated coagulation factor X (FXa) (0.1 nM) was purchased from EMD Biosciences (La Jolla, CA), FXIIa (0.1 nM) was purchased from Haematologic Technologies Inc. (Essex Junction, VT), kallikrein (0.08 nM) was purchased from Fitzgerald Industries International (Concord, MA), elastase (0.18 nM) was purchased from Elastin Products (Owensville, MO), FXIa (0.06 nM), urokinase-type plasminogen activator (u-PA) (0.5 nM) and tissue plasminogen activator (t-PA) (0.06 nM) from Molecular Innovations (Southfield, MI), matriptase (0.2 nM) from R&D Systems (Minneapolis, MN), cathepsin G (6.7 nM) from Enzo Life Sciences (Plymouth Meeting, PA) and sequencing grade trypsin (0.1 nM) was purchased from

Roche (Chicago, IL). The amount of enzyme used in each of the IC₅₀ estimation assays is shown also in the Table 1.

Assay buffer for elastase and chymase was 50 mM HEPES buffer pH 7.4, 100 mM NaCl, and 0.01% Triton X-100. Assay buffer for trypsin, a-chymotrypsin, factor XIa, factor XIIa and thrombin was 50 mM Tris buffer pH 8.0, 150 mM NaCl, 20 mM CaCl₂, and 0.01% Triton X-100. Assay buffer for β -tryptase was 50 mM Tris pH 8.0, 50 mM NaCl, and 0.05% Triton X-100. Assay buffer for kallikrein, matriptase and plasmin was 20 mM Tris buffer pH 8.5, 150 mM NaCl, and 0.02% triton X-100. Assay buffer for factor Xa was 20 mM Tris buffer pH 8.0, 200 mM NaCl, 5 mM CaCl₂, and 0.1% BSA. Assay buffer for u-PA and t-PA was 20 mM Tris buffer pH 8.5, and 0.05% Triton X-100. Assay buffer for cathepsin G was 50 mM Tris buffer pH 7.4, 150 mM NaCl, and 0.01% Triton X-100. Peptidyl substrates used were: Suc-A-A-P-V-AMC for elastase, a-chymotrypsin and chymase (EMD Biosciences, La Jolla, CA); Boc-D-P-R-AMC for thrombin and plasmin; Boc-Q-A-R-AMC for trypsin, factor XIa and u-PA (Sigma, St. Louis, MO); Boc-F-S-R-AMC for β -tryptase; Suc-L-L-V-Y-AMC for chymase (Bachem, King of Prussia, PA); and methylsulfonyl-D-cyclohexylalanyl-G-R-AMC acetate for factor Xa, factor XIIa, t-PA, matriptase and kallikrein (American Diagnostica Inc., Stamford, CT). All substrates were used at 250 μ M final concentration in all the assays.

Surface plasmon resonance (SPR) assays. SPR experiments were conducted using a T100 instrument (Biacore Inc., Uppsala, Sweden) following the manufacturer's instructions. Sensor CM5, amine coupling reagents, and buffers were also purchased from Biacore Inc. (Piscataway, NJ, USA). HBS P (10 mM Hepes, pH 7.4, 150 mM NaCl, and 0.005% (v/v) P20 surfactant) was used as the running buffer for all SPR experiments, which were carried out as previously described (Albrecht et al., 1983, *Hoppe Seylers Z Physiol Chem*; 364:1697–1702). For analytes, FVIIa (recombinant) was purchased from American Diagnostica (Stamford, CT); FX, FXa (human, bovine, mouse), DEGR-FXa (human), des-Gla-FXa (human), FIXa, FXIa, FXIIIa, and thrombin were purchased from Haematologic Technologies, Inc. (Essex Junction, VT); elastase (purified from human sputum) was purchased from Molecular Innovations, Inc. (Novi, MI). All enzymes were of the highest available purity. For immobilization and kinetic analysis, rSimukunin (10 μ g/ml) in acetate buffer pH 5.0 was immobilized over a CM5 sensor via amine coupling, resulting in a final immobilization of 475.3 RU. Kinetic experiments were carried out with a contact time of 180 seconds at a flow rate of 30

5 $\mu\text{l}/\text{minute}$ at 25°C . rSimukunin-FXa complex dissociation was monitored for 1800 seconds, and the sensor surface was regenerated by a pulse of 30 seconds of 20 mM HCl at 30 $\mu\text{l}/\text{minute}$. In other experiments, other coagulation factors or enzymes were used 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 (k_a) and dissociation (k_d) rate constants were obtained by global fitting of data to a 1:1 interaction model (Langmuir) using BIAevaluation™ (Biacore, Inc.) software (Morton et al., 1995, *Anal Biochem*; 227:176–185):



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Values were then used to calculate the dissociation constant (K_D). Conditions were chosen so that the contribution of mass transport to the observed values of K_D was negligible. Also, models in the T100 evaluation software fit for mass transfer coefficient to mathematically extrapolate the true k_a and k_d .

15 Fig. 7 shows visualization of rSV-66 and rSV-170 following separation by SDS-PAGE (left panel) and immunoblotting (right panel). The SDS-PAGE gel was stained with Coomassie Brilliant Blue, while the immunoblot was probed with an anti-His primary antibody and visualized by chemiluminescence. Lanes were loaded with bacterial lysate (L), Ni^{2+} resin purified protein, (Ni^{2+}), or protein further purified by RP-HPLC (HPLC). Western blotting of the bacterial lysates was done separately from the
 20 Ni^{2+} and RP-HPLC purified proteins. The figure is therefore a composite, with the lysate lanes aligned with the others based on protein standards, but blotting protocols were identical for all samples.

Fig. 8 shows determination of rSimukunin IC_{50} values for selected serine
 25 proteinases. Enzymes, at the concentrations given in Table 1, were incubated with the indicated concentration of rSimukunin for five minutes at 30°C , followed by addition of substrate (250 μM final concentration). The amount of enzyme used in the assays was the lowest possible to give a linear substrate hydrolysis rate in the assays ($r^2 > 0.95$). Substrate hydrolysis was followed in a Tecan Infinite M200 96-well plate fluorescence
 30 reader (Tecan group Ltd, Switzerland) using 365 nm excitation and 450 nm emission wavelength with a cutoff at 435 nm for 20 min at 30°C . Wells without enzyme were used to monitor spontaneous substrate hydrolysis and protease contamination in the inhibitor

preparation. All experiments were performed in triplicate (for each enzyme and each concentration of the inhibitor). The mean percentage of enzymatic activity in the presence of various rSimukunin concentrations was then compared with enzymatic activity in the absence of rSimukunin. The sigmoidal fit of the data then yielded the estimate for the IC₅₀ of rSimukunin for the various enzymes reported in Table 1.

Example 2

Anti-inflammatory Effects of Simukunin

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The effects of simukunin on inflammatory responses will be studied using various in vitro and in vivo assays. For the in vitro assays, macrophage cell cultures or mouse spleen cell cultures will be exposed to simukunin (or a control treatment, including, but not limited to, saline alone or saline plus an irrelevant protein such as bovine serum albumin (BSA)). Cells will then be challenged with a variety of inflammation-inducing substances including, but not limited to, ATP, endotoxin, and keyhole limpet hemacyanogen (KLH). Murine splenocyte proliferation, including T-cell proliferation, and the secretion of various immune modulators, including, but not limited to, tumor necrosis factor alpha (TNF α), interleukin-2 (IL-2), interferon gamma (IFN- γ), nitric oxide (NO), and superoxide, will be measured. The effect of simukunin on LPS-stimulated B-cell proliferation and mitogen-stimulated mouse splenocyte proliferation, including proliferation of both CD4(+) and CD8(+) T cells, will be determined. The impact of simukunin on the secretion of Th1 cytokines (such as IL-2, IFN- γ , and TGF- β) and Th2 cytokines (such as IL-4, IL-5, IL-6, IL-10, and IL-13) will be determined. The effect of simukunin in both antigen-specific and non-specific immune responses will be assayed. The effect of simukunin on macrophage and dendritic cell viability will be determined. In vivo assays will include the mouse footpad assay. Briefly, mice will be injected in the footpad with either an irritant such as carrageenan, or simukunin plus carrageenan, and inflammation measured by monitoring footpad thickness (a measure of edema and swelling due to cellular infiltration) and myeloperoxidase enzyme activity (a measure of activated neutrophil activity).

Procedures to be followed include, but are not limited to, those described in Wanasen et al., 2004, *Med Vet Entomol*; 18(2):191-9; Wasserman et al., 2004, *Parasite Immunol*; 26(6-7):295-306; Champagne, 2004, *Curr Drug Targets Cardiovasc Haematol*

Disord; 4(4):375-96; Champagne, 2005, *Pathophysiol Haemost Thromb*; 34(4-5):221-7; and Tsujimoto et al., 2010, *Parasite Immunol*; 32(4):275-84.

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Example 3

Further Characterization of the Effect of Simukunin
on Blood Clotting

A rat model of arterial thrombosis may be used to further characterize the effects
10 of simukunin on blood clotting in vivo. Such a rat artery assay may be used to monitor
clot formation in response to ADP injection, in the presence and absence of simukunin.
Simukunin may be administered intravenously. Procedures to be followed include, for
example, those described in more detail by Li et al., 1999, *Arterioscler Thromb Vasc*
Biol; 19:378–383; Saldeen et al., 1999, *J Am Coll Cardiol*; 34:1208–1215; Zancan and
15 Mourao, 2004, *Blood Coagul Fibrinolysis*; 15(1): 45-54.

Example 4

Effect of Simukunin on Pathogen Killing
20 by Neutrophils and Macrophages

The effect of simukunin on pathogen killing by neutrophils and macrophages will
be determined in various in vitro assays. Neutrophils and macrophages grown in culture
will be exposed to various concentrations of simukunin, and their subsequent ability to
25 phagocytose and kill potential pathogens (including, but not limited to, *E. coli*,
Salmonella, and *Listeria monocytogenes*) will be measured. Simukunin inhibits
cathepsin G and elastase. Both cathepsin G and elastase are involved in pathogen killing
as well as phagocytosis. Thus, whether simukunin inhibits the activation and/or the
function of innate immune protection against infection will also be investigated. The
30 inhibitory effect of simukunin on elastase and cathepsin G will be determined in various
in vivo systems, such as, for example, rat emphysema models (see, for example, Kuraki
et al., 2002, *Am J Respir Crit Care Med*; 166(4):496-500) and mouse models of collagen
induced arthritis (see, for example, Raptis et al., 2005, *Immunity*; 22(6):679-691).

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

15

Sequence Listing Free Text

	SEQ ID NO:1	Nucleotide sequence of <i>Simulium vittatum</i> SV-66
	SEQ ID NO:2	Translated amino acid sequence of <i>Simulium vittatum</i> SV-66
5	SEQ ID NO:3	Amino acid sequence of <i>Simulium vittatum</i> SV-66, without 19 amino acid N-terminal signal sequence
	SEQ ID NO:4	Nucleotide sequence of <i>Simulium vittatum</i> SV-170
	SEQ ID NO:5	Translated amino acid sequence of <i>Simulium vittatum</i> SV-170
	SEQ ID NO:6	Amino acid sequence of <i>Simulium vittatum</i> SV-170, without 22 amino acid N-terminal signal sequence
10	SEQ ID NO:7	Kunitz domain sequence of SV-66
	SEQ ID NO:8	Kunitz domain sequence of SV-170
	SEQ ID NO:9-11	Kunitz domain sequences of human TFPI
	SEQ ID NO:12	Kunitz domain sequences <i>Bos taurus</i> BPTI
	SEQ ID NO:13-14	Kunitz domain sequences of <i>Amblyomma hebraeum</i> Amblin
15	SEQ ID NO:15-16	Kunitz domain sequences of <i>Rhipicephalus microplus</i> : Boophilin
	SEQ ID NO:17-36	Synthetic Oligonucleotide Primers

What is claimed is:

1. A method of inhibiting plasma coagulation and inhibiting elastase and/or cathepsin G in a subject, the method comprising administering to the subject an isolated simukunin polypeptide, wherein the isolated simukunin polypeptide comprises an amino acid sequence at least about 90% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G.
2. An isolated simukunin polypeptide comprising an amino acid sequence at least about 80% identical to SEQ ID NO:3 and fragments thereof, wherein the simukunin polypeptide inhibits Factor Xa.
3. An isolated simukunin polypeptide comprising an amino acid sequence at least about 95% identical to SEQ ID NO:3 and fragments thereof, wherein the simukunin polypeptide inhibits Factor Xa.
4. The isolated simukunin polypeptide of claim 2 or 3, wherein the simukunin polypeptide inhibits elastase and/or cathepsin G.
5. An isolated simukunin polypeptide comprising an amino acid sequence at least about 95% identical to SEQ ID NO:3 and fragments thereof, wherein the isolated simukunin polypeptide inhibits Factor Xa, inhibits elastase, and inhibits cathepsin G.
6. The isolated simukunin polypeptide of any one of claims 2 to 5, wherein the simukunin polypeptide inhibits Factor IXa, Factor XIa, and/or plasmin.
7. The isolated simukunin polypeptide of any one of claims 2 to 6, wherein the simukunin polypeptide inhibits kallikrein, trypsin, and/or β -tryptase.
8. A composition comprising the isolated simukunin polypeptide of any one of claims 2 to 7.
9. The composition of claim 8, comprising a pharmaceutically acceptable carrier.

10. The composition of claim 9, wherein the pharmaceutically acceptable carrier is pyrogen free.
11. The composition of any one of claims 8 to 10, wherein the composition is pyrogen free.
12. A composition comprising the isolated simukunin polypeptide of claim 5, wherein the composition is pyrogen free.
13. A composition of any one of claims 8 to 12 formulated for parenteral application or formulated for topical application.
14. An isolated polynucleotide sequence encoding a simukunin polypeptide of any one of claims 2 to 7.
15. A vector or host cell comprising the isolated polynucleotide sequence of claim 14.
16. A method of producing a polypeptide, the methods comprising culturing a host cell of claim 15 and purifying an expression product from the cell mass and/or the culture medium.
17. A method of inhibiting plasma coagulation, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.
18. A method of inhibiting Factor Xa, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.
19. A method of inhibiting one or more serine proteases, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

20. A method of inhibiting elastase and/or cathepsin G, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

21. A method of inhibiting Factor IXa, Factor XIa, and/or plasmin, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

22. A method of inhibiting kallikrein, trypsin, and/or β trypsin, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

23. A method of modulating inflammation, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

24. A method of modulating the function of neutrophils, macrophages, and/or mast cells, the method comprising providing an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

25. The method of any one of claims 17 to 24, wherein the isolated polypeptide is provided in vitro.

26. The method of any one of claims 17 to 24, wherein the isolated polypeptide is provided in vivo to a subject.

27. A method of inhibiting plasma coagulation in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

28. A method of inhibiting Factor Xa in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.

29. A method of inhibiting elastase and/or cathepsin G in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.
30. A method of inhibiting Factor Xa and elastase in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.
31. A method of modulating inflammation in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.
32. A method of modulating the function of neutrophils, macrophages, and/or mast cells in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.
33. A method of inhibiting plasma coagulation and inhibiting elastase and/or cathepsin G in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any one of claims 2 to 7 or a composition of any one of claims 8 to 13.
34. The method of any one of claims 17 to 33, wherein the isolated simukunin polypeptide comprises an amino acid sequence at least about 95% identical to SEQ ID NO:3 and wherein the isolated simukunin polypeptide inhibits Factor Xa, elastase, and cathepsin G.
35. The method of any one of claims 26 to 34, wherein administration is parenteral or topical.
36. A method of inhibiting plasma coagulation, the method comprising providing an isolated simukunin polypeptide of claim 5.
37. A method of inhibiting Factor Xa, the method comprising providing an isolated simukunin polypeptide of claim 5.

38. A method of inhibiting elastase and/or cathepsin G, the method comprising providing an isolated simukunin polypeptide of claim 5.
39. A method of modulating inflammation, the method comprising providing an isolated simukunin polypeptide of claim 5.
40. A method of modulating the function of neutrophils, macrophages, and/or mast cells, the method comprising providing an isolated simukunin polypeptide of claim 5.
41. The method of any one of claims 36 to 40, wherein the isolated polypeptide is provided in vitro.
42. The method of any one of claims 36 to 42, wherein the isolated polypeptide is provided in vivo to a subject.
43. A method of inhibiting plasma coagulation in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of claim 5.
44. A method of inhibiting Factor Xa in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of claim 5.
45. A method of inhibiting elastase and/or cathepsin G in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of claim 5.
46. A method of inhibiting Factor Xa and inhibiting elastase and/or cathepsin G in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of claim 5.
47. A method of modulating inflammation in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of claim 5.
48. A method of modulating the function of neutrophils, macrophages, and/or mast cells in a subject, the method comprising administering to the subject an isolated simukunin polypeptide of any claim 5.

TGAATTGGATCGAAATGCAATATACTTCCAATAAGTGCTTTTCCTCCTATATCTTTGGCCATTCTTTGGCCCCAA
M N I L P I S A F F L L Y L G H S L A Q
-19 -10 1

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60 70

ATACCAATTATTTTCGTTGGACCTA AAAAGGACATTCAA ACTAAGTTATAGACAAACATTTATATTTTCA CAATTACT
I P I I S L D -
80

TGAAAAATAAAA TC GAAC TG TGA AAAATTTTAATTTG ACCAGAAAAAAAAAAAAAAAAAAAAA

GCATTAATCATTAGTACACCTGAGAGAATCTTCTGCGTCAAAGTATGCTTGAAAAACAATCATCTCGGAACAATC
M L K T I I L G T I
-22
GCCATACTGATATGSTATGGCAAACAATTCTGAAGCAAAGTCAGCTGACATCTGCAGACTTCCAATGGATAAAGGT
A I L I C M A N N S E A K S A D I C R L P M D K G
-10 1 10
ATTTCGCACTCCAACAGAATGGCGTTATCATTTTGACCCAGCGAAAAACAAGTGCTTTATGTTTCCGTGGGGATGC
I C T P T E W R Y H F D F A K N K C F M F P W G C
20 30
CTTGGGAATGCGAACAATTTCAAAAACCAGACAAGARTGTAAAGCCAAGTGTATGTTTCAAGACAGCACAAGAGGA
L G N A N N F K T R Q E C K A K C M -
40 50
TAAAAATGTATTGACCGATTGTTATTAAAAATAAGGCATAAACAATTTGATAGACAAAACCTGAAAAAAAAAAAAAA
AAAAAA

Figure 2A

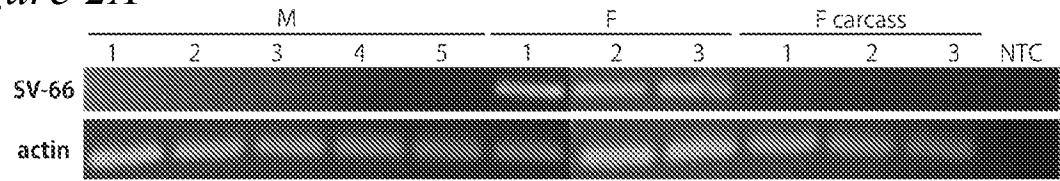


Figure 2B

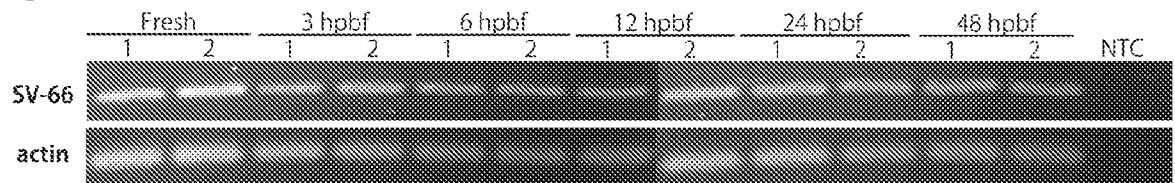


Figure 3

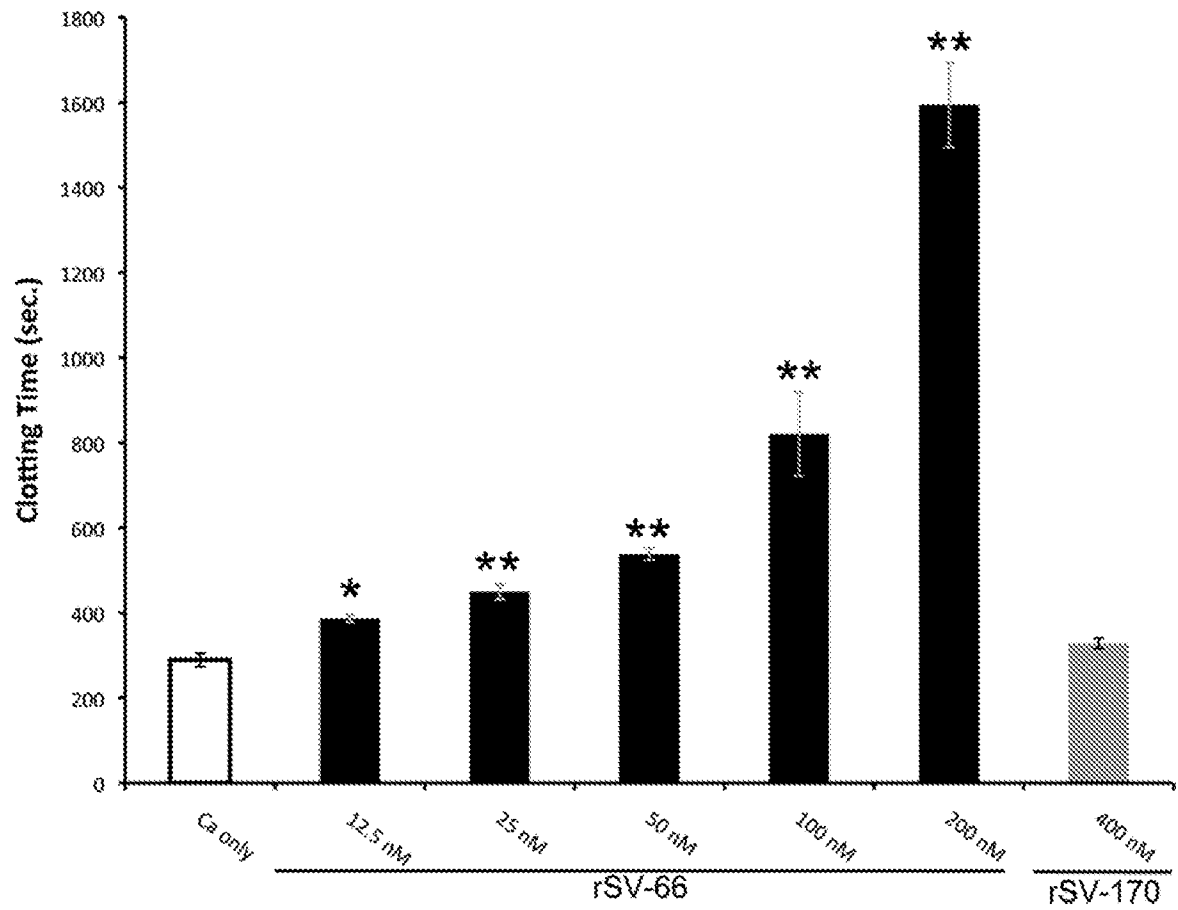


Figure 4

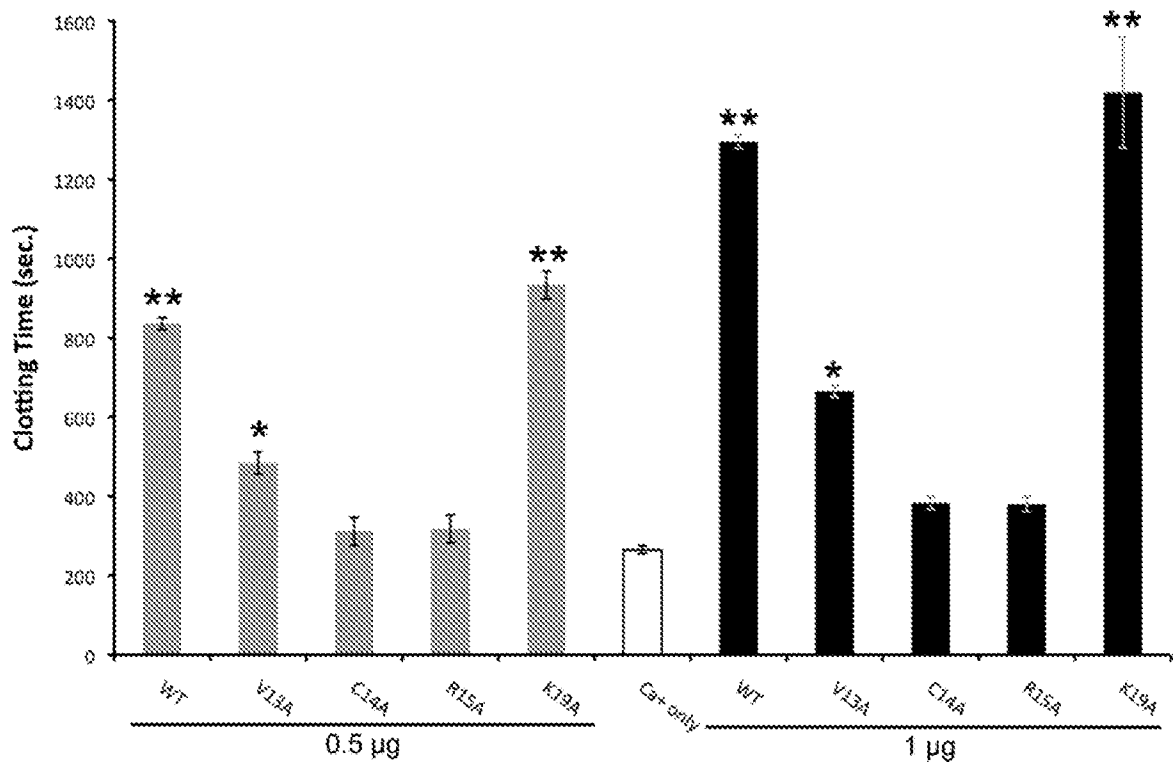


Figure 5

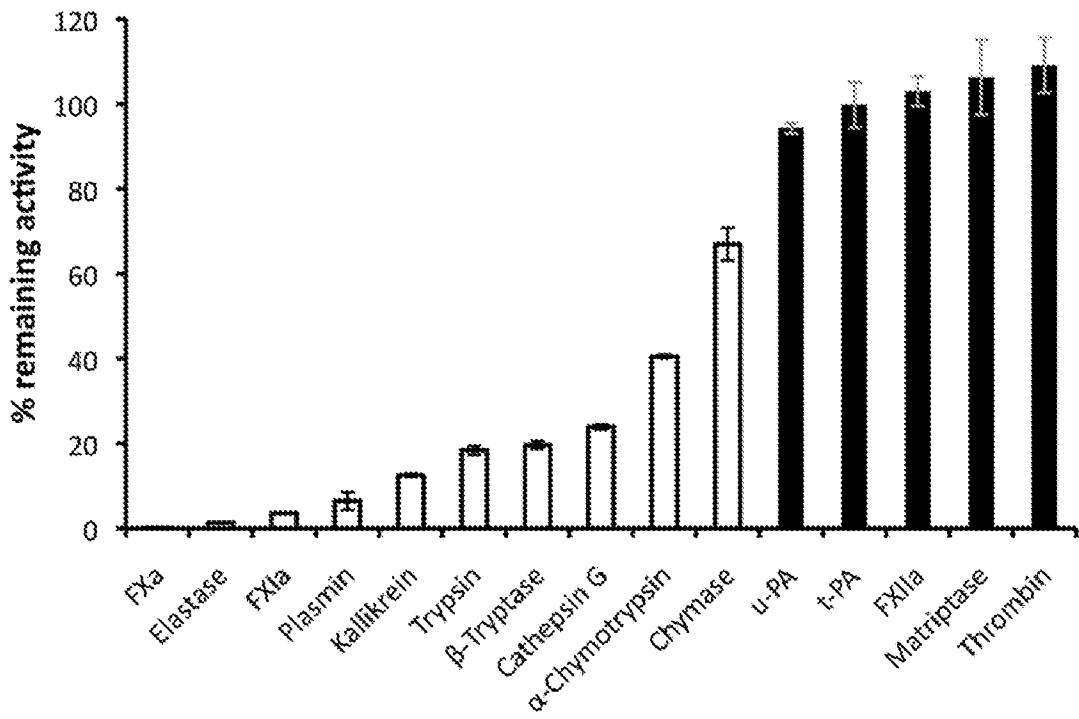


Figure 6A

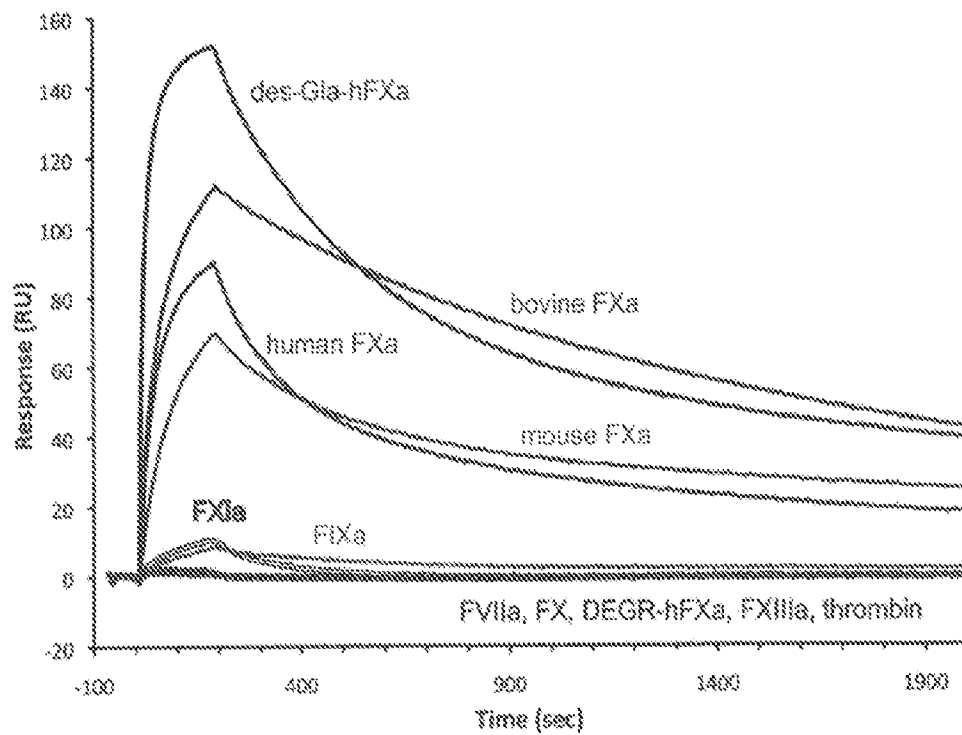


Figure 6B

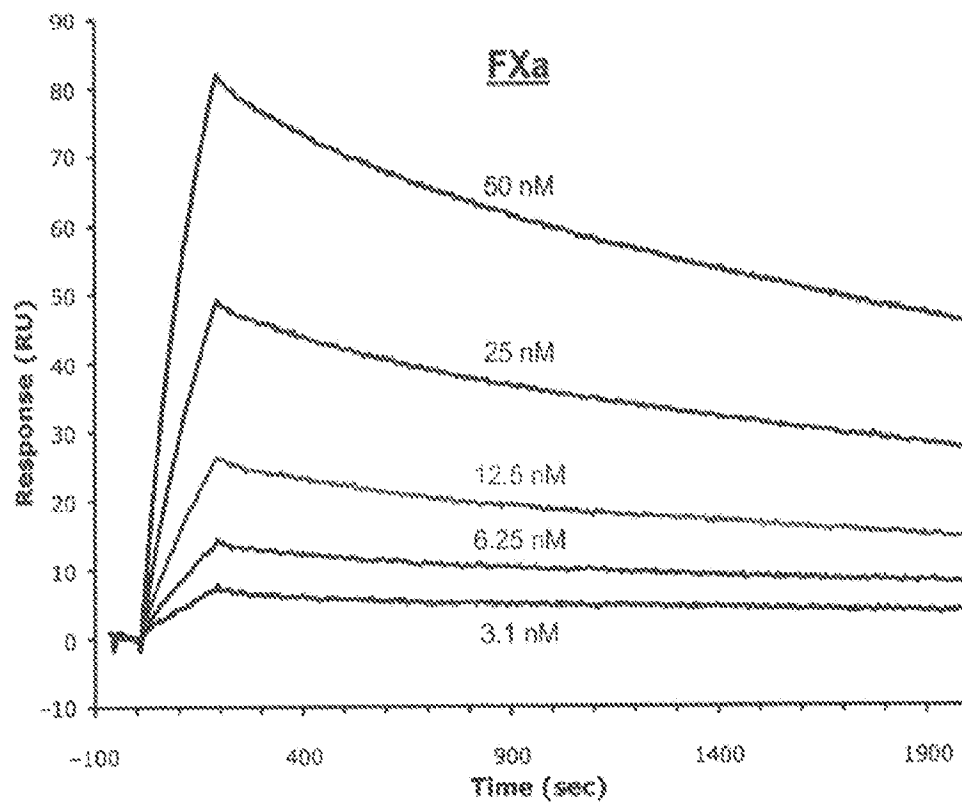


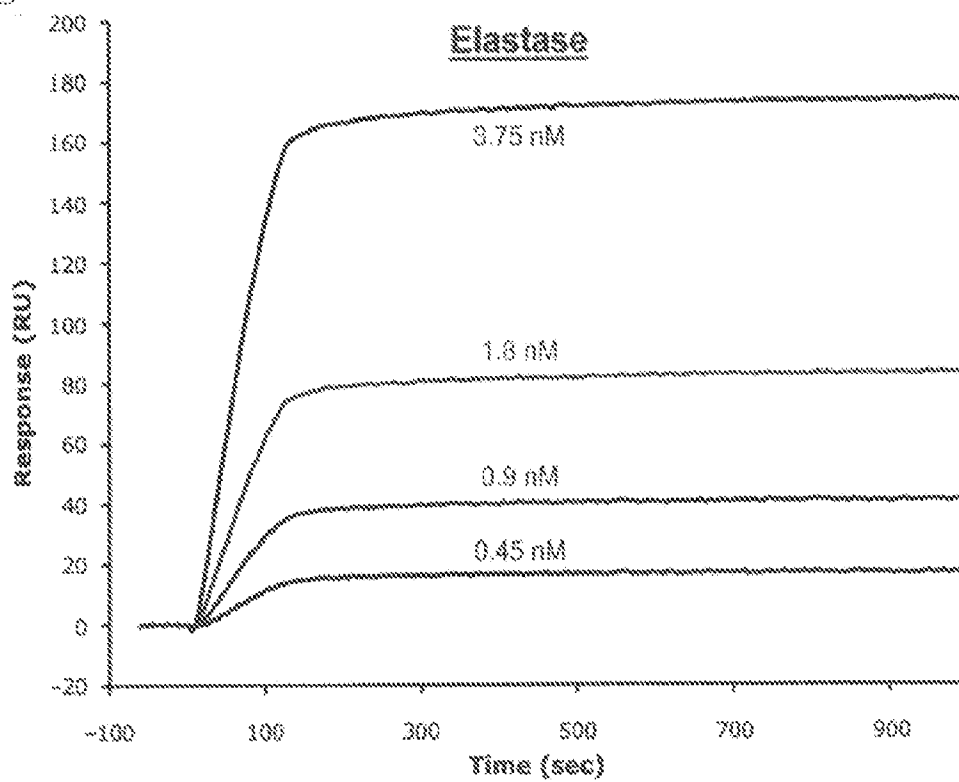
Figure 6C

Figure 7

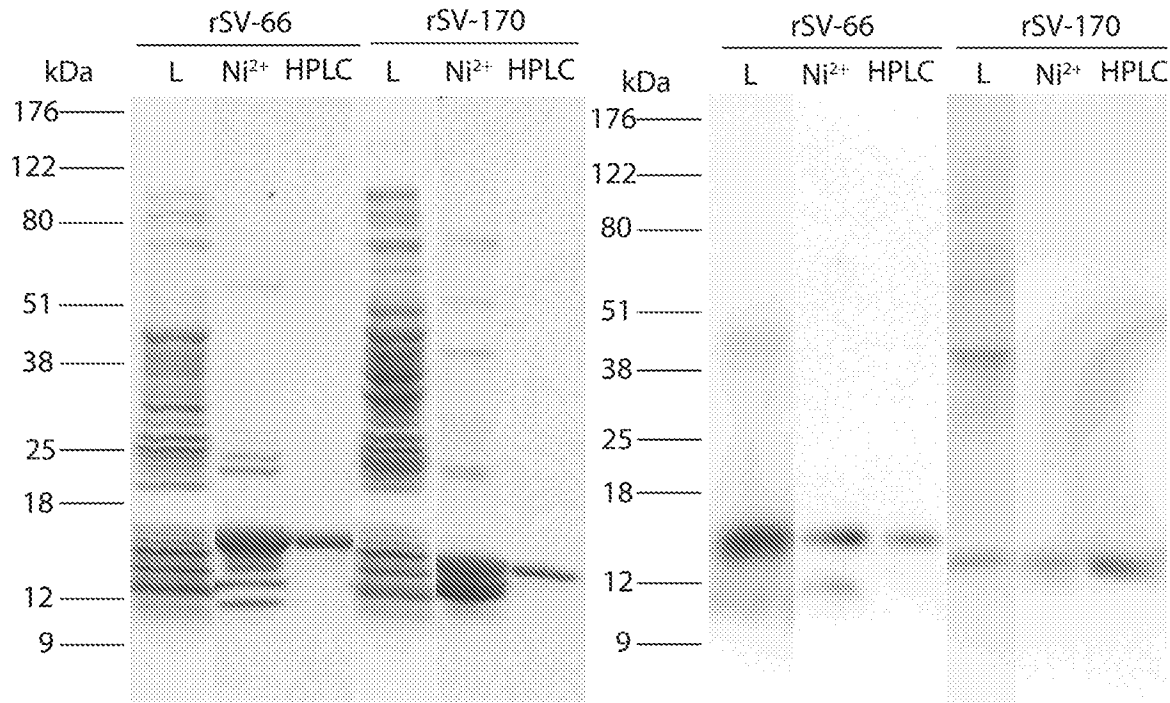
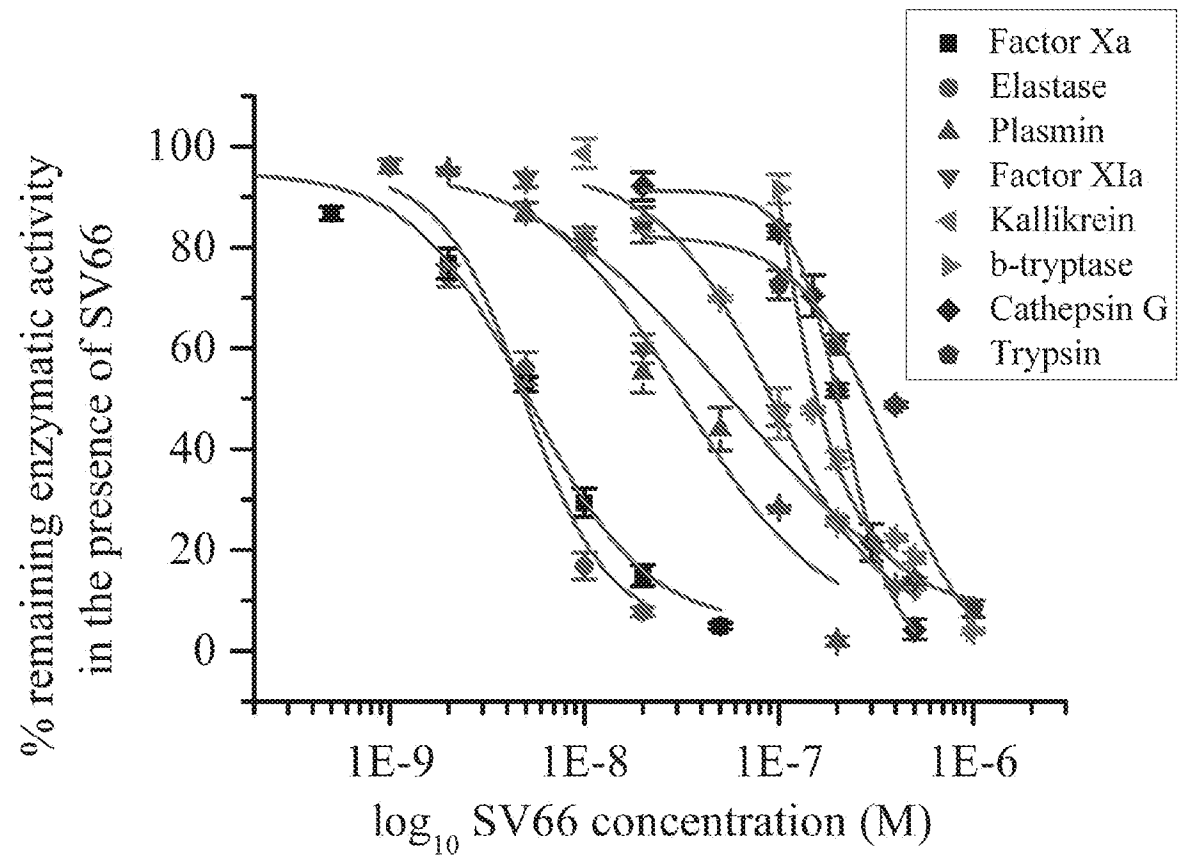


Figure 8



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/39585

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/36; A61P 7/02; C07K 14/745 (2012.01)

USPC - 514/13.7, 514/21.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 514/13.7, 514/21.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 530/324 (text search, see term below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog Classic, Google Scholar, Free Patents Online

simukunin polypeptides, kunitz domain polypeptide, plasma coagulation, cathepsin G, elastase, Factor Xa, inflammation, neutrophils, macrophages,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	"An insight into the sialome of the black fly, Simulium vittatum."; Uniprot/TrEMBL ID NO: B5M0W4-SIMV1; Submitted (JUL-2008) to the EMBL/GenBank/DBJ databases (Anderson et al.) [retrieved from http://www.uniprot.org/uniprot/b5m0w4.txt on 07 August 2012]; pg 1	1-6, 12, 36-41 and 43-48
Y	US 2011/0092413 A1(Hauser et al.) 21 April 2011 (21.04.2011), [0004]-[0006], [0034], [0040] [0045]-[0046], [0103], [0207], claim 73	1-6, 12, 36-41 and 43-48

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 August 2012 (04.08.2012)

Date of mailing of the international search report

31 AUG 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/39585

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 7-11, 13-35, 42
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.