(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 August 2014 (07.08.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/118677 A1

(51) International Patent Classification:
A61K 38/48 (2006.01) *A61K 31/5377* (2006.01)
A61K 31/437 (2006.01) *A61P 7/04* (2006.01)

(21) International Application Number:
PCT/IB2014/058494

(22) International Filing Date:
23 January 2014 (23.01.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/759,332 31 January 2013 (31.01.2013) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,

[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR COUNTERACTING FACTOR XA INHIBITION

(57) Abstract: The disclosure provides compositions and methods for counteracting the effects of direct activated Factor X (FXa) inhibitors in a subject by administering a variant of FXa.

FIG. 13A

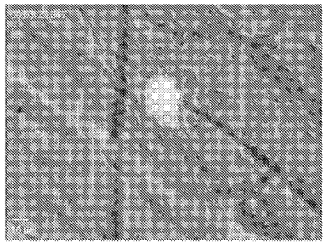


FIG. 13B

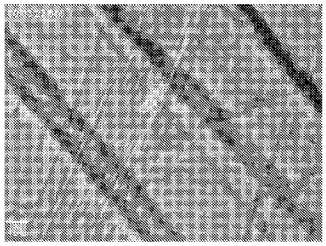
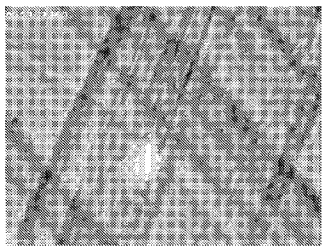


FIG. 13C



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HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

COMPOSITIONS AND METHODS FOR COUNTERACTING FACTOR XA INHIBITION

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/759,332, filed 31 Jan 2013, the contents of which are incorporated herein by reference in its entirety.

REFERENCE TO SEQUENCE LISTING

[0002] The Sequence Listing submitted concurrently herewith under 37 CFR §1.821 in a computer readable form (CRF) via EFS-Web as file name PC072006_SEQLIST_ST25.txt is incorporated herein by reference. The electronic copy of the Sequence Listing was created on 18 Dec 2013, with a file size of 7 kilobytes.

BACKGROUND OF THE INVENTION

[0003] Pharmacological anticoagulation is the mainstay of treatment for patients with prothrombotic conditions. For over fifty years, the only oral anticoagulant available was warfarin, an inhibitor of the vitamin K epoxide reductase (VKOR) that recycles oxidized vitamin K. Warfarin has many drawbacks, including unpredictable pharmacokinetics that necessitate frequent monitoring of coagulation parameters and dose adjustment. However, in the event of emergency bleeding or the need for urgent surgery, antidotes exist that allow rapid and complete reversal.

[0004] Oral direct FXa inhibitors are emerging anticoagulants that have the potential to simplify dosing schemes and hemostatic monitoring in patients with prothrombotic diseases when compared to standard treatments, such as warfarin. Although these drugs have many advantages over warfarin, no fully efficacious reversal agent is available for these novel anticoagulants.

[0005] The lack of a specific countermeasure to their effects, however, is a critical unmet clinical need that could limit the widespread adoption of these agents due to fears of unmanageable bleeding.

SUMMARY OF THE INVENTION

[0006] Applicants have addressed this critical unmet clinical need by providing compositions and methods for counteracting the effects of direct activated Factor X (FXa) inhibitors.

[0007] According to some embodiments, the disclosure provides methods for reducing or preventing bleeding in a subject being treated with a direct Factor Xa (FXa) inhibitor by administering a composition comprising a Factor Xa variant containing at least one modification including substitution for the wild-type amino acid at position 16 (using the chymotrypsin numbering system) with Thr, Leu, Phe, Asp or Gly, or substitution for the wild-type amino acid at position 17 (using the chymotrypsin numbering system) with Leu, Ala, or Gly. In certain embodiments, treatment with a composition comprising a FXa variant results in at least a 50% reduction in bleeding. According to certain embodiments, direct Factor Xa inhibitors include rivaroxaban or apixaban. In some embodiments, the plasma concentration of the direct FXa inhibitor is a typical therapeutic amount or a supratherapeutic amount. For example, in some embodiments, the plasma concentration of rivaroxaban can be about 500 nM, or greater, and the plasma concentration of apixaban can be about 250 nM, or greater. According to certain embodiments the FXa variant contains the substitution I16L. In some embodiments, the FXa variant is capable of countering the effect of the direct Factor Xa inhibitor at a plasma concentration that is at least 100-fold lower than the plasma concentration of the Factor Xa inhibitor. In certain embodiments, the composition comprising the FXa variant is administered before a planned surgery, after an injury, or after an intentional or accidental overdose with a direct FXa inhibitor. In some embodiments, hemostasis in the subject is monitored using a hemostasis assay after a first dose with a FXa variant and, if adequate hemostasis is not attained by a predetermined time, at least one second dose of FXa variant is administered to achieve sufficient hemostasis. According to some embodiments, the predetermined time is about 15 mins, 30 mins, 45 mins or 60 mins after the first dose of FXa variant is administered. Other times are also possible. In some other embodiments, at least a second procoagulant is administered in addition to FXa variant, including for example, a different FXa

variant, factor IX, factor XIa, factor XIIa, factor VIII, factor VIIa, FEIBA or prothrombin complex concentrate (PCC).

[0008] According to some embodiments, the disclosure provides methods for increasing the amount of thrombin produced in response to activation of the extrinsic or intrinsic clotting pathway in a subject being treated with a direct Factor Xa (FXa) inhibitor by administering a composition comprising a Factor Xa variant containing at least one modification including substitution for the wild-type amino acid at position 16 (using the chymotrypsin numbering system) with Thr, Leu, Phe, Asp or Gly, or substitution for the wild-type amino acid at position 17 (using the chymotrypsin numbering system) with Leu, Ala, or Gly. According to certain embodiments, direct Factor Xa inhibitors include rivaroxaban or apixaban. In some embodiments, the plasma concentration of the direct FXa inhibitor is a typical therapeutic amount or a supratherapeutic amount. For example, in some embodiments, the plasma concentration of rivaroxaban can be about 500 nM, or greater, and the plasma concentration of apixaban can be about 250 nM, or greater. According to certain embodiments the FXa variant contains the substitution I16L. According to certain embodiments, the amount of thrombin produced increases by about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, or more. In some embodiments, the FXa variant is capable of countering the effect of the direct Factor Xa inhibitor at a plasma concentration that is at least 100-fold lower than the plasma concentration of the Factor Xa inhibitor. In certain embodiments, the composition comprising the FXa variant is administered before a planned surgery, after an injury, or after an intentional or accidental overdose with a direct FXa inhibitor. In some embodiments, hemostasis in the subject is monitored using a hemostasis assay after a first dose with a FXa variant and, if adequate hemostasis is not attained by a predetermined time, at least one second dose of FXa variant is administered to achieve sufficient hemostasis. According to some embodiments, the predetermined time is about 15 mins, 30 mins, 45 mins or 60 mins after the first dose of FXa variant is administered. Other times are also possible. In some other embodiments, at least a second procoagulant is administered in addition to FXa variant, including for example, a different FXa variant, factor IX, factor XIa, factor XIIa, factor VIII, factor VIIa, FEIBA or prothrombin complex concentrate (PCC).

[0009] According to some embodiments, the disclosure provides methods for decreasing clotting time (as measured, for example, using PT or INR, or some other assay) in a subject being treated with a direct Factor Xa (FXa) inhibitor by administering a composition comprising a Factor Xa variant containing at least one modification including substitution for the wild-type amino acid at position 16 (using the chymotrypsin numbering system) with Thr, Leu, Phe, Asp or Gly, or substitution for the wild-type amino acid at position 17 (using the chymotrypsin numbering system) with Leu, Ala, or Gly. According to certain embodiments, direct Factor Xa inhibitors include rivaroxaban or apixaban. In some embodiments, the plasma concentration of the direct FXa inhibitor is a typical therapeutic amount or a supratherapeutic amount. For example, in some embodiments, the plasma concentration of rivaroxaban can be about 500 nM, or greater, and the plasma concentration of apixaban can be about 250 nM, or greater. According to certain embodiments the FXa variant contains the substitution I16L. According to certain embodiments, clotting time is reduced by about 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more. In some embodiments, the FXa variant is capable of countering the effect of the direct Factor Xa inhibitor at a plasma concentration that is at least 100-fold lower than the plasma concentration of the Factor Xa inhibitor. In certain embodiments, the composition comprising the FXa variant is administered before a planned surgery, after an injury, or after an intentional or accidental overdose with a direct FXa inhibitor. In some embodiments, hemostasis in the subject is monitored using a hemostasis assay after a first dose with a FXa variant and, if adequate hemostasis is not attained by a predetermined time, at least one second dose of FXa variant is administered to achieve sufficient hemostasis. According to some embodiments, the predetermined time is about 15 mins, 30 mins, 45 mins or 60 mins after the first dose of FXa variant is administered. Other times are also possible. In some other embodiments, at least a second procoagulant is administered in addition to FXa variant, including for example, a different FXa variant, factor IX, factor XIa, factor XIIa, factor VIII, factor VIIa, FEIBA or prothrombin complex concentrate (PCC).

BRIEF DESCRIPTION OF THE DRAWINGS

[00010] Figures 1A-B show inhibition of free wt-FXa or FXa^{I16L} by rivaroxaban. The initial velocity of peptidyl substrate (SpecXa; 200 uM) hydrolysis by A) wt-FXa (2 nM) or B) FXa^{I16L} (6 nM) was determined at increasing concentrations of rivaroxaban. The Ki value is given on each graph.

[00011] Figures 2A-B show rivaroxaban inhibition of wt-FXa or FXa^{I16L} assembled in prothrombinase. The initial velocity of peptidyl substrate (SpecXa; 200 uM) hydrolysis by A) wt-FXa (2 nM) or B) FXa^{I16L} (6 nM) in the presence of PCPS (20 uM) and FVa (40 nM) was determined at increasing concentrations of rivaroxaban.

[00012] Figure 3 shows the effect of different concentrations of FXa^{I16L} on reversing the effects on thrombin generation of rivaroxaban.

[00013] Figures 4A-D show the effect of FXa^{I16L} on reversing the effects of rivaroxaban. Normal human plasma was incubated with 500 nM rivaroxaban and in the absence or presence of increasing concentrations of FXa^{I16L}. Following data analysis, peak thrombin (A and C) and total thrombin generated (ETP; B and D) were plotted.

[00014] Figures 5A-B show FXa^{I16L} reverses the effects of high dose rivaroxaban. Normal human plasma was incubated with 7.5 uM rivaroxaban and in the absence or presence of increasing concentrations of FXa^{I16L}. Following data analysis, peak thrombin (A) and total thrombin generated (ETP; B) were plotted.

[00015] Figures 6A-B show FXa^{I16L} or FXa^{I16T} reverses the effects of 250 nM apixaban. Normal human plasma was incubated with 250 nM apixaban and in the absence or presence of increasing concentrations of FXa^{I16L} or FXa^{I16T}. Following data analysis, peak thrombin (A) and total thrombin generated (ETP; B) were plotted.

[00016] Figures 7A-B show FXa^{I16L} or FXa^{I16T} reverses the effects of high dose apixaban. Normal human plasma was incubated with 2.0 uM Apixaban and in the absence or presence of increasing concentrations of FXa^{I16L} or FXa^{I16T}. Following data analysis, peak thrombin (A) and total thrombin generated (ETP; B) were plotted.

[00017] Figures 8A-B show FXa^{I16L} corrects whole blood clotting in the presence of rivaroxaban. Whole blood thromboelastography was used to assess the ability of FXa^{I16L} to reverse the effects of rivaroxaban at a typical (A) and a high (B) dose.

[00018] Figures 9A-B show FXa^{I16L} corrects whole blood clotting in the presence of apixaban. Whole blood thromboelastography was used to assess the ability of FXa^{I16L} to reverse the effects of apixaban at a typical (A) and a high (B) dose.

[00019] Figures 10A-B show that FXa^{I16L} counteracts rivaroxaban in a thrombin generation assay. Figure 10A shows a dose response of rivaroxaban and Figure 10B shows a dose response of FXa^{I16L} in the presence of rivaroxaban.

[00020] Figure 11 shows that FXa^{I16L} counteracts rivaroxaban in a mouse tail clip bleeding model.

[00021] Figure 12 demonstrates that rivaroxaban administered to mice delays clotting time of whole blood measured using ROTEM and that administration with FXa^{I16L} dose-responsively counteracts the effect of rivaroxaban.

[00022] Figure 13 shows that rivaroxaban administered to a mouse prevents clot formation at a site of vascular injury in the cremaster muscle caused by laser and that administration with FXa^{I16L} counteracts the effect of rivaroxaban. Clot formation was visualized using intravital microscopy and fluorescently labeled antibodies against fibrin and platelets. Figure 13A shows clot formation in an untreated mouse. Figure 13B shows that rivaroxaban delayed and reduced platelet accumulation and prevented fibrin deposition. By contrast, Figure 13C shows that in a mouse administered rivaroxaban and FXa^{I16L} clot formation occurred at the site of injury.

[00023] Figure 14 is the amino acid sequence of wild-type human Factor X preprotein (SEQ ID NO:1). The signal peptide corresponds to amino acids 1-23. The propeptide corresponds to amino acids 24-40. The mature light chain of activated Factor X (FXa) corresponds to amino acids 41-179. The mature heavy chain of activated FXa (after removal of the activation peptide) corresponds to amino acids 235-488. The activation peptide (AP) corresponds to amino acids 183-234.

[00024] Figure 15 is the nucleotide sequence of the cDNA encoding wild-type human Factor X preprotein (SEQ ID NO:2). The coding sequence corresponds to nucleotides 58 to 1524.

DETAILED DESCRIPTION

[00025] The disclosure provides compositions and methods for counteracting the anti-coagulant effect of a direct FXa inhibitor in a subject in need thereof. Applicants have discovered that certain FXa variants rapidly and completely counteract the effect of a direct FXa inhibitor in a dose dependent manner. More specifically, applicants have discovered that a relatively small amount of an FXa variant restores normal coagulation activity *in vivo* in the presence of FXa inhibitor at therapeutic concentrations and even at supratherapeutic concentrations. By providing fast and effective antidotes for the anti-coagulation effects of direct FXa inhibitors, Applicants' disclosure therefore contributes to fulfilling the promise of these advantageous anti-coagulants.

[00026] Coagulation factor X (FX) is a zymogen which, upon activation by the intrinsic factor IX/factor VIII or extrinsic pathway (tissue factor/factor VIIa), becomes FXa, which is the protease moiety of prothrombinase. Following proteolytic cleavage of the Arg-Ile scissile bond, releasing an activation peptide (AP), a series of well defined structural changes in the zymogen drives the activation process to the mature active serine protease (See Toso et al., (2008) *J. Biol. Chem.* **283,** 18627-18635; Bunce et al., (2011) *Blood* **117,** 290-298; and Ivanciu et al., (2011) *Nat. Biotechnol.* **29,** 1028-1033, incorporated by reference herein in their entirety). The mature FXa has a light chain and a heavy chain that contains the catalytic domain. The mature FXa becomes an active serine protease upon formation of the prothrombinase complex, which includes binding of an activated cofactor, Factor Va (FVa).

[00027] Variant forms of FX have been developed that upon activation cleavage yield a zymogen-like FXa variant. That is, once cleaved, the resulting FXa variant has poor active site function and is more resistant to inactivation by circulating inhibitors (i.e. antithrombin III and TFPI). The FXa variants, thus, have longer half-lives in plasma than wild-type FXa. The FXa variant binds FVa, lipid

membrane and calcium to form a fully active prothrombinase complex that efficiently activates prothrombin.

[00028] The zymogen-like variants of FXa circulate in the zymogen-like conformation and do not seem to be thrombogenic (See Toso et al., (2008) *J. Biol. Chem.* **283,** 18627-18635 and Ivanciu et al., (2011) *Nat. Biotechnol.* **29,** 1028-1033, incorporated by reference herein in their entirety). Examples of such FXa variants are described in International patent publication WO2007/059513, incorporated herein by reference in its entirety.

[00029] The enzymes of coagulation are trypsin-like enzymes that belong to the S1 peptidase family of proteases that bear a chymotrypsin-like fold. The coagulation proteases contain catalytic domains that are highly homologous to each other and to the ancestral serine proteases of digestion. The structural homology/identity is so great (>70%) that residues in the catalytic domains of the coagulation enzymes (including Factor Xa) are numbered according to the corresponding residues in chymotrypsinogen. (Chymotrypsin numbering system; see Bajaj and Birktoft, Methods Enzymol. 1993; 222:96-128, Table 2, and Bode W, Mayr I, Bauman Y, et al. The refined 1.9 Å crystal structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Trp insertion segment. EMBO J 1989;8(11):3467-3475, both of which are incorporated by reference herein in their entireties). Accordingly, amino acids may be referred to herein according to the chymotrypsin numbering system, which is well-known to those of skill in the art.

[00030] According to the disclosure, an FXa variant may be an FXa protein comprising an amino acid substitution that makes the variant more zymogen-like compared to a wild-type FXa protein *in vivo* or *in vitro*. FXa variants of the disclosure substantially regain wild-type FXa activity upon formation of prothrombinase. Examples of FXa variants that are useful in methods of the disclosure are variants comprising a modification selected from the group consisting of: a) Ile at position 16 is Thr, Leu, Phe, Asp or Gly and b) Val at position 17 is Leu, Ala, or Gly, according to the chymotrypsin numbering system. Amino acids 16 and 17 in the chymotrypsin numbering system occur at amino acids 235 and 236, respectively, of SEQ ID NO:1 (human Factor X preproprotein). In certain embodiments, FXa variants are FXa^{I16L} and FXa^{I16T} (the nomenclature

used herein for the FXa variants recites the original amino acid at the numbered position according to the chymotrypsin numbering system followed by the substituted amino acid). The FXa variants can be variants of any mammalian FXa. Of particular interest, however, are FXa variants of human FXa.

[00031] In certain embodiments, the FX variant that is activated into a variant FXa of the disclosure may be further modified by inserting a non-native intracellular proteolytic cleavage site. In a non-limiting example, to express "activated" zymogen-like FXa variants in mammalian cells, a non-native intracellular proteolytic cleavage site can be inserted between the Arg at position 234 of SEQ ID NO:1 (position 15 in the chymotrypsin numbering system) and the amino acid at the position corresponding to position 235 of SEQ ID NO:1 (position 16 in the chymotrypsin numbering system) in the variant FX zymogen. In certain embodiments, the non-native intracellular protease cleavage site is Arg-Lys-Arg or Arg-Lys-Arg-Arg-Lys-Arg (SEQ ID NO:3). These cleavage sites are efficiently recognized by proteases (PACE/furin-like enzymes) within the cell and are removed. This cleavage may result in a processed variant FXa in which the mature heavy chain of the molecule now begins at the amino acid at the position corresponding to position 235 of SEQ ID NO:1 (position 16 in the chymotrypsin numbering system). Introduction of this cleavage site at said position allows for the intracellular conversion of FX to FXa.

[00032] In certain embodiments the entire amino acid sequence of the FX variant activation peptide (AP) (i.e., amino acids 183-234 of SEQ ID NO:1) is replaced with a non-native intracellular protease cleavage site. According to certain embodiments, the non-native intracellular protease cleavage site is Arg-Lys-Arg or Arg-Lys-Arg-Arg-Lys-Arg (SEQ ID NO:3). As explained above, this modification allows for intracellular cleavage of the FX variant expressed by cells. The intracellular cleavage converts FX variant to activated zymogen-like FXa variant which is then secreted by cells for subsequent purification. This approach obviates the need for extracellular cleavage that would otherwise be required to activate the variant clotting factor, for example, after isolating the protein or just before blood clotting.

[00033] In certain embodiments, FXa variants of the disclosure are derived from FX variant preproteins comprising native wild-type human signal sequence and/or

propeptide sequence. In other embodiments, FX signal sequences and/or propeptide from non-human species can be used in place of the corresponding native amino acid sequences. And in yet other embodiments, signal sequence and/or propeptide sequence from other human or non-human secreted proteins can be used in place of the corresponding native amino acid sequences.

[00034] In an exemplary embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 235 (isoleucine in the wild-type sequence) is substituted with a different amino acid selected from the group consisting of threonine (Thr), leucine (Leu), phenylalanine (Phe), aspartic acid (Asp), or glycine (Gly). These substitutions can respectively be written using the nomenclature I235T, I235L, I235F, I235D and I235G, where the first letter is the single letter code for isoleucine and the last letter is the single letter code for the amino acid being substituted into the wild-type sequence. Because position 235 of SEQ ID NO:1 is equivalent to position 16 in the chymotrypsin numbering system, the same substitutions can be written I16T, I16L, I16F, I16D and I16G. In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 235 is substituted with Thr (i.e., I235T or I16T). In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 235 is substituted with Leu (i.e., I235L or I16L). In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 235 is substituted with Phe (i.e., I235F or I16F). In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 235 is substituted with Asp (i.e., I235D or I16D). In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 235 is substituted with Gly (i.e., I235G or I16G).

[00035] According to another exemplary embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 236 (valine in the wild-type sequence) is substituted with a different amino acid selected from the group consisting of leucine (Leu), alanine (Ala), or glycine (Gly). These substitutions can respectively be written using the

nomenclature V236L, V236A, and V236G, where the first letter is the single letter code for valine and the last letter is the single letter code for the amino acid being substituted into the wild-type sequence. Because position 236 of SEQ ID NO:1 is equivalent to position 17 in the chymotrypsin numbering system, the same substitutions can be written V17L, V17A, and V17G. In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 236 is substituted with Leu (i.e., V236L or V17L). In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 236 is substituted with Ala (i.e., V236A or V17A). In an embodiment, a FXa variant comprises amino acids 41-179 and amino acids 235-488 of SEQ ID NO:1, wherein the amino acid at position 236 is substituted with Gly (i.e., V236G or V17G).

[00036] In other embodiments, FXa variants of the disclosure, including those specific variants described in the preceding paragraphs, can include various isoforms of the light and/or mature heavy chain of the protein. Non-limiting exemplary isoforms of the FXa variant mature heavy chain include the alpha and beta versions of the mature heavy chain. Jesty et al., J Biol Chem. 1975 Jun 25;250(12):4497-504, incorporated by reference herein. Compositions of the disclosure can include FXa variant proteins in which one or the other or both alpha and beta mature heavy chain isoforms are represented.

[00037] According to yet other embodiments, isoforms of FXa variant proteins, including those specific variants described in the preceding paragraphs, can include isoforms in which a variable number of amino acids (for example, 1, 2, 3, 4, 5, 6, or more amino acids) are either missing from or added to the carboxy terminus of the light chain and/or mature heavy chains of the protein.

[00038] According to certain embodiments, FXa variants of the disclosure include proteins with a certain minimal degree of homology or sequence identity compared to the amino acid sequence of wild-type FXa in SEQ ID NO:1. Thus, for example, FXa variants include proteins that contain a light and mature heavy chain that are at least 60%, 70%, 80%, 85%, 90%, 95%, 98%, or 99% homologous or identical in sequence with the wild-type FXa light and mature heavy chains in SEQ ID NO:1, wherein such FXa variants also include a

substitution at the amino acid position corresponding to position 235 of SEQ ID NO:1 with Thr, Leu, Phe, Asp, or Gly, or a substitution at the amino acid position corresponding to position 236 of SEQ ID NO:1 with Leu, Ala, or Gly, and further wherein such FXa variants are zymogenic until incorporated into prothrombinase complex. In the amino acid sequence of SEQ ID NO:1, the wild-type FXa light chain sequence corresponds to amino acids 41 to 179 and the wild-type FXa mature heavy chain sequence corresponds to amino acids 235 to 488. Percentage amino acid sequence homology or identity can readily be determined using software such as Protein BLAST available at the website of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

[00039] According to other non-limiting embodiments, FXa variants of the disclosure can also include FXa variants containing one or more post-translational modifications including, without limitation, one or more O-linked or N-linked carbohydrate groups or a variable number of gamma-carboxyglutamic acid (Gla) residues. FXa variants of the disclosure can further include chemically modified FXa variant proteins. Other FXa variants useful in the methods of the disclosure are also possible.

[00040] As used herein, the term FXa^{I16x} refers to a variant of activated Factor X wherein the amino acid corresponding to position 235 in SEQ ID NO:1 (corresponding to position 16 in the chymotrypsin numbering system) is changed from the amino acid in the wild-type sequence (isoleucine) to a different amino acid denoted "x". In some non-limiting exemplary embodiments, amino acid "x" can be threonine (Thr or T), leucine (Leu or L), phenylalanine (Phe or F), aspartic acid (Asp or D), or glycine (Gly or G).

[00041] As used herein, the term FXa^{V17y} refers to a variant of activated Factor X wherein the amino acid corresponding to position 236 in SEQ ID NO:1 (corresponding to position 17 in the chymotrypsin numbering system) is changed from the amino acid in the wild-type sequence (valine) to a different amino acid denoted "y". In some non-limiting exemplary embodiments, amino acid "y" can be leucine (Leu or L), alanine (Ala or A), or glycine (Gly or G).

[00042] The terms FXa^{I16x} and FXa^{V17y} are not limited by the protein sequence set forth in SEQ ID NO:1. Rather these terms additionally include the variety of isoforms and homologous proteins described herein with the specified substitution

mutations at positions 16 or 17 in the chymotrypsin numbering system that behave as zymogens until incorporated into prothrombinase complex.

[00043] An FXa variant of the disclosure may be produced by any technique for expressing a protein.

[00044] An "isolated protein," "isolated polypeptide" or "isolated variant" is a protein, polypeptide or variant that by virtue of its origin or source of derivation (1) is not associated with naturally associated components that accompany it in its native state, (2) is free of other proteins from the same species, (3) is expressed by a cell from a different species, or (4) does not occur in nature. Thus, a polypeptide that is chemically synthesized or synthesized in a cellular system different from the cell from which it naturally originates will be "isolated" from its naturally associated components. A protein may also be rendered substantially free of naturally-associated components by isolation, using protein purification techniques well known in the art.

[00045] A protein or polypeptide is "substantially pure," "substantially homogeneous," or "substantially purified" when at least about 60 to 75% of a sample exhibits a single species of polypeptide. The polypeptide or protein may be monomeric or multimeric. A substantially pure polypeptide or protein will typically comprise about 50%, 60%, 70%, 80% or 90% W/W of a protein sample, more usually about 95%, and may be over 99% pure. Protein purity or homogeneity may be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band upon staining the gel with a stain well known in the art. For certain purposes, higher resolution may be provided by using HPLC or other means well known in the art for purification.

[00046] The methods of the disclosure are useful to counteract a direct FXa inhibitor. A direct FXa inhibitor is an inhibitor that binds directly to FXa and selectively binds FXa over other proteases. Direct FXa inhibitors are noncompetitive inhibitors of FXa with respect to prothrombin. They bind the substrate binding cleft and inhibit FXa competitively with respect to small peptide substrates that also bind this region. They inhibit FXa with high picomolar affinity and are highly protein bound in plasma. Examples of direct FXa inhibitors are rivaroxaban, apixaban, betrixaban, darexaban, edoxaban and otamixaban. In

certain embodiments, direct FXa inhibitors are selected from rivaroxaban and apixaban.

[00047] According to the disclosure, an FXa variant can be used to counteract a direct FXa inhibitor that binds FXa or that binds FXa that has formed prothrombinase. The direct FXa inhibitors may or may not require cofactors of FXa for inhibition. According to the methods of the disclosure, an FXa variant, such as FXa^{I16L} and FXa^{I16T}, are administered to a subject whose blood contains a direct FXa inhibitor.

[00048] The disclosure encompasses the use of a FXa variant to counteract direct FXa inhibitors, including but not limited to synthetic inhibitors, small molecule inhibitors, orally available inhibitors, or reversible inhibitors. The FXa inhibitor may be any combination of these features, such as an orally available, synthetic, reversible, small molecule inhibitor. In certain embodiments, the direct FXa inhibitors may be selected from rivaroxaban, apixaban, betrixaban, darexaban, edoxaban and otamixaban (see Perzborn et al., Nat Rev Drug Discov. 2011 Jan;10(1):61-75; Turpie, Arterioscler Thromb Vasc Biol. 2007 Jun;27(6):1238-47; Pinto et al., Expert Opin. Ther. Patents 22:645-661 (2012); Pinto, et al., J. Med. Chem. 50:5339-5356 (2007), each of which is incorporated by reference herein). In certain embodiments, direct FXa inhibitors are selected from rivaroxaban or apixaban.

[00049] In some embodiments, a FXa variant of the disclosure can be administered to a subject to reverse the effects of a direct FXa inhibitor where such inhibitor occurs at therapeutic concentrations. In other embodiments, a FXa variant of the disclosure can be administered to a subject to reverse the effects of a direct FXa inhibitor where such inhibitor occurs at supratherapeutic concentrations. A supratherapeutic concentration is one that is higher than that ordinarily considered required to safely achieve anti-coagulation in a particular subject or class of subjects. Supratherapeutic concentrations of a direct FXa inhibitor can result from accidental or intentional overdose. Supratherapeutic concentrations of a direct FXa inhibitor can also result from unexpected effects in particular subjects, such as an unexpectedly high sensitivity to these drugs, or unexpectedly slow rate of clearance, due for example to drug interactions or other factors. Determination of what would be a therapeutic concentration or

supratherapeutic concentration of direct FXa inhibitor in a particular subject or class of subjects is within the knowledge of those ordinarily skilled in the art.

[00050] According to the disclosure, an FXa variant is used to counteract a direct FXa inhibitor or inhibitors that selectively bind FXa over other trypsin-like proteases by at least 5-fold, at least 6-fold, at least 7-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 30-fold, at least 50-fold, at least 100-fold, at least, 500-fold, at least 1,000-fold, at least 5,000-fold or at least 10,000-fold.

[00051] The direct FXa inhibitor may bind an FXa variant with a K_i of about 2 x 10^{-7} M or less. "K_i" refers to the inhibitor constant of a particular inhibitor-target interaction, which is the concentration required to produce half maximum inhibition. One can determine the K_i by using methods known in the art. The disclosure contemplates, thus, counteracting a direct FXa inhibitor that binds an FXa variant free of the prothrombinase complex with a K_i of about 2 x 10^{-8} M or less, about 1 x 10^{-8} M or less, about 9 x 10^{-9} M or less, about 8 x 10^{-9} M or less, about 7 x 10^{-9} M or less, about 6 x 10^{-9} M or less, about 5 x 10^{-9} M or less, about 4 x 10^{-9} M or less, about 3 x 10^{-9} M or less, about 2 x 10^{-9} M or less , about 1 x 10^{-9} M or less, about 9 x 10^{-10} M or less, about 8 x 10^{-10} M or less, about 7 x 10^{-10} M or less, about 6 x 10^{-10} M or less, about 5 x 10^{-10} M or less, about 4 x 10^{-10} M or less, about 3 x 10^{-10} M or less, about 2 x 10^{-10} M or less, about 1 x 10^{-10} M or less, about 9 x 10^{-11} M or less, about 8 x 10^{-11} M or less, about 7 x 10^{-11} M or less, about 6 x 10^{-11} M or less, about 5 x 10^{-11} M or less, about 4 x 10^{-11} M or less, about 3 x 10^{-11} M or less, about 2 x 10^{-11} M or less, about 1 x 10^{-11} M or less, about 9 x 10^{-12} M or less, about 8 x 10^{-12} M or less, about 7 x 10^{-12} M or less, about 6 x 10^{-12} M or less, about 5 x 10^{-12} M or less, about 4 x 10^{-12} M or less, about 3 x 10^{-12} M or less, about 2 x 10^{-12} M or less, or about 1 x 10^{-12} M or less, or less. The direct FXa inhibitor to be counteracted by an FXa variant according to the methods of the disclosure may bind a wild-type FXa with a K_i at least 1.5 fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 30-fold, or at least 50-fold less than it binds the FXa variant. The direct FXa inhibitor may bind a wild-type FXa with a K_i of at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least

95%, or at least 99% less than the K_i with an FXa variant free of the prothrombinase complex. The direct FXa inhibitor may bind a prothrombinase complex comprising a wild-type FXa with about the same K_i as it binds a prothrombinase complex comprising an FXa variant.

[00052] In one aspect, the disclosure provides methods for counteracting the effects of a direct FXa inhibitor in a subject who is bleeding (internally or externally) or is at risk of bleeding (e.g., in the course of a planned surgery) by administering a FXa variant. In some embodiments, the direct FXa inhibitor may be present in the subject at a therapeutic concentration or a higher concentrations (i.e., a supratherapeutic concentration). In some embodiments, the therapeutic concentration may be an overdose in sensitive individuals. The methods of the disclosure, thus, are useful for providing an antidote to an overdose of a direct FXa inhibitor. In various embodiments, the subject of treatment may be a human or a veterinary subject.

[00053] Direct inhibitor overdose can be detected based on existence of symptoms or signs of excessively reduced clotting ability. Non-limiting examples include evidence of gastrointestinal bleeding, including dark tarry stools, bloody stools, and vomiting of blood. Other examples include nosebleeds, and increased tendency to, or severity of, bruising or bleeding from minor cuts and scrapes.

[00054] In a clinical setting, direct inhibitor overdose can be detected directly or by measuring the ability of subject blood to clot and detecting deviations from the expected degree of anti-coagulation. Blood clotting potential can be measured in ways familiar to those ordinarily skilled in the art. For example, overdose may be suspected when a subject's prothrombin time is excessively prolonged. In certain embodiments, overdose is confirmed when the prothrombin time expressed as an International Normalized Ratio (INR) is measured to be greater than about 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, 12, 14, 16, 18, 20, or greater.

[00055] The FXa variant may be administered whenever it is desired to counteract the effects of the direct FXa inhibitor, including but not limited to before a planned surgery, after an injury resulting in external or internal bleeding or after a direct FXa inhibitor overdose. According to the disclosure, the FXa variant may be administered at least about 12 hours, at least about 6 hours, at least about 3

hours, at least about 2 hours, at least about 1 hour, at least about 30 minutes, at least about 10 minutes, or at least about 5 minutes of when the desired counteracting effect is needed, such as before a planned surgery, after an injury resulting in external or internal bleeding or after a direct FXa inhibitor overdose.

[00056] According to another embodiment, the disclosure provides a method of administering a FXa variant to effect the urgent reversal of acquired coagulopathy due to FXa inhibition therapy in a subject with acute major bleeding. In some embodiments, subjects are adult human patients. In other embodiments, subjects are pediatric human patients.

[00057] In some embodiments, acute major bleeding is caused by trauma. In other embodiments, acute major bleeding occurs during surgery or other type of interventional procedure. Exemplary non-limiting interventional procedures include incisions, drainage, vascular surgery, appendectomy, herniotomy or hernioplasty, abdominal surgery, cholecystectomy, trephination (burr hole), lumbar puncture, cardiac pacemaker insertion, hip fracture surgery, and others. In yet other embodiments, acute major bleeding can be spontaneous bleeding with no apparent cause.

[00058] Without limitation, sites of acute major bleeding include gastrointestinal bleeding, subcutaneous or intramuscular bleeding, bladder bleeding, hemarthrosis, subdural hematoma, nasal bleeding, peritoneal bleeding, uterine bleeding, and other sites of bleeding.

[00059] Effective treatment with FXa variants of the disclosure can reverse the effects of a direct FXa inhibitor. Successful reversal of such effects by a FXa variant can be determined in a variety of ways and be measured or monitored using different assays, methods, or endpoints.

[00060] In some embodiments, treatment with a FXa variant to reverse the effects of a direct FXa inhibitor is monitored using tests or assays performed on blood or plasma from a subject treated with FXa variant. A blood sample can be taken from a subject at a predetermined time after treatment with FXa variant. The blood, or plasma prepared from it, is then subjected to one or more tests to determine if certain hemostatic pharmacodynamic parameters have been normalized despite the presence of direct FXa inhibitor. If normalization is found then the subject need not be further treated with FXa variant. If normalization is

not found, however, then further treatment with FXa variant in accordance with the methods of the disclosure may be required to reverse the effect of a direct FXa inhibitor. Tests for monitoring the effectiveness of treatment with a FXa variant include tests that directly or indirectly measure the ability to clot or that measure the activity of a direct FXa inhibitor. Non-limiting exemplary tests include prothrombin time or the related International Normalized Ratio, the prothrombinase-induced clotting time assay, thromboelastometry, thromboelastography, chromogenic anti-FXa assay, thrombin generation assay, level of prothrombin fragment 1 + 2, level of thrombin-antithrombin III complex, activated partial thromboplastin time, and partial thromboplastin time. Other tests are also possible within the knowledge of those of ordinary skill in the art.

[00061] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant reduces bleeding in the subject. In some embodiments, treatment with FXa variant reduces bleeding in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in the presence of a direct FXa inhibitor compared to absence of treatment with FXa variant. In other embodiments, treatment with FXa variant reduces bleeding in a subject about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

[00062] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant reduces the activity of a direct FXa inhibitor in the subject. In some embodiments, treatment with FXa variant reduces activity of the direct FXa inhibitor in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in the presence of a direct FXa inhibitor compared to absence of treatment with FXa variant. In other embodiments, treatment with FXa variant reduces the activity of a direct FXa inhibitor in a subject about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

[00063] Activity of a direct FXa inhibitor can be monitored using a chromogenic anti-FXa assay, such as that described in Asmis, et al., Thromb Res., 129:492-

498 (2012), or Barrett, et al., Thromb Haemost. 104:1263-71 (2010), each of which are incorporated by reference herein.

[00064] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant increases the amount of thrombin produced in the blood or plasma of the subject. In some embodiments, treatment with FXa variant increases thrombin production in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 1.5 fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, at least 50-fold, or more in the presence of a direct FXa inhibitor compared to the absence of an FXa variant. Thrombin production in the blood or plasma of a subject can be determined using the thrombin generation assay (TGA) or other technique familiar to those of ordinary skill in the art.

[00065] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant increases clotting in the subject. In some embodiments, treatment with FXa variant increases clotting in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 1.5 fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, at least 50-fold, or more in the presence of a direct FXa inhibitor compared to the absence of an FXa variant.

[00066] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant reduces clotting time in the subject. In some embodiments, treatment with FXa variant reduces clotting time in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in the presence of a direct FXa inhibitor compared to absence of treatment with FXa variant. In other embodiments, treatment with FXa variant reduces clotting time in a subject about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

[00067] According to some embodiments, clotting time is determined by measuring the subject's prothrombin time (PT) which decreases as hemostasis is restored. PT is the amount of time it takes for serum to clot after addition of tissue factor. PT therefore measures the capability of the extrinsic clotting system to

support clotting. PT can vary depending on the particular reagents a lab uses to run the test, but a normal PT is about 11 to 13 seconds. Clotting time can also be expressed using the International Normalized Ratio (INR), which eliminates lab to lab variability in clotting time measurements. Using the INR, a ratio of 0.8 to 1.1 indicates normal clotting. PT or INR can be determined at a predetermined time after a FXa variant is administered to a subject in need of reversal of the effects of a direct FXa inhibitor.

[00068] In some embodiments, treatment with a FXa variant to reverse the effects of a direct FXa inhibitor reduces the PT of a subject to about 25 seconds, 24 seconds, 23 seconds, 22 seconds, 21 seconds, 20 seconds, 19 seconds, 18 seconds, 17 seconds, 16 seconds, 15 seconds, 14 seconds, 13 seconds, 12 seconds, 11 seconds, 10 seconds, or less. In other embodiments, treatment with a FXa variant reduces the INR or a subject to about 4.0, 3.9, 3.8, 3.7, 3.6, 3.5, 3.4, 3.3, 3.2, 3.1, 3.0, 2.9, 2.8, 2.7, 2.6, 2.5, 2.4, 2.3, 2.2, 2.1, 2.0, 1.9, 1.8, 1.7, 1.6, 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, or less. According to other embodiments, treatment with FXa variant reduces PT or INR in a subject about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

[00069] Prothrombin time can be measured at a predetermined after administration of a FXa variant. Thus, in some non-limiting embodiments, PT is measured 15 mins, 20 mins, 30 mins, 40 mins, 45 mins, 50 mins, 60 mins or more after administration of FXa. Other times are also possible according to the knowledge of those of ordinary skill in the art.

[00070] Clotting time can also be measured using the one-step prothrombinase-induced clotting time (PiCT) assay as described in Graff, et al., Monitoring effects of direct FXa-inhibitors with a new one-step prothrombinase-induced clotting time (PiCT) assay: comparative in vitro investigation with heparin, enoxaparin, fondaparinux and DX 9065a, Int J Clin Pharmacol Ther., 45:237-43 (2007) and Harder, et al., Monitoring direct FXa-inhibitors and fondaparinux by Prothrombinase-induced Clotting Time (PiCT): relation to FXa-activity and influence of assay modifications, Thromb Res.,123:396-403 (2008), each of which are incorporated by reference.

[00071] In yet other embodiments, the methods of thromboelastometry or thromboelastography may be used to analyze clot formation or clotting time.

[00072] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant increases the level of prothrombin fragment 1 + 2 (PF1 + 2) in the blood or plasma of the subject. In some embodiments, treatment with FXa variant increases PF1 + 2 in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, at least 50-fold, or more in the presence of a direct FXa inhibitor compared to the absence of an FXa variant.

[00073] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant increases the level of thrombin-antithrombin III complex (TAT) in the blood or plasma of the subject. In some embodiments, treatment with FXa variant increases TAT in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, at least 50-fold, or more in the presence of a direct FXa inhibitor compared to the absence of an FXa variant.

[00074] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant reduces activated partial thromboplastin time (aPTT) in the subject. In some embodiments, treatment with FXa variant reduces activated partial thromboplastin time (aPTT) in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in the presence of a direct FXa inhibitor compared to absence of treatment with FXa variant. In other embodiments, treatment with FXa variant reduces aPTT in a subject about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

[00075] According to some embodiments, reversing the effects of a direct FXa inhibitor in a subject by administering a FXa variant reduces partial thromboplastin time (PTT) in the subject. In some embodiments, treatment with FXa variant reduces partial thromboplastin time (PTT) in a subject at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% in the presence of a direct FXa

inhibitor compared to absence of treatment with FXa variant. In other embodiments, treatment with FXa variant reduces PTT in a subject about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

[00076] In other embodiments, clinical endpoints can be relied upon to determine if hemostasis has been adequately restored in a subject treated with a FXa variant to reverse the effects of a direct FXa inhibitor. For example, where a subject presents with acute bleeding, clinical hemostatic efficacy can be scored "very good" where prompt cessation of existing bleeding occurs after treatment with FXa variant; "satisfactory" where there is a 1-2 hr delay in bleeding cessation; "questionable" where there is a >2 hr delay in bleeding cessation; and "none" where an effect on bleeding is absent. Where treatment with FXa variant is determined to be less than satisfactory, then an additional dose of FXa variant can be administered to effect adequate hemostasis. In a further example, where a subject is undergoing an interventional procedure, clinical hemostatic efficacy can be scored "very good" where normal hemostasis is attained during the procedure; "satisfactory" where intraprocedural hemostasis is mildly abnormal as judged by quantity or quality of blood loss (e.g., slight oozing); "questionable" where intraprocedural hemostasis is moderately abnormal as judged by quantity or quality of blood loss (e.g., controllable bleeding); and "none" where intraprocedural hemostasis is severely abnormal as judged by quantity or quality of blood loss (e.g., severe refractory hemorrhage).

[00077] A therapeutically effective dose of a direct FXa inhibitor depends upon numerous factors that are well known to a medical practitioner of skill in the art. A typical therapeutic plasma concentration of rivaroxaban is about 500 nM. However, according to the disclosure, an FXa variant can be administered to counteract lower or higher concentrations of inhibitor. The plasma concentration of rivaroxaban in a subject to be treated with an FXa variant may be lower or higher than the typical therapeutic concentration, for example about 100 nM, about 200 nM, about 300 nM, about 400 nM, about 500 nM, about 600 nM, about 700 nM, about 800 nM, about 900 nM or about 1,000 nM.

[00078] A typical therapeutic plasma concentration of apixaban is about 250 nM. In certain embodiments, the FXa variant is administered to a subject with a plasma concentration of apixaban of about 100 nM, about 200 nM, about 300 nM, about 400 nM, about 500 nM, about 600 nM, about 700 nM, about 800 nM, about 900 nM or about 1,000 nM.

[00079] Likewise, according to the disclosure, an FXa variant can be used to counteract a direct FXa inhibitor in cases of overdose, such as when the plasma concentration of the inhibitor is at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or at least 1.5 fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 30-fold, or at least 50-fold higher than the typical therapeutic plasma concentration.

[00080] The FXa variants are surprisingly effective in counteracting a direct FXa inhibitor at a plasma concentration that is lower than the plasma concentration of the direct FXa inhibitor. According to the disclosure, the FXa variant counters the effect of a direct FXa inhibitor at a plasma concentration ratio of variant to inhibitor of about 1 to 10, about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 250, about 1 to 500, about 1 to 1,000, about 1 to 2,500, about 1 to 5,000 or about 1 to 10,000. In certain embodiments, the FXa variant counters the effect of a direct FXa inhibitor at a plasma concentration of at least 10-fold, at least 25-fold, at least 50-fold, at least 100-fold, at least 250-fold, at least 500-fold, at least 1,000-fold, at least 2,500-fold, at least 5,000-fold, or at least 10,000-fold lower than the plasma concentration of the direct FXa inhibitor.

[00081] In other embodiments, the plasma concentration of an FXa variant sufficient to reverse the effect of a direct FXa inhibitor is calculated by multiplying the plasma concentration of the direct inhibitor by a conversion factor ranging from about 0.1×10^{-4} to about 1000×10^{-4}, about 4×10^{-4} to about 40×10^{-4}, about 20×10^{-4} to about 200×10^{-4}, or other ranges. In yet other embodiments, the conversion factor can be about 0.1×10^{-4}, 0.5×10^{-4}, 1×10^{-4}, 2×10^{-4}, 3×10^{-4}, 4×10^{-4}, 5×10^{-4}, 6×10^{-4}, 7×10^{-4}, 8×10^{-4}, 9×10^{-4}, 10×10^{-4}, 11×10^{-4}, 12×10^{-4}, 13×10^{-4}, 14×10^{-4}, 15×10^{-4}, 16×10^{-4}, 17×10^{-4}, 18×10^{-4}, 19×10^{-4}, 20×10^{-4}, 21×10^{-4}, 22×10^{-4}, 23×10^{-4}, 24×10^{-4}, 25×10^{-4}, 26×10^{-4}, 27×10^{-4}, 28×10^{-4}, $29 \times$

10^{-4}, 30 x 10^{-4}, 31 x 10^{-4}, 32 x 10^{-4}, 33 x 10^{-4}, 34 x 10^{-4}, 35 x 10^{-4}, 36 x 10^{-4}, 37 x 10^{-4}, 38 x 10^{-4}, 39 x 10^{-4}, 40 x 10^{-4}, 45 x 10^{-4}, 50 x 10^{-4}, 55 x 10^{-4}, 60 x 10^{-4}, 65 x 10^{-4}, 70 x 10^{-4}, 75 x 10^{-4}, 80 x 10^{-4}, 85 x 10^{-4}, 90 x 10^{-4}, 95 x 10^{-4}, 100 x 10^{-4}, 110 x 10^{-4}, 120 x 10^{-4}, 130 x 10^{-4}, 140 x 10^{-4}, 150 x 10^{-4}, 160 x 10^{-4}, 170 x 10^{-4}, 180 x 10^{-4}, 190 x 10^{-4}, 200 x 10^{-4}, 250 x 10^{-4}, 300 x 10^{-4}, 350 x 10^{-4}, 400 x 10^{-4}, 450 x 10^{-4}, 500 x 10^{-4}, 550 x 10^{-4}, 600 x 10^{-4}, 650 x 10^{-4}, 700 x 10^{-4}, 750 x 10^{-4}, 800 x 10^{-4}, 850 x 10^{-4}, 900 x 10^{-4}, 950 x 10^{-4}, or 1000 x 10^{-4}, and ranges among these numbers. Plasma concentration of FXa direct inhibitor can be measured according to the knowledge of the skilled artisan, for example, by radio-immuno assay (RIA) or other method.

[00082] Achieving a target plasma concentration of FXa variant sufficient to reverse overdose of a direct FXa inhibitor is within the knowledge of those ordinarily skilled in the art. In a non-limiting example, estimates of relevant pharmacokinetic parameters, such as subject plasma volume or other parameters, can be made based on upon subject sex, height and weight, or other factors, and used to calculate how much FXa variant needs be administered to achieve the target concentration. After administering FXa variant, plasma concentrations can be monitored according to the knowledge of those ordinarily skilled in the art and this information used to maintain the concentration in any desired range.

[00083] The compositions and methods of the disclosure include a "therapeutically effective amount" or a "prophylactically effective amount" of an FXa variant. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the FXa variant may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the FXa variant to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the FXa variant are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. For example, a dose may be given prior to a planned surgery.

[00084] Dosage regimens can be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single

bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the disclosure are dictated by and directly dependent on (a) the unique characteristics of the FXa variant and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an FXa variant for the treatment of individuals.

[00085] In certain embodiments, a therapeutically or prophylactically-effective amount of an FXa variant administered is about 0.0001 to 50 mg/kg, about 0.001 to 50 mg/kg, about 0.001 to 5 mg/kg, about 0.001 to 0.5 mg/kg, about 0.001 to 0.05 mg/kg, about 0.01 to 5 mg/kg or about 0.01 to 0.5 mg/kg.

[00086] In certain embodiments, a therapeutically or prophylactically-effective serum concentration of an FXa variant of the disclosure is about 0.0003 to 300 nM, about 0.003 to 300 nM, about 0.03 to 300 nM, about 0.003 to 30 nM, about 0.03 to 30 nM or about 0.3 to 3 nM. The concentration of the FXa variant, for example in blood or plasma, may be measured by any method known in the art.

[00087] It is to be noted that dosage values may vary with FXa inhibitor concentration. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

[00088] Another aspect of the present disclosure provides kits comprising an FXa variant or a composition comprising such an FXa variant. A kit may include, in addition to the FXa variant or composition, diagnostic or additional therapeutic agents. A kit can also include instructions for use in a therapeutic method, as well

as packaging material such as, but not limited to, ice, dry ice, styrofoam, foam, plastic, cellophane, shrink wrap, bubble wrap, cardboard and starch peanuts. In one embodiment, the kit includes the FXa variant or a composition comprising it and one or more therapeutic agents that can be used in a method described herein.

[00089] The FXa variant may be administered, for example in a composition comprising it, once or multiple times to a subject until adequate hemostasis is restored or the direct FXa inhibitor or inhibitors are no longer effective. Where multiple administrations are used they may administered hourly, daily, or at any other appropriate interval, including for example multiple daily doses. Multiple doses may be administered on a schedule such as every 10 minutes, every 15 minutes, every 20 minutes, every 30 minutes, every hour, every two hours, every three hours, every four hours, three times daily, twice daily, once daily, once every two days, once every three days, and once weekly. The FXa variant may also be administered continuously, *e.g.* via a minipump. The FXa variant may be administered, for example, via a parenteral route (*e.g.*, intravenously, subcutaneously, intraperitoneally, or intramuscularly). The FXa variant will generally be administered as part of a pharmaceutical composition as described below.

[00090] In another embodiment, the FXa variant may be co-administered with another procoagulant including another FXa variant, Factor IX, Factor XIa, Factor XIIa, Factor VIII, Factor VIIa, FEIBA and prothrombin complex concentrate (PCC).

[00091] Co-administration of an FXa variant of the disclosure with an additional therapeutic agent (combination therapy) encompasses administering a pharmaceutical composition comprising the FXa variant and the additional therapeutic agent, as well as administering two or more separate pharmaceutical compositions, i.e., one comprising the FXa variant and the other(s) comprising the additional therapeutic agent(s). Co-administration or combination therapy further includes administering the FXa variant and additional therapeutic agent(s) simultaneously or sequentially, or both. For instance, the FXa variant may be administered once every three days, while the additional therapeutic agent is administered once daily at the same as the FXa variant, or at a different time. An FXa variant may be administered prior to or subsequent to treatment with the

additional therapeutic agent. Similarly, administration of an FXa variant of the disclosure may be part of a treatment regimen that includes other treatment modalities including surgery. The combination therapy may be administered to prevent recurrence of the condition. The combination therapy may be administered from multiple times hourly to weekly. The administrations may be on a schedule such as every 10 minutes, every 15 minutes, every 20 minutes, every 30 minutes, every hour, every two hours, every three hours, every four hours, three times daily, twice daily, once daily, once every two days, once every three days, once weekly, or may be administered continuously, *e.g.* via a minipump. The combination therapy may be administered, for example, via a parenteral route (*e.g.*, intravenously, subcutaneously, intraperitoneally, or intramuscularly).

[00092] In a further aspect, the disclosure provides a composition comprising an FXa variant for use in counteracting a direct FXa inhibitor in a subject. The composition may comprise a pharmaceutically acceptable carrier, vehicle or other ingredients that are physiologically compatible. Non-limiting examples of such carriers, vehicles and other ingredients include solvents (e.g., water, ethanol, saline, phosphate buffered saline), detergents, surfactants, dispersion media, coatings, antibacterial or antifungal agents, isotonifying agents, absorption delaying agents, sugars (e.g., sucrose, dextrose, lactose), polyalcohols (e.g., glycerol, mannitol, sorbitol), salts (e.g., sodium chloride, potassium chloride), wetting agents, emulsifying agents, preservatives, buffers, and agents capable of enhancing the stability or effectiveness of the FXa variant.

[00093] A composition for use according to the disclosure may be in any suitable form for administration to a subject, such as liquid solutions (*e.g.*, injectable and infusible solutions). Compositions can be provided in a pre-mixed format ready for administration to a subject, for example, in a vial or pre-filled syringe. Such formats do not require reconstitution with diluent before administration. Alternatively, compositions can be provided in lyophilized form requiring reconstitution with diluent (e.g., sterile water or saline) before administration. If the latter, diluent can be provided with the lyophilisate in a separate container. According to the knowledge of those of ordinary skill in the art, compositions can be formulated for storage under refrigeration or at room temperature. The form of the composition depends, at least in part, on the

intended mode of administration. In certain embodiments, the mode of administration is parenteral, including for example intravenous, subcutaneous, intraperitoneal, or intramuscular administration.

[00094] Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, in liposomes, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the FXa variant in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

[00095] It is further contemplated by the present disclosure that any of the compositions herein may be administered to a subject being treated with a direct FXa inhibitor.

[00096] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be apparent to persons skilled in the art and are to be included within the and can be made without departing from the true scope of the invention.

EXAMPLES

Example 1 - FXa^{I16L} Sensitivity Toward Rivaroxaban

[00097] To test the sensitivity of FXa^{I16L} toward rivaroxaban, inhibition assays were established. Rivaroxaban was an efficient inhibitor of wild-type FXa

exhibiting an inhibition constant (K_i) of 0.582 nM (**Figure 1A**). Due to the zymogen-like nature of FXa^{I16L}, rivaroxaban bound with a ~15-fold reduced affinity to this variant (K_i = 9.3 nM) (**Figure 1B**). In contrast, when the variant was assembled in the prothrombinase complex (i.e. upon addition of FVa and phospholipid vesicles), the K_i for rivaroxaban was nearly restored to a value comparable to the wild-type enzyme (wt FXa, K_i = 2.67 nM (**Figure 2A**); FXa^{I16L}, K_i = 3.4 nM (**Figure 2B**).

Example 2 - FXa Variants Counteract Rivaroxaban and Apixaban

[00098] The thrombin generation assay (TGA) was used to assess whether the zymogen-like FXa variants can reverse the effects of direct FXa inhibitors in a more physiologic environment. The TGA measures thrombin production in plasma over time following the initiation of coagulation and was performed as previously described (See Bunce et al., (2011) *Blood* **117,** 290-298, incorporated by reference herein in their entirety). Thrombin generation in normal human plasma was measured for 90 min at 37°C in the presence or absence of 500 nM rivaroxaban. To evaluate if FXa^{I16L} can reverse the effect of rivaroxaban, increasing amounts of FXa^{I16L} was added to plasma containing 500 nM rivaroxaban. Thrombin generation was initiated with 2.0 pM tissue factor/4 uM phospholipid as well as $CaCl_2$ and a thrombin fluorogenic substrate.

[00099] The data demonstrated that the thrombin generation profile of plasma in the presence of 500 nM rivaroxaban was substantially reduced compared to plasma in the absence of rivaroxaban. In contrast, increasing concentrations of FXa^{I16L} from 0.03 to 1 nM restored thrombin generation (**Figure 3**). These data show that unexpectedly low concentrations of FXa^{I16L} in the nanomolar and subnanomolar range can reverse the effects of the inhibitor. Dose response analysis of FXa^{I16L} in the presence of 500 nM rivaroxaban (a typical therapeutic plasma concentration) shows that the peak height of thrombin generation (**Figure 4A and C**) and total thrombin produced (ETP) (**Figure 4 B and D**) essentially reached a maximum and was completely restored to normal levels between 1-3 nM of FXa^{I16L} under these conditions. Further experiments showed that even in the presence of high concentration of rivaroxaban (7.5 μM; supratherapeutic),

FXa^{I16L} was still quite effective at a relatively low dose (≤ 3.0 nM) in restoring peak thrombin (**Figure 5A**) as well as total thrombin generated (**Figure 5B**).

[000100] Similar experiments were also performed to evaluate whether FXa zymogen-like variants could reverse the effects of another direct FXa inhibitor, apixaban. In these experiments, the effectiveness of FXa^{I16L} with another zymogen-like FXa variant, FXa^{I16T}, was also compared. FXa^{I16T} is similar to FXa^{I16L}, however it has intrinsically less activity, has a longer plasma half-life, and has ~3-5-fold reduced activity compared to FXa^{I16L} when assembled in the prothrombinase complex. Consistent with the rivaroxaban data, FXa^{I16L} could restore peak thrombin (**Figure 6A**) and total thrombin (**Figure 6B**) generated in the presence of 250 nM apixaban (a typical therapeutic plasma concentration) in a dose-dependent manner, which appears to reach a maximum between 1-3 nM of FXa^{I16L}. FXa^{I16T} was also effective at reversing the effects of apixaban; however, it appears that higher concentrations of this variant are needed to fully restore thrombin generation (**Figure 6**). Both variants were still effective even in the presence of a higher concentration of apixaban (2 μM). However, under these conditions it appears that higher concentrations of both variants are needed to fully restore thrombin generation (**Figure 7A and B**).

Example 3 - FXa^{I16L} Counteracts Rivaroxaban and Apixaban in Whole Blood

[000101] Whole blood thromboelastometry was used to assess the ability of the FXa^{I16L} variant to reverse the effects of the direct FXa inhibitors in whole blood. In this system, blood is drawn from healthy volunteers. The first 2 mL of blood were discarded and the subsequent 5 mL of blood was collected into a vacutainer (BD, Franklin Lakes, NJ). Corn trypsin inhibitor and sodium citrate were in the collection tube, prior to collection of the blood sample, to achieve a final concentration of 0.105 M citrate and 25 μg/mL corn trypsin inhibitor (Haematologic Technologies, Burlington, VT) in the blood. Two sets of reactions were analyzed for each donor. The initial reaction initiated 5 minutes post blood collection. The second reaction initiated 1 hour post initiation of first reaction (1 hour 5 minutes after collection).

[000102] Blood was analyzed using Thromboelastometry ROTEM®delta (Tem International GmbH, Munich, Germany). For the reaction: (1) 6 μL of vehicle, protein, and/or inhibitor were added to the empty cup, (2) 20 μl of 0.2 M $CaCl_2$

(final concentration 11.6 mM), and (3) 20 μl of Innovin (final concentration in reaction 1:10,000; source of tissue factor) were added to the cup. Whole blood collected as described above was added to the reaction (300 μL) and recordings were allowed to proceed for approximately 30-60 minutes. The data collected was analyzed using the manufacture's software (Rotem Gamma Software Version 1.1.1).

[000103] The ability of FXa^{I16L} to accelerate whole blood clot formation in the presence of rivaroxaban or apixaban was examined using rotational thromboelastometry (ROTEM). Both direct FXa inhibitors alone and at two different concentrations have a substantial effect on whole blood clot formation: at low doses (therapeutic concentrations), whole blood clot formation is partially eliminated (**Figures 8A and Figure 9A**), while at high doses (supratherapeutic concentrations), whole blood clot formation is almost completely eliminated (**Figures 8B and Figure 9B**). The effects of either rivaroxaban or apixaban on whole blood clot formation could be reversed by FXa^{I16L}. In the presence of either 500 nM rivaroxaban or 250 nM apixaban, 0.3 nM FXa^{I16L} could fully or nearly fully restore whole blood coagulation (**Figures 8A and Figure 9A**). When higher concentrations of the direct FXa inhibitors were used (~ 2 uM), 0.3 nM FXa^{I16L} partially restored whole blood coagulation and 3 nM FXa^{I16L} fully restored it (**Figures 8B and Figure 9B**). These data demonstrated that an FXa zymogen-like variant can effectively reverse the anticoagulant effect of rivaroxaban or apixaban in plasma-based and whole-blood coagulation assays at both therapeutic and supratherapeutic concentrations of the inhibitor.

[000104] The results of these studies were confirmed and extended by testing if FXa^{I16L} could counteract the anti-coagulant effect of rivaroxaban when both agents were administered in vivo. In these experiments, C57BL/6 mice were infused with rivaroxaban (1 mg/kg) or buffer via the tail vein. Mice were then prepared to expose the jugular vein and the vena cava. Approximately 10 min later FXa^{I16L} (1 or 2 mg/kg) was infused by direct injection into the jugular vein. Five minutes post injection blood was collected via the vena cava into citrate and corn trypsin inhibitor. Collected blood was then analyzed by ROTEM using dilute tissue factor (Innovin, 1:42,000 dilution). Whole blood from mice administered buffer only clotted by about 2 min (**Figure 12**). Administration of 1 mg/kg

rivaroxaban substantially prolonged the clot time to about 10 min (**Figure 12**). Further administration of FXa^{I16L} shortened clotting time in the presence of rivaroxaban in a dose dependent manner (**Figure 12**).

Example 4 – FXa^{I16L} Counteracts Rivaroxaban in a Thrombin Generation Assay

[000105] The effect of FXa^{I16L} on reversing rivaroxaban in plasma was examined in a thrombin generation assay (TGA) using the calibrated automated thrombography (CAT) system (Thrombinoscope BV, Maastricht, The Netherlands). Normal human plasma was obtained from George King Biomedical (Overland Park, KS). In the reaction, 20 µL of PPP-Reagent LOW containing 4 µM phospholipids and 1 pM tissue factor was added to 70 µL of pooled citrated normal human plasma (treated with 250 nM rivaroxaban, within the therapeutic plasma concentration range) in an Immulon 2HB round bottom 96 well plate with reactions duplicated. Immediately preceding reaction initiation, 10 µL of vehicle or FXa^{I16L} was added to plasma at final concentrations ranging from 0.03125 nM to 0.5 nM FXa^{I16L}, given a 120 µL total reaction volume. Reactions were initiated by addition of 20 µL FluCa buffer containing calcium chloride and fluorogenic substrate. Fluorescence of plasma reactions was read at 37°C at 20 second intervals on a Fluoroskan Ascent fluorometer and compared to those of reference thrombin calibrator reactions to determine thrombin concentrations. The intensity of the fluorescence signal (FU) was continuously monitored at 37ºC using the CAT. Thrombin generation curves (nM thrombin vs. time) were analyzed to extract lag time, peak height, time to peak, and the area under the curve representing the endogenous thrombin potential (ETP) using the Thromboscope software (Thrombinoscope BV version).

[000106] A dose dependent inhibition of thrombin generation in normal human plasma was observed with *in vitro* rivaroxaban treatment (5-200 nM) (**Figure 10A**). Rivaroxaban resulted in an increase in the lag time coupled with a decrease in the peak thrombin and a decrease in the ETP. The addition of FXa^{I16L} to rivaroxaban (250 nM) inhibited human plasma resulted in a dose dependent reversal of thrombin inhibition (**Figure 10B**): peak thrombin generation was restored, the lag phase was shorter, and the ETP increased. At a low dose of

0.03125 nM FXa^{I16L}, thrombin generation was restored to levels comparable to vehicle treated normal human plasma.

Example 5 – FXa^{I16L} Counteracts Rivaroxaban in a Mouse Tail Clip Bleeding Model

[000107] The ability of FXa^{I16L} to overcome the effects of rivaroxaban *in vivo* was assessed in an acute bleeding model in normal mice. The results demonstrated that a zymogen-like FXa variant could reverse the anticoagulant effect of a direct FXa inhibitor.

[000108] To establish a dose of rivaroxaban that would prolong bleeding, male C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) received a single intravenous injection of rivaroxaban at a dose of 10, 25 or 50 mg/kg. Thirty minutes later, mice were anesthetized with isoflurane and placed on a heated platform, and the body temperature of the mice was maintained at 37ºC prior to the tail cut. The tails were immersed in 50 mL pre-warmed phosphate buffered saline (PBS) at 37ºC for 2 minutes. A 3 mm tail cut was made and blood was collected into PBS for a 10 minute period. A quantitative assessment of the amount of bleeding was determined by hemoglobin content of the blood collected into PBS. Tubes were centrifuged to collect erythrocytes, resuspended in 5 mL lysis buffer (8.3 g/L NH_4Cl, 1.0 g/L $KHCO_3$, and 0.037 g/L EDTA), and the absorbance of the sample was measured at 575 nm. The absorbance values were converted to total blood loss (µL) using a standard curve. The administration of rivaroxaban resulted in a dose dependent increase in blood loss following a tail cut (**Figure 11**).

[000109] In this model, a dose of 50 mg/kg rivaroxaban resulted in an increase in blood loss following the tail transection. Mice were dosed with 50 mg/kg rivaroxaban and 30 minutes later 50 or 200 ug/kg of FXa^{I16L} was dosed intravenously at 37ºC prior to the tail cut. Mice were then anesthetized with isoflurane and placed on a heated platform, and the body temperature of the mice was maintained at 37ºC prior to the tail cut. The tails were immersed in 50 mLs pre-warmed phosphate buffered saline (PBS) at 37ºC for 2 minutes. A 3 mm tail cut was made and blood was collected into PBS for a 10 minute period and the assessment of the amount of bleeding was determined by hemoglobin content as

described. In this model, the administration of the hemostatic FXa^{I16L} variant decreased the excessive bleeding loss induced with rivaroxaban (**Figure 11**).

[000110] Example 6 – FXa^{I16L} Counteracts Rivaroxaban in a Mouse Bleeding Model Demonstrated Using Intravital Microscopy

[000111] As visualized using intravital microscopy, rivaroxaban was demonstrated to inhibit thrombus formation in the microcirculation of the mouse cremaster muscle after laser-induced injury. Further administration of FXa^{I16L} could counteract the anti-coagulant effect of rivaroxaban in this system.

[000112] Using standard techniques, the cremaster muscle of mice was exposed and visualized using intravital microscopy. A vascular injury in the muscle was then induced using a laser. After injury, clot formation was visualized using different fluorescently labeled antibodies that specifically recognize fibrin and platelets. Clotting is indicated by the presence of fluorescent signal from both types of antibodies.

[000113] After laser injury, an untreated mouse rapidly formed a clot at the site of injury that was stable for several minutes (**Figure 13A**). In the video frame, the clot is visible as the coincidence of fluorescent signal associated with antibodies against fibrin and platelets (light gray center region overlapping darker gray region). Administration of 1 mg/kg rivaroxaban to a mouse, however, delayed the accumulation of platelets at the injury site and eliminated any signs of fibrin (**Figure 13B**). In the video frame, only a reduced extent of platelets can be seen as indicated by the dark gray region, which reflects presence of fluorescent signal associated with anti-platelet antibodies. By contrast, when a mouse was administered 1 mg/kg rivaroxaban followed by 0.5 mg/kg FXa^{I16L}, a clot rapidly formed at the injury site (**Figure 13C**). In the video frame, the clot is indicated by the characteristic pattern of fluorescent signal associated with antibodies against platelets and fibrin.

[000114] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural

terms shall include the singular. Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art.

[000115] The methods and techniques of the present disclosure are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Sambrook J. & Russell D.. *Molecular Cloning: A Laboratory Manual,* 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000); Ausubel et al., *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Wiley, John & Sons, Inc. (2002); Harlow and Lane *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1998); and Coligan et al., *Short Protocols in Protein Science,* Wiley, John & Sons, Inc. (2003), incorporated herein by reference. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclature used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art.

[000116] All publications, patents, patent applications or other documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document was individually indicated to be incorporated by reference for all purposes.

[000117] Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

What is claimed is:

1. A method for reducing or preventing bleeding in a subject being treated with a direct Factor Xa inhibitor, comprising administering to said subject a Factor Xa variant that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

2. A method for reducing or preventing bleeding in a subject being treated with rivaroxaban or apixaban, comprising administering to said subject a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

3. A pharmaceutical composition for reducing or preventing bleeding in a subject being treated with a direct Factor Xa inhibitor, comprising a Factor Xa variant that that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

4. A pharmaceutical composition for reducing or preventing bleeding in a subject being treated with rivaroxaban or apixaban, comprising a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

5. Use of a Factor Xa variant in the production of a medicament for reducing or preventing bleeding in a subject being treated with a direct Factor Xa inhibitor,

wherein the Factor Xa variant contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

6. Use of a Factor Xa variant in the production of a medicament for reducing or preventing bleeding in a subject being treated with rivaroxaban or apixaban, wherein the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

7. The method, composition or use of any one of claims 1-6, wherein there is a reduction in bleeding of at least about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

8. A method for increasing the amount of thrombin produced in the presence of a direct Factor Xa inhibitor in a subject in need thereof, comprising administering to said subject a Factor Xa variant that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

9. A method for increasing the amount of thrombin produced in the presence of rivaroxaban or apixaban in a subject in need thereof, comprising administering to said subject a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

10. A pharmaceutical composition for increasing the amount of thrombin produced in the presence of rivaroxaban or apixaban in a subject in need thereof, comprising a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

11. A pharmaceutical composition for increasing the amount of thrombin produced in the presence of a direct Factor Xa inhibitor in a subject in need thereof, comprising a Factor Xa variant that that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

12. Use of a Factor Xa variant in the production of a medicament for increasing the amount of thrombin produced in the presence of rivaroxaban or apixaban, wherein the Factor Xa variant contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

13. Use of a Factor Xa variant in the production of a medicament for increasing the amount of thrombin produced in the presence of a direct Factor Xa inhibitor, wherein the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

14. The method, composition or use of any one of claims 8-13, wherein the amount of thrombin produced increases at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100%, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, or 50-fold.

15. A method for decreasing clotting time in the presence of a direct Factor Xa inhibitor in a subject in need thereof, comprising administering to said subject a Factor Xa variant that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

16. A method for decreasing clotting time in the presence of rivaroxaban or apixaban in a subject in need thereof, comprising administering to said subject a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

17. A pharmaceutical composition for decreasing clotting time in the presence of a direct Factor Xa inhibitor in a subject in need thereof, comprising a Factor Xa variant that that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

18. A pharmaceutical composition for decreasing clotting time in the presence of rivaroxaban or apixaban in a subject in need thereof, comprising a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

19. Use of a Factor Xa variant in the production of a medicament for decreasing clotting time in the presence of a direct Factor Xa inhibitor in a subject in need thereof, wherein the Factor Xa variant contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and
b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

20. Use of a Factor Xa variant in the production of a medicament for decreasing clotting time in the presence of rivaroxaban or apixaban in a subject in need thereof, wherein the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

21. The method, composition or use of any one of claims 15-20, wherein there is a reduction in clotting time of at least about 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-45%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, or 95%-100%.

22. The method, composition or use of any one of claims 15-20, wherein the reduction in clotting time is measured using prothrombin time (PT).

23. The method of claim 22, wherein said PT in said subject is about 25 seconds, 24 seconds, 23 seconds, 22 seconds, 21 seconds, 20 seconds, 19 seconds, 18 seconds, 17 seconds, 16 seconds, 15 seconds, 14 seconds, 13 seconds, 12 seconds, 11 seconds, or 10 seconds.

24. The method of claim 22, wherein the International Normalized Ratio (INR) in said subject is about 4.0, 3.9, 3.8, 3.7, 3.6, 3.5, 3.4, 3.3, 3.2, 3.1, 3.0, 2.9, 2.8, 2.7, 2.6, 2.5, 2.4, 2.3, 2.2, 2.1, 2.0, 1.9, 1.8, 1.7, 1.6, 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, or 0.7.

25. The method of any one of claims 22, 23, or 24, wherein PT is determined 15 mins, 20 mins, 30 mins, 40 mins, 45 mins, 50 mins, 60 mins, 75 min, or 90 min after administration of the FXa variant.

26. A pharmaceutical composition comprising a Factor Xa variant that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

wherein said Factor Xa variant counters the effect of a direct Factor Xa inhibitor at a plasma concentration of at least 100-fold lower than the plasma concentration of the direct Factor Xa inhibitor.

27. A pharmaceutical composition comprising a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr, and wherein the Factor Xa variant counters the effect of rivaroxaban or apixaban at a plasma concentration of at least 100-fold lower than the plasma concentration of the rivaroxaban or apixaban.

28. The method, composition or use of any of the previous claims, wherein the Factor Xa variant counters the effect of a direct Factor Xa inhibitor at a plasma concentration of at least 100-fold lower than the plasma concentration of the direct Factor Xa inhibitor.

29. The method, composition or use of any of the previous claims, wherein the Factor Xa variant is administered before a planned surgery, after an injury or after a direct Factor Xa inhibitor overdose.

30. The method, composition or use of any of the previous claims, wherein Factor Xa variant is administered more than one time.

31. The method, composition or use of any of the previous claims, wherein at least one additional procoagulant is administered.

32. The method, composition or use of claim 30, wherein the procoagulant is selected from the group consisting of: a different Factor Xa variant, Factor IX,

Factor XIa, Factor XIIa, Factor VIII, Factor VIIa, FEIBA and prothrombin complex concentrate (PCC).

33. The method, composition or use of any of the previous claims, wherein the plasma concentration of the direct FXa inhibitor is a supratherapeutic amount.

34. The method, composition or use of any of the previous claims, wherein the direct FXa inhibitor is rivaroxaban and wherein the plasma concentration of rivaroxaban is at least about 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 600 nM, 700 nM, or 800 nM.

35. The method, composition or use of any of the previous claims, wherein the direct FXa inhibitor is apixaban and wherein the plasma concentration of apixaban is at least about 50 nM, 100 nM, 150 nM, 200nM, 250 nM, 300 nM, 350 nM, or 400 nM.

36. A method for effecting the urgent reversal of acquired coagulopathy due to FXa inhibition therapy in a subject with acute major bleeding, comprising administering to said subject a Factor Xa variant that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and
b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

37. A method for effecting the urgent reversal of acquired coagulopathy due to FXa inhibition therapy in a subject with acute major bleeding, comprising administering to said subject a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

38. A pharmaceutical composition for effecting the urgent reversal of acquired coagulopathy due to FXa inhibition therapy in a subject with acute major bleeding,

comprising a Factor Xa variant that that contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

39. A pharmaceutical composition for effecting the urgent reversal of acquired coagulopathy due to FXa inhibition therapy in a subject with acute major bleeding, comprising a Factor Xa variant in which the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

40. Use of a Factor Xa variant in the production of a medicament for effecting the urgent reversal of acquired coagulopathy due to FXa inhibition therapy in a subject with acute major bleeding, wherein the Factor Xa variant contains at least one modification selected from the group consisting of:

a) the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Thr, Leu, Phe, Asp or Gly; and

b) the amino acid at the position corresponding to 236 in SEQ ID NO:1 is substituted with Leu, Ala, or Gly.

41. Use of a Factor Xa variant in the production of a medicament for effecting the urgent reversal of acquired coagulopathy due to FXa inhibition therapy in a subject with acute major bleeding, wherein the amino acid at the position corresponding to 235 in SEQ ID NO:1 is substituted with Leu or Thr.

FIG. 1A

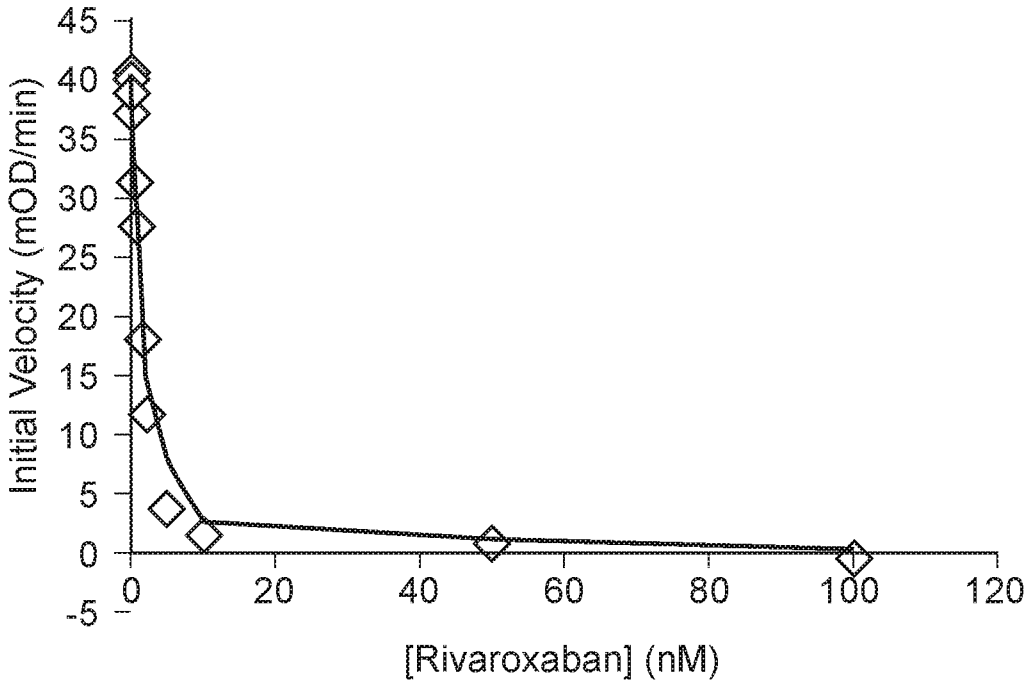

Initial Velocity (mOD/min)
45
40
35
30
25
20
15
10
5
0
-5
0
20
40
60
80
100
120
[Rivaroxaban] (nM)

FIG. 1B

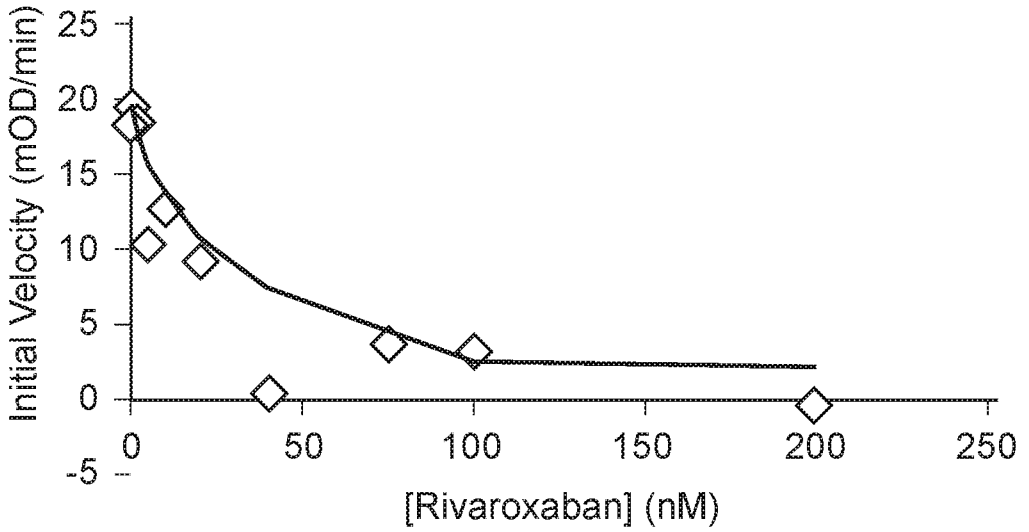

Initial Velocity (mOD/min)
25
20
15
10
5
0
-5
0
50
100
150
200
250
[Rivaroxaban] (nM)

FIG. 2A

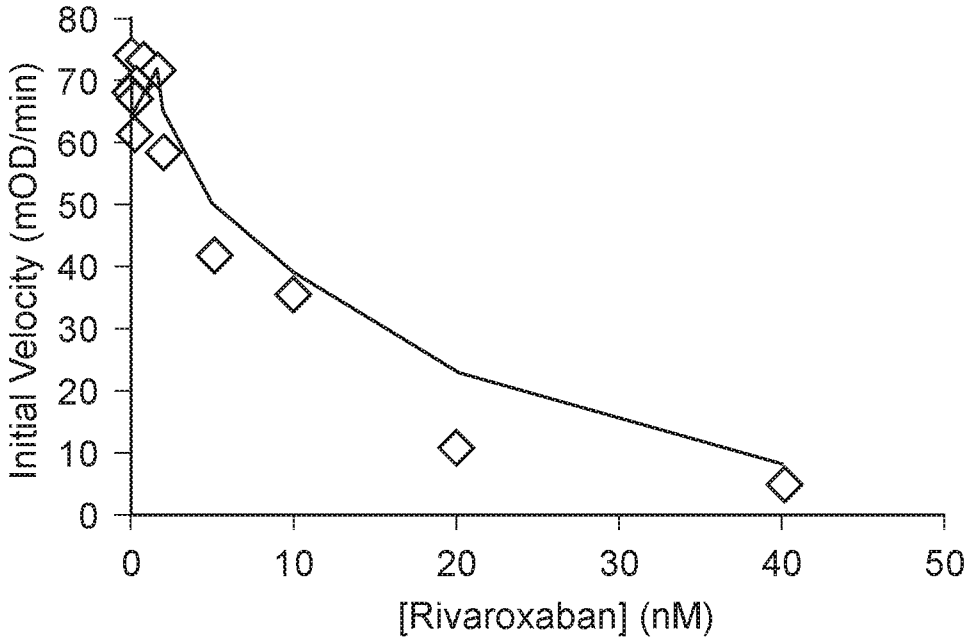

Initial Velocity (mOD/min)
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
[Rivaroxaban] (nM)

FIG. 2B

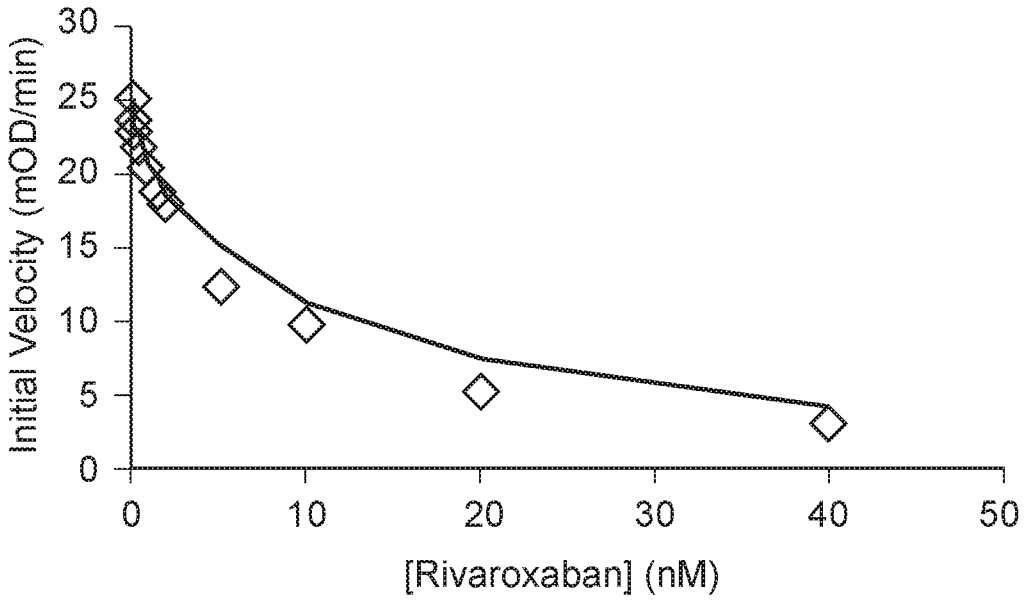

Initial Velocity (mOD/min)
30
25
20
15
10
5
0
0
10
20
30
40
50
[Rivaroxaban] (nM)

FIG. 3

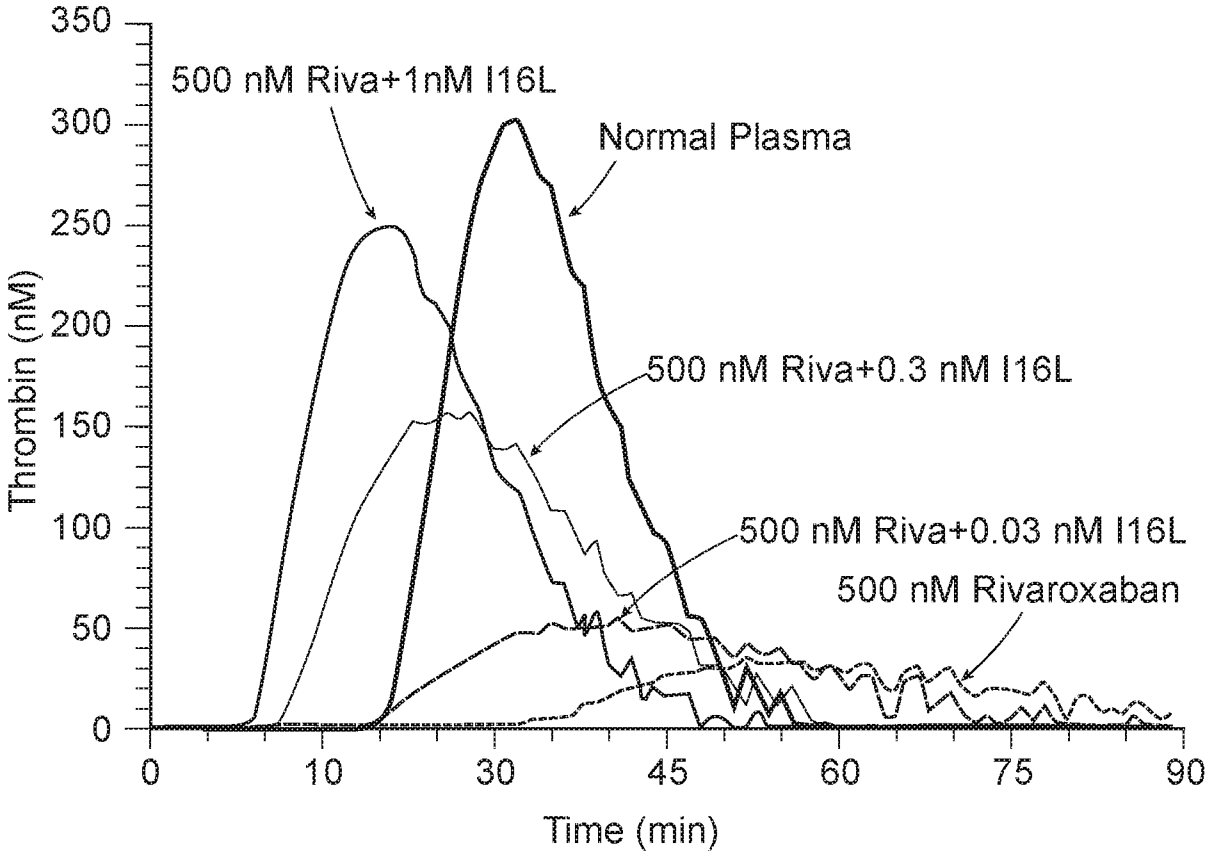

350
300
250
200
150
100
50
0
Thrombin (nM)
0
10
30
45
60
75
90
Time (min)
500 nM Riva+1nM I16L
Normal Plasma
500 nM Riva+0.3 nM I16L
500 nM Riva+0.03 nM I16L
500 nM Rivaroxaban

FIG. 4A

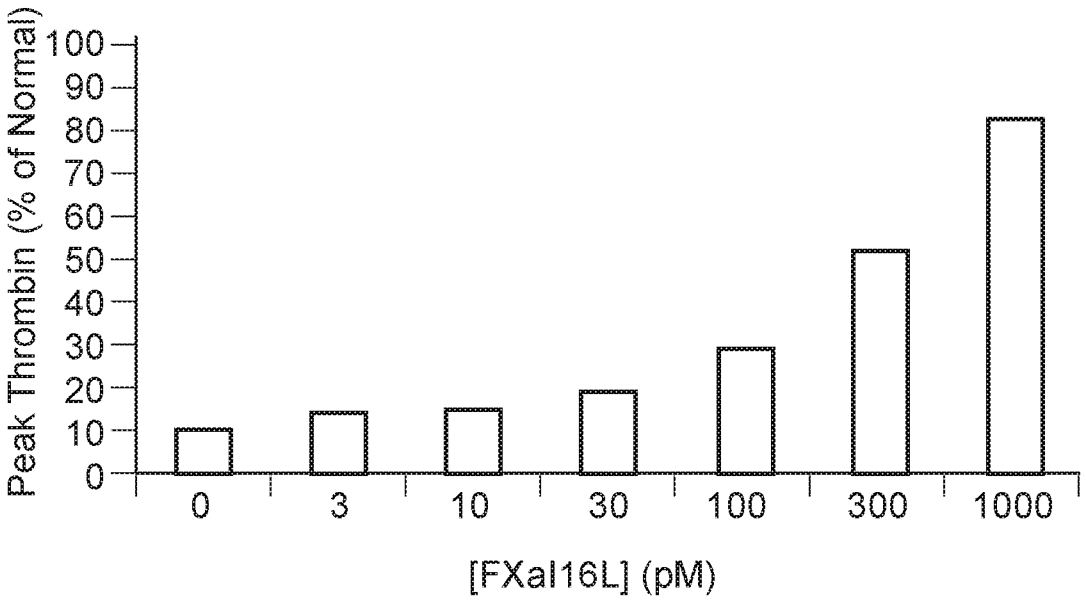

Peak Thrombin (% of Normal)
100
90
80
70
60
50
40
30
20
10
0
0
3
10
30
100
300
1000
[FXaI16L] (pM)

FIG. 4B

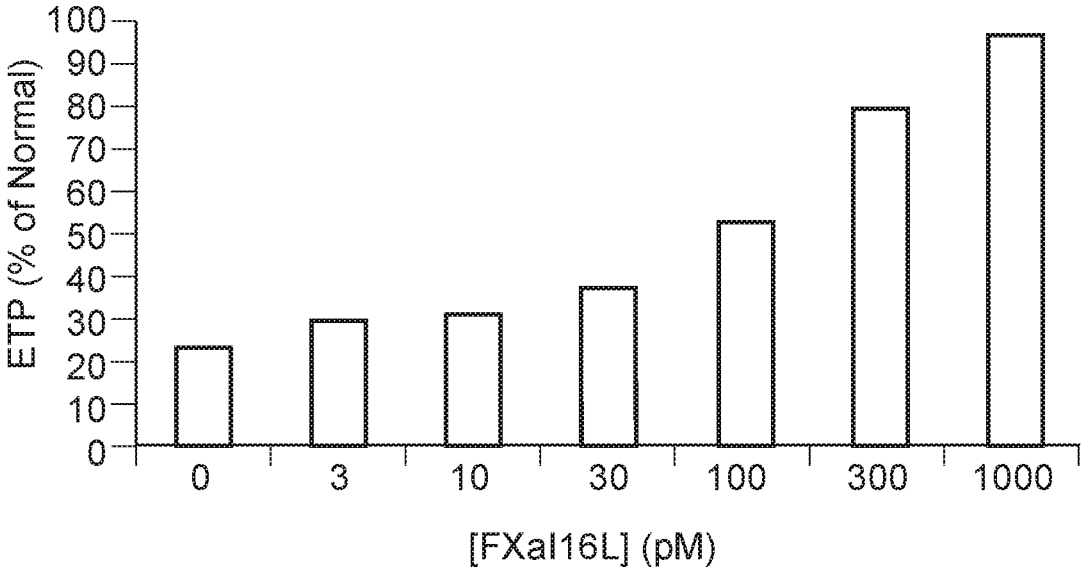

ETP (% of Normal)
100
90
80
70
60
50
40
30
20
10
0
0
3
10
30
100
300
1000
[FXaI16L] (pM)

FIG. 4C

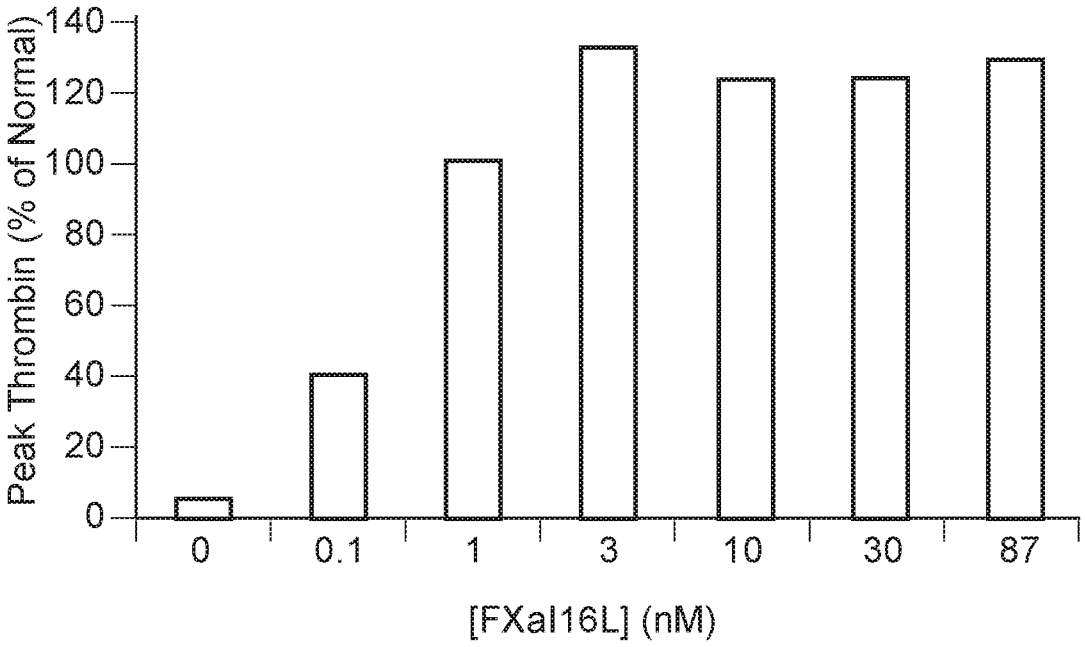

Peak Thrombin (% of Normal)
140
120
100
80
60
40
20
0
0
0.1
1
3
10
30
87
[FXaI16L] (nM)

FIG. 4D

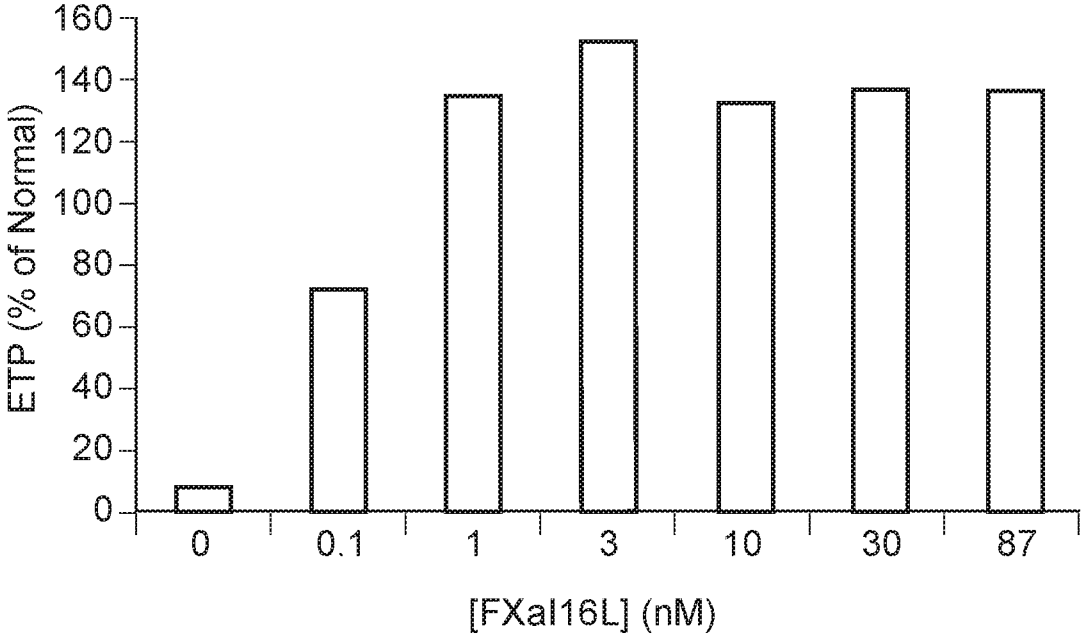

ETP (% of Normal)
160
140
120
100
80
60
40
20
0
0
0.1
1
3
10
30
87
[FXaI16L] (nM)

FIG. 5A

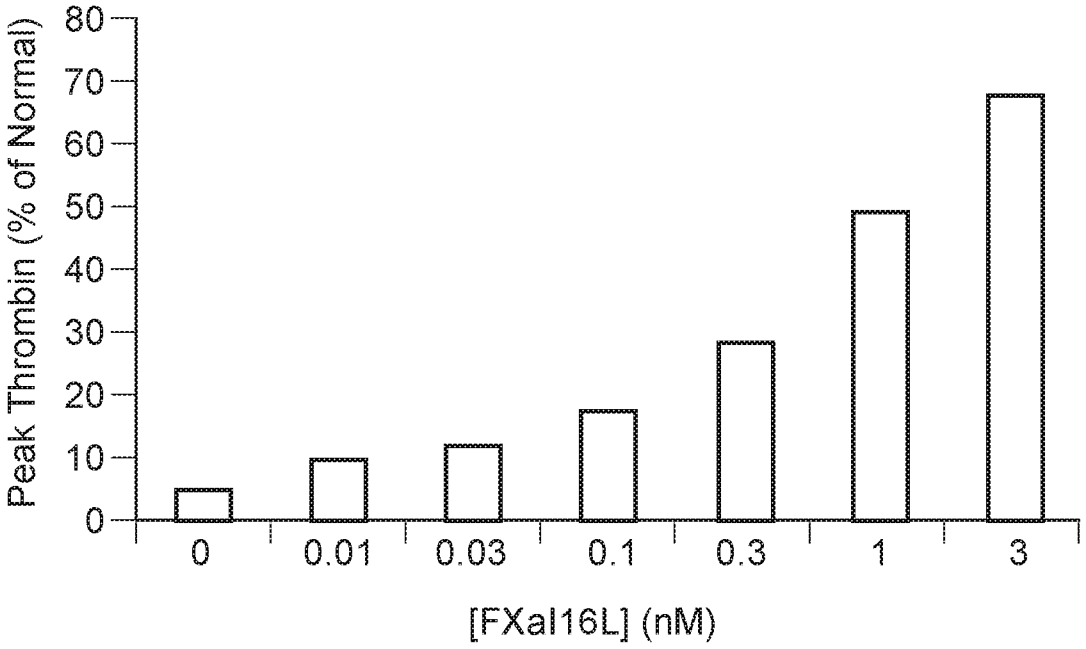

Peak Thrombin (% of Normal)
80
70
60
50
40
30
20
10
0
0
0.01
0.03
0.1
0.3
1
3
[FXaI16L] (nM)

FIG. 5B

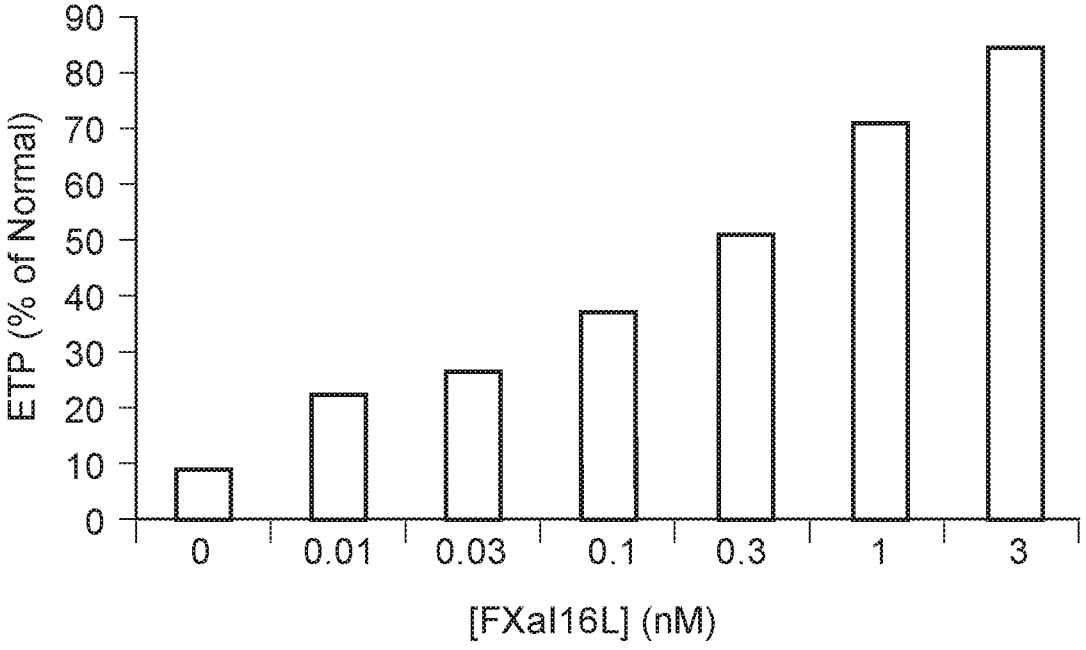

ETP (% of Normal)
90
80
70
60
50
40
30
20
10
0
0
0.01
0.03
0.1
0.3
1
3
[FXaI16L] (nM)

FIG. 6A

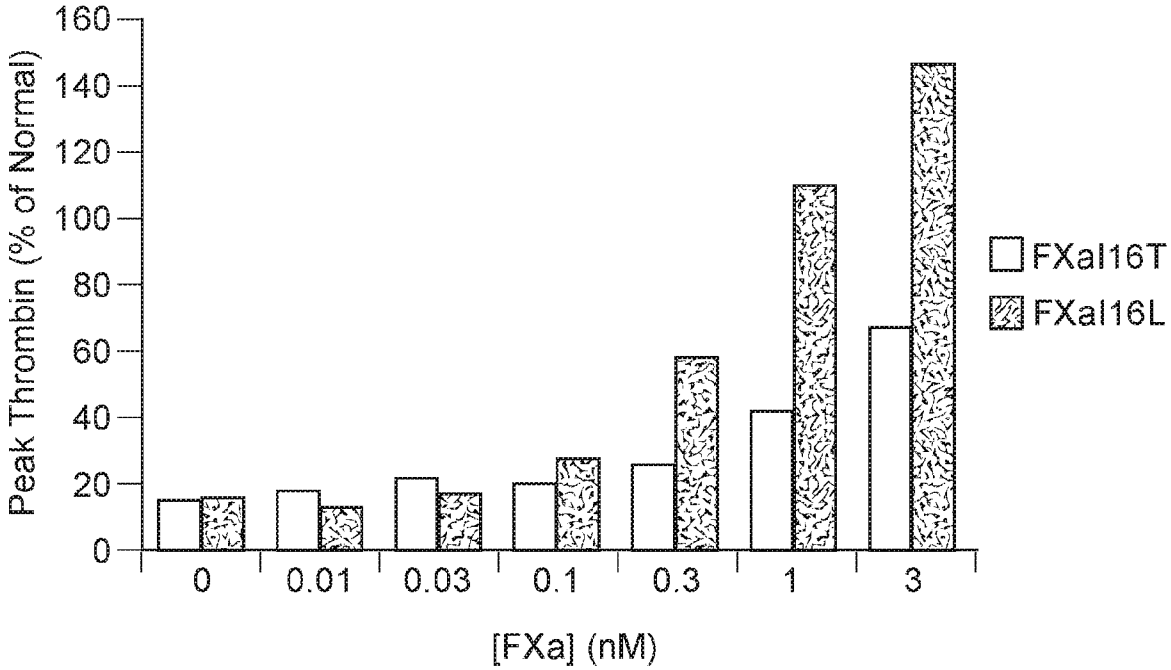

160
140
120
100
80
60
40
20
0
Peak Thrombin (% of Normal)
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXaI16T
FXaI16L

FIG. 6B

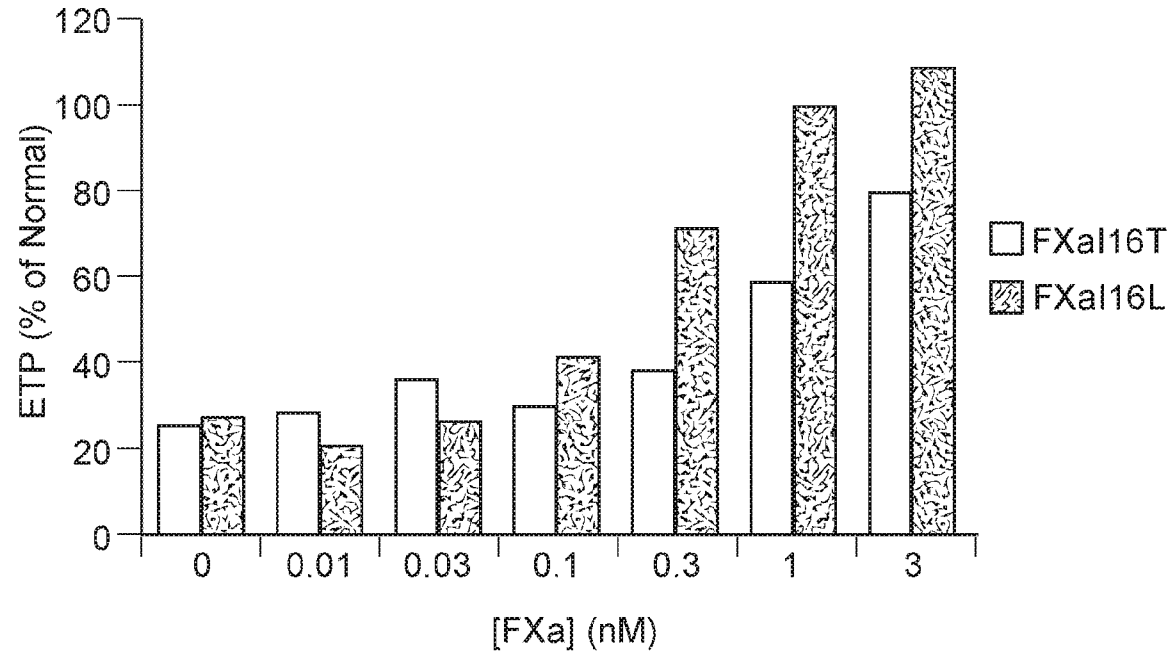

120
100
80
60
40
20
0
ETP (% of Normal)
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXaI16T
FXaI16L

FIG. 7A

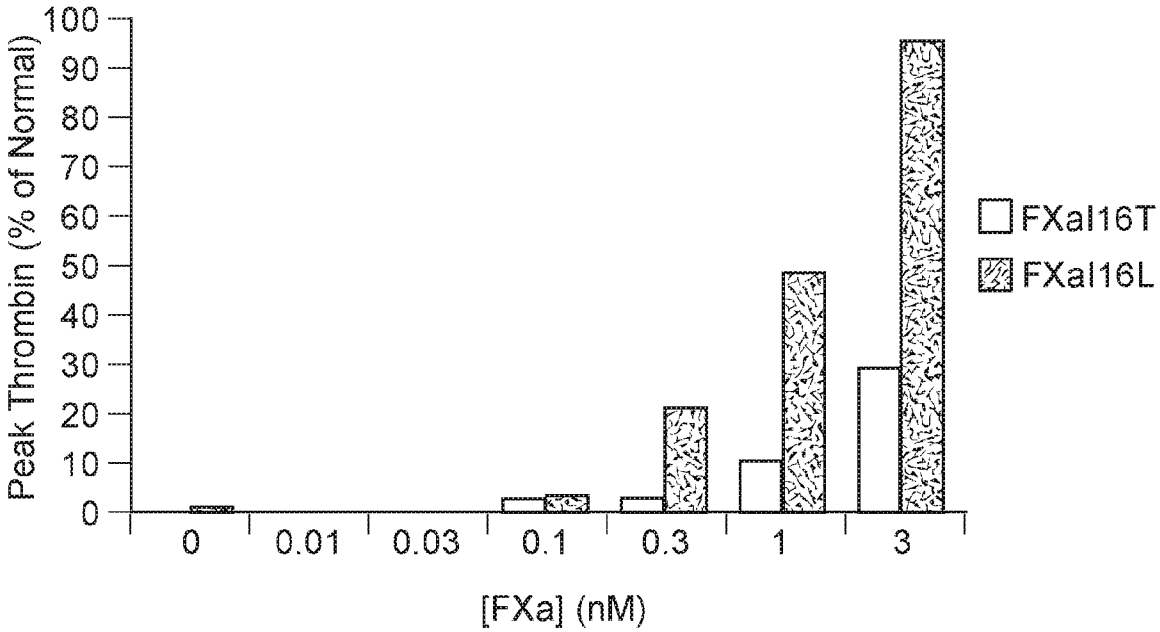
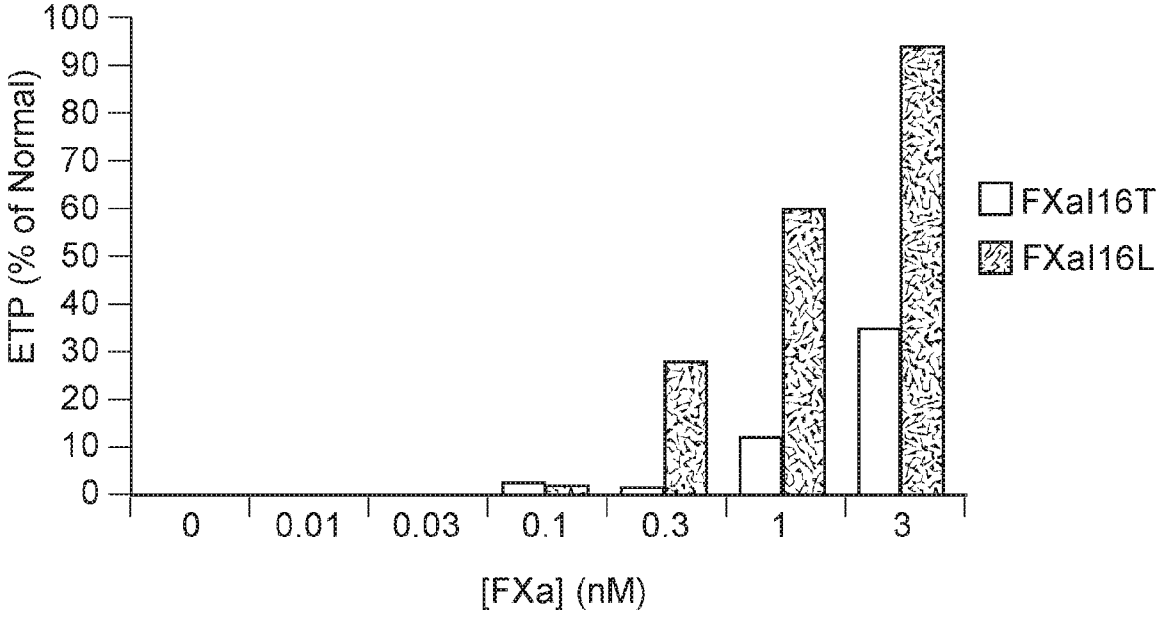

Peak Thrombin (% of Normal)
100
90
80
70
60
50
40
30
20
10
0
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXal16T
FXal16L

FIG. 7B


ETP (% of Normal)
100
90
80
70
60
50
40
30
20
10
0
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXal16T
FXal16L

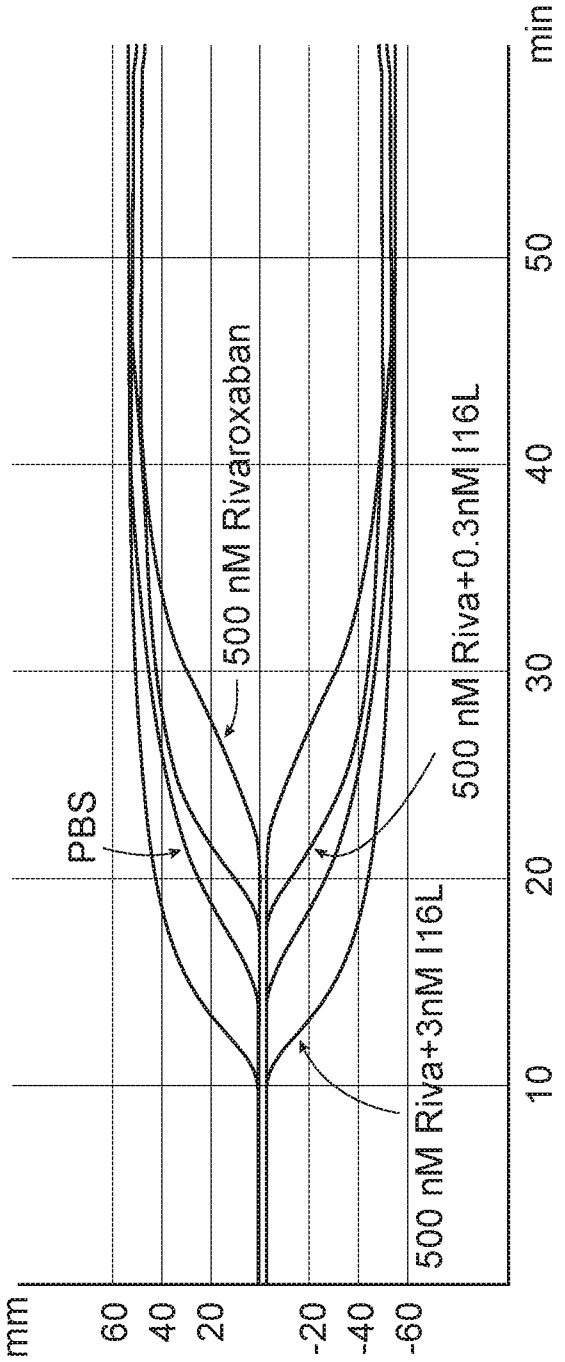

mm
60
40
20
-20
-40
-60
10
20
30
40
50
min
PBS
500 nM Rivaroxaban
500 nM Riva+0.3nM I16L
500 nM Riva+3nM I16L

FIG. 8A

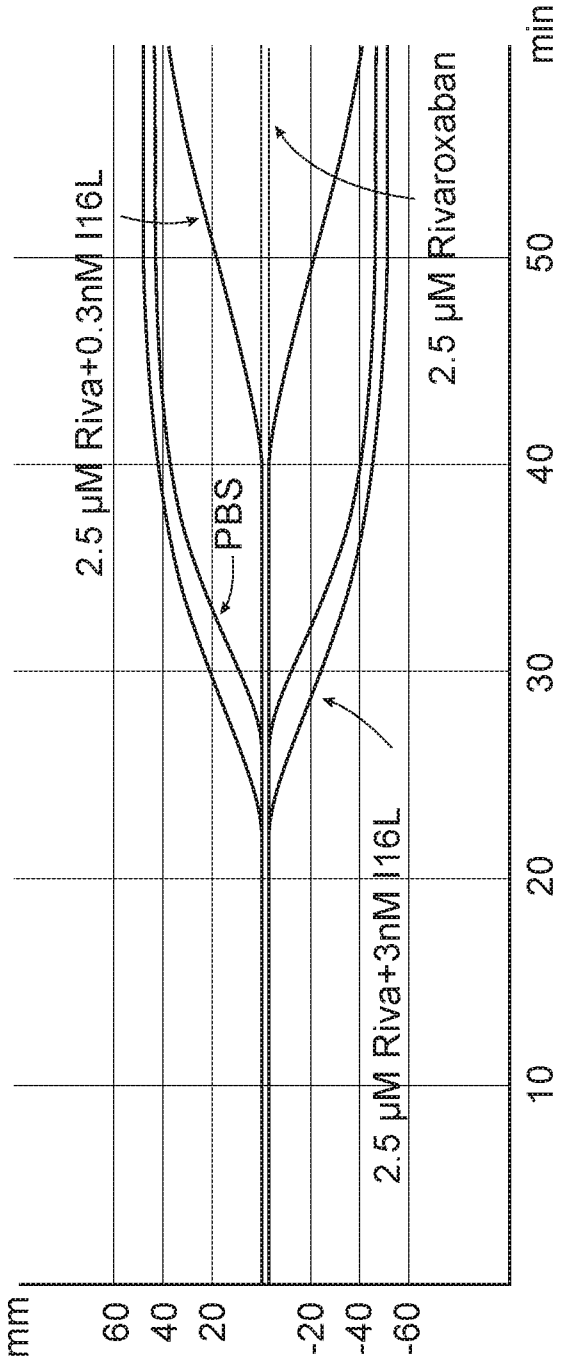

mm
60
40
20
-20
-40
-60
10
20
30
40
50
min
2.5 µM Riva+0.3nM I16L
PBS
2.5 µM Rivaroxaban
2.5 µM Riva+3nM I16L

FIG. 8B

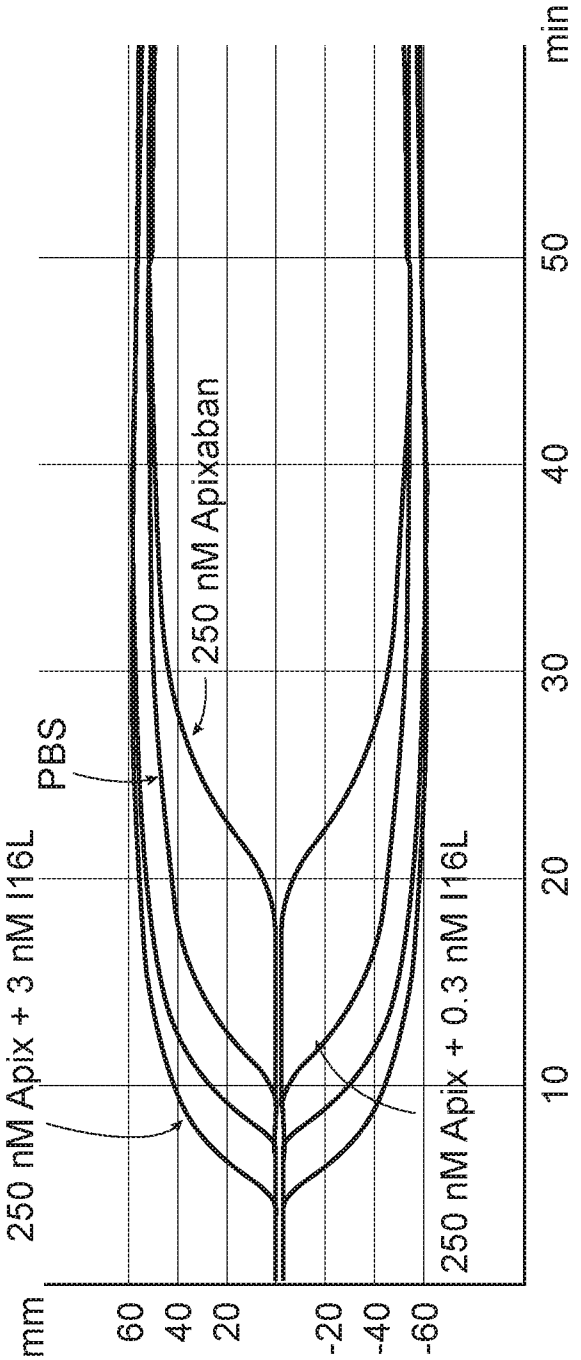

mm
60
40
20
-20
-40
-60
10
20
30
40
50
min
250 nM Apix + 3 nM I16L
PBS
250 nM Apixaban
250 nM Apix + 0.3 nM I16L

FIG. 9A

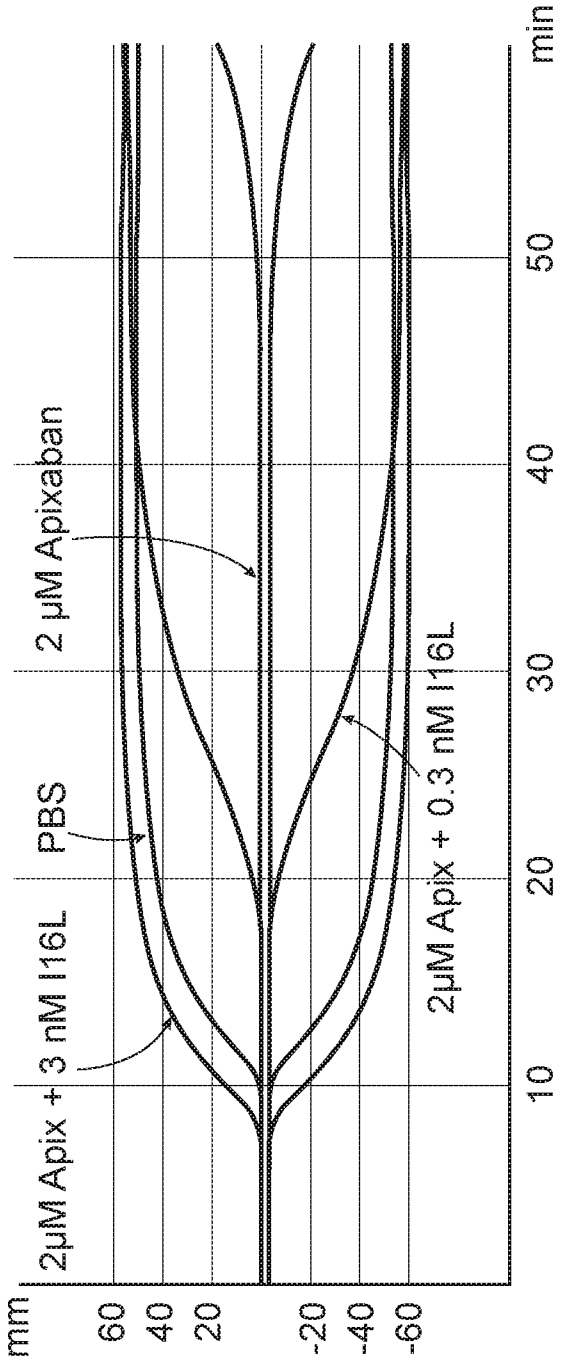

mm
60
40
20
-20
-40
-60
10
20
30
40
50
min
2µM Apix + 3 nM I16L
PBS
2 µM Apixaban
2µM Apix + 0.3 nM I16L

FIG. 9B

FIG. 10A

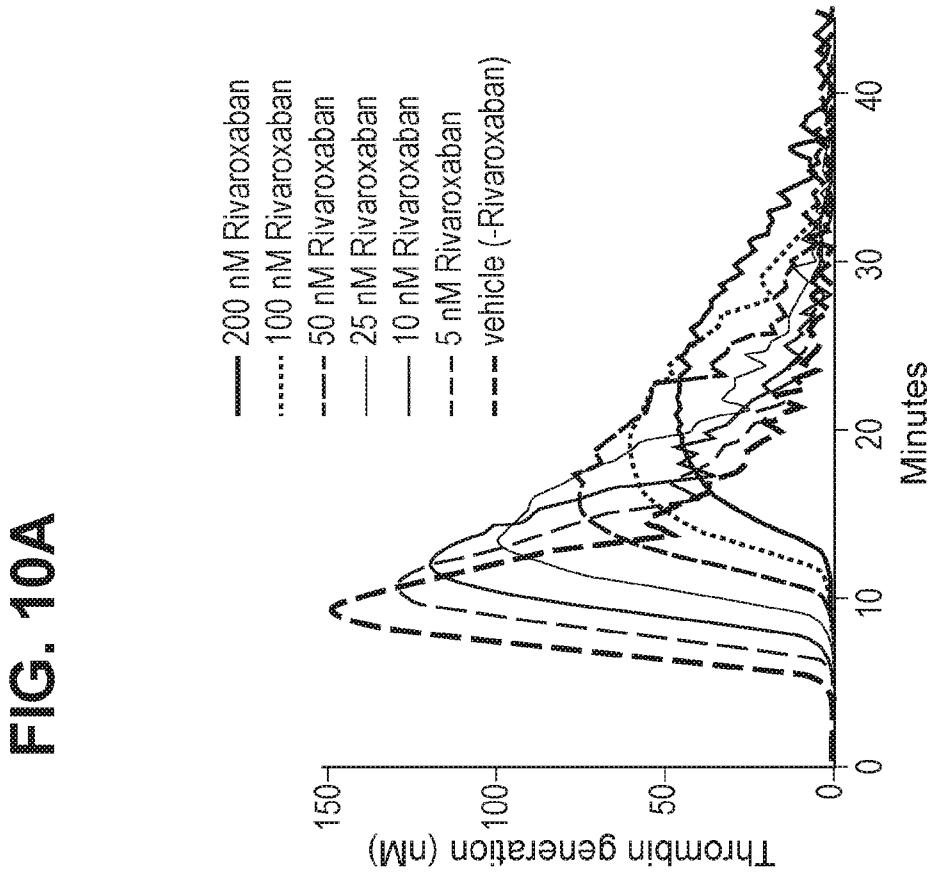


FIG. 10B

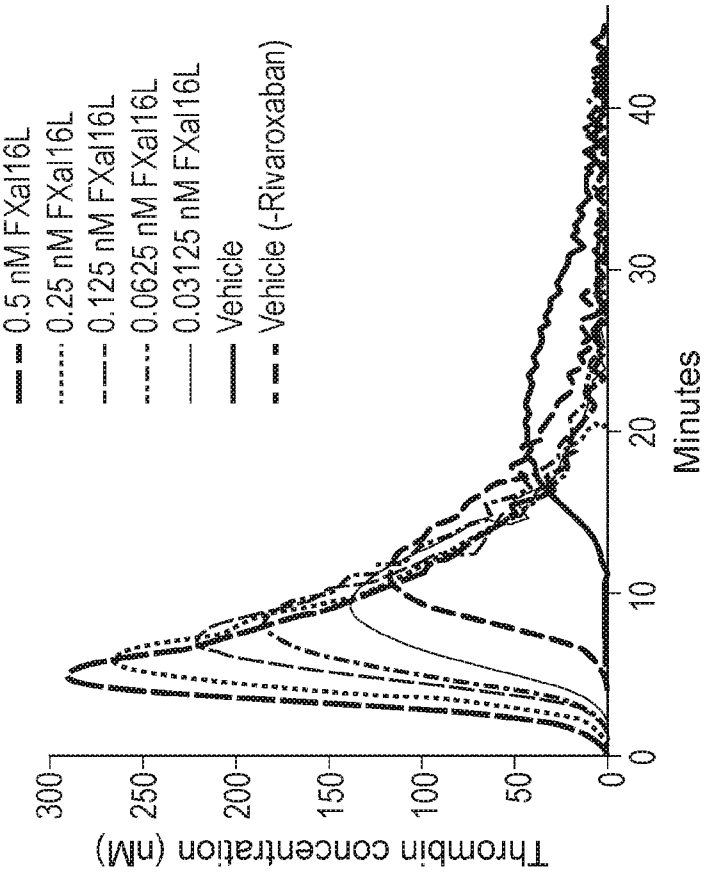


FIG. 11

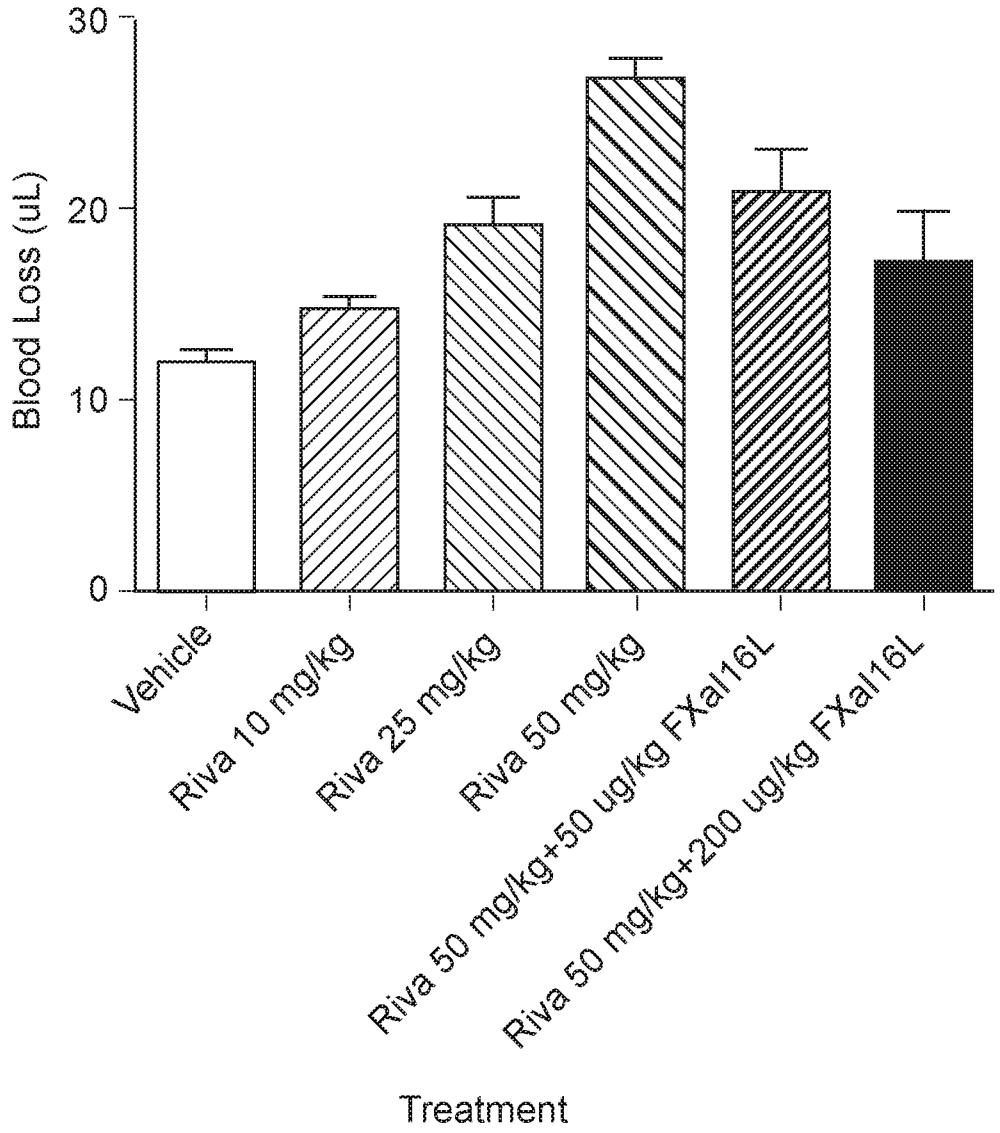

30
20
10
0
Blood Loss (uL)
Vehicle
Riva 10 mg/kg
Riva 25 mg/kg
Riva 50 mg/kg
Riva 50 mg/kg+50 ug/kg FXaI16L
Riva 50 mg/kg+200 ug/kg FXaI16L
Treatment

FIG. 12

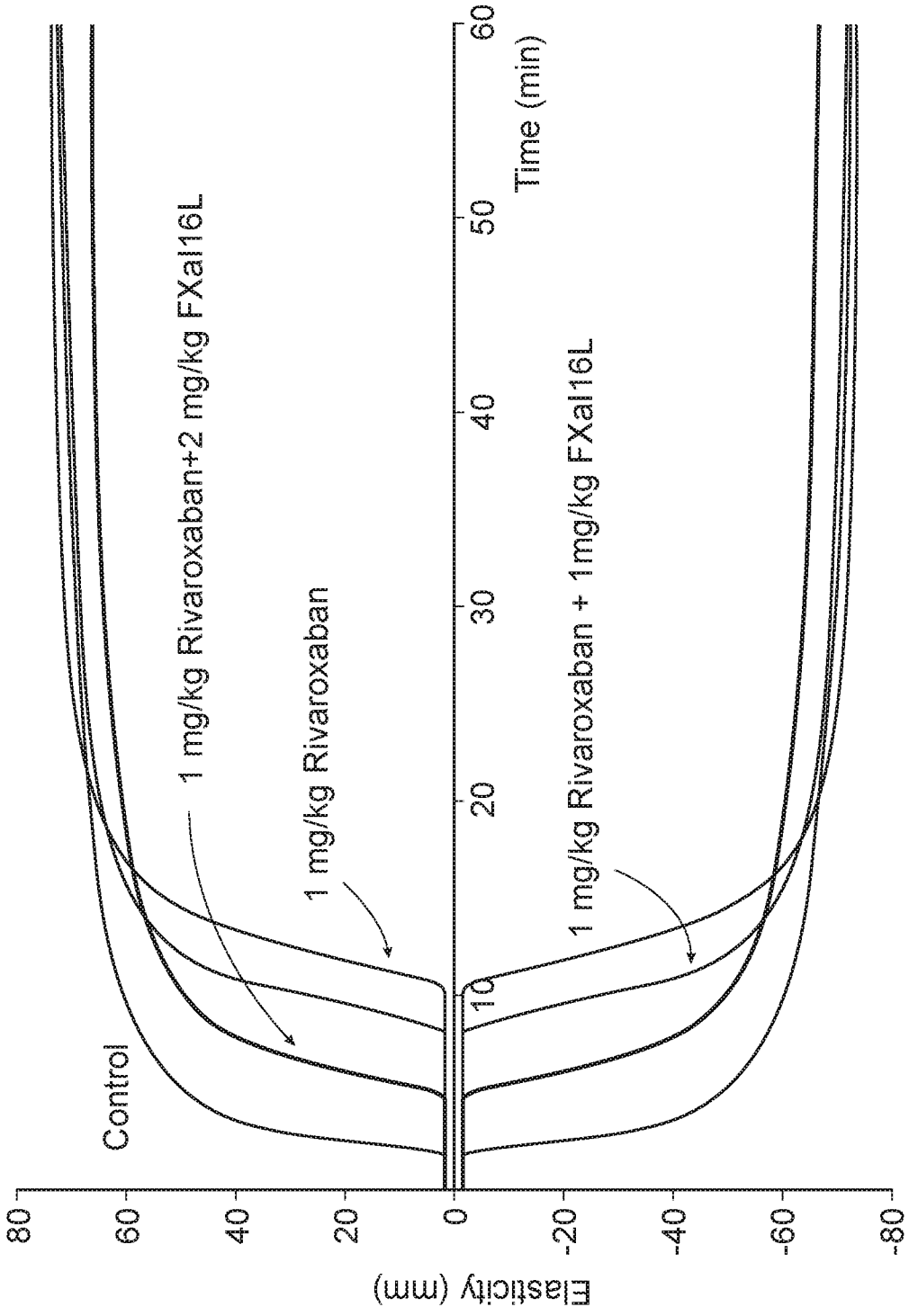

Elasticity (mm)
80
60
40
20
0
-20
-40
-60
-80
10
20
30
40
50
60
Time (min)
Control
1 mg/kg Rivaroxaban
1 mg/kg Rivaroxaban+2 mg/kg FXaI16L
1 mg/kg Rivaroxaban + 1mg/kg FXaI16L

FIG. 13A

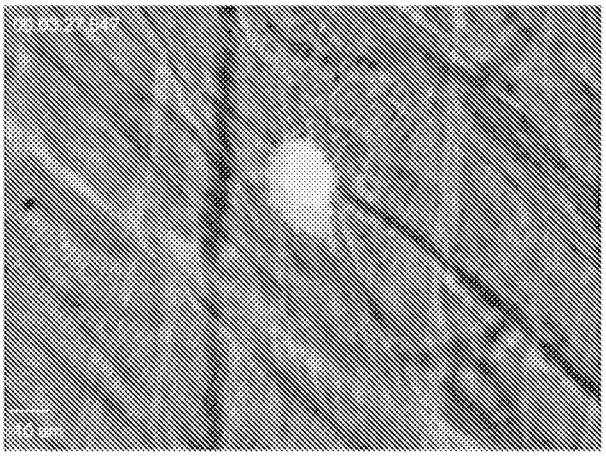


FIG. 13B

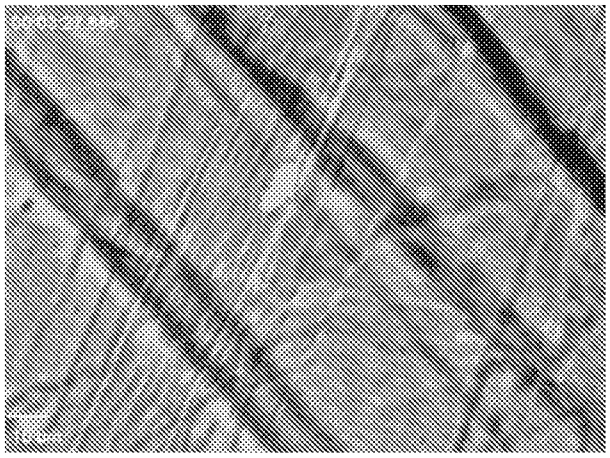


FIG. 13C

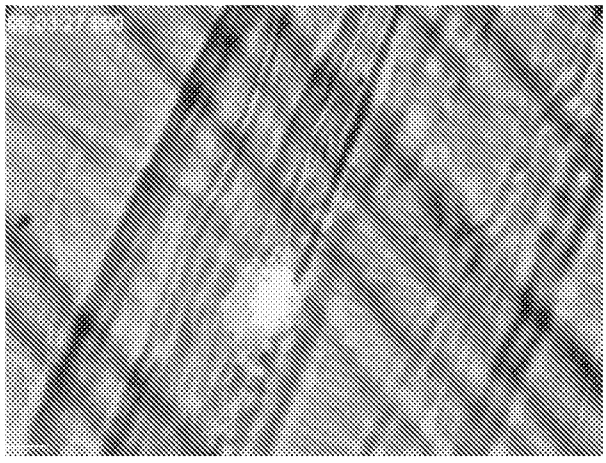


FIG. 14

Human Factor X Preprotein (SEQ ID NO:1)

```
  1 MGRPLHLVLL SASLAGLLLL GESLFIRREQ ANNILARVTR ANSFLEEMKK GHLERECMEE
 61 TCSYEEAREV FEDSDKTNEF WNKYKDGDQC ETSPCQNQGK CKDGLGEYTC TCLEGFEGKN
121 CELFTRKLCS LDNGDCDQFC HEEQNSVVCS CARGYTLADN GKACIPTGPY PCGKQTLERR
181 KRSVAQATSS SGEAPDSITW KPYDAADLDP TENPFDLLDF NQTQPERGDN NLTRIVGGQE
241 CKDGECPWQA LLINEENEGF CGGTILSEFY ILTAAHCLYQ AKRFKVRVGD RNTEQEEGGE
301 AVHEVEVVIK HNRFTKETYD FDIAVLRLKT PITFRMNVAP ACLPERDWAE STLMTQKTGI
361 VSGFGRTHEK GRQSTRLKML EVPYVDRNSC KLSSSFIITQ NMFCAGYDTK QEDACQGDSG
421 GPHVTRFKDT YFVTGIVSWG EGCARKGKYG IYTKVTAFLK WIDRSMKTRG LPKAKSHAPE
481 VITSSPLK
```

FIG. 15

Human Factor X Preprotein cDNA (SEQ ID NO:2)

```
   1 GACTTTGCTC CAGCAGCCTG TCCCAGTGAG GACAGGGACA CAGTACTCGG CCACACCATG
  61 GGGCGCCCAC TGCACCTCGT CCTGCTCAGT GCCTCCCTGG CTGGCCTCCT GCTGCTCGGG
 121 GAAGTCTGT TCATCCGCAG GGAGCAGGCC AACAACATCC TGGCGAGGGT CACGAGGCC
 181 AATTCCTTTC TTGAAGAGAT GAAGAAAGGA CACCTCGAAA GAGAGTGCAT GGAAGACC
 241 TGCTCATACG AAGAGGCCCG CGAGGTCTTT GAGGACAGCG ACAAGACGAA TGAATTCTGG
 301 AATAAATACA AAGATGGCGA CCAGTGTGAG ACCAGTCCTT GCCAGAACCA GGGCAAATGT
 361 AAAGACGGCC TCGGGGAATA CACCTGCACC TGTTTAGAAG GATTCGAAGG CAAAAACTGT
 421 GAATTATTCA CACGGAAGCT CTGCAGCCTG GACAACGGGG ACTGTGACCA GTTCTGCCAC
 481 GAGGAACAGA ACTCTGTGGT GTGCTCCTGC GCCCGCGGGT ACACCCCTGGC TGACAACGGC
 541 AAGGCCTGCA TTCCCACAGG GCCCTACCCC TGTGGGAAAC AGACCCTGGA ACGCAGGAAG
 601 AGGTCAGTGG CCCAGGCCAC CAGCAGCAGC GGGGAGGCCC CTGACAGCAT CACATGGAAG
 661 CCATATGATG CAGCCGACCT GGACCCCACC GAGAACCCCT TCGACCTGCT TGACTTCAAC
 721 CAGACGCAGC CTGAGAGGGG CGACAACAAC CTCACCAGGA TCGTGGGAGG CCAGGAATGC
 781 AAGGACGGGG AGTGTCCCTG GCAGGCCCTG CTCATCAATG AGGAAAACGA GGGTTTCTGT
```

FIG. 15 continued

```
 841 GGTGGAACCA TTCTGAGCGA GTTCTACATC CTAACGGCAG CCCACTGTCT CTACCAAGCC
 901 AAGAGATTCA AGGTGAGGGT AGGGGACCGG AACACGGAGC AGGAGGAGGG CGGTGAGGCG
 961 GTGCACGAGG TGGAGGTGGT CATCAAGCAC AACCGGTTCA CAAAGGAGAC CTATGACTTC
1021 GACATCGCCG TGCTCCGGCT CAAGACCCCC ATCACCTTCC GCATGAACGT GGCGCCTGCC
1081 TGCCTCCCCG AGCGTGACTG GGCCGAGTCC ACGCTGATGA CGCAGAAGAC GGGGATTTGTG
1141 AGCGGCTTCG GGCGCACCCA CGAGAAGGGC CGGCAGTCCA CCAGGCTCAA GATGCTGGAG
1201 GTGCCCTACG TGGACCGCAA CAGCTGCAAG CTGTCCAGCA GCTTCATCAT CACCCAGAAC
1261 ATGTTCTGTG CCGGCTACGA CACCAAGCAG GAGGATGCCT GCCAGGGGGA CAGCGGGGGC
1321 CCGCACGTCA CCCGCTTCAA GGACACCTAC TTCGTGACAG GCATCGTCAG CTGGGGAGAG
1381 GGCTGTGCCC GTAAGGGAA GTACGGGATC TACACCAAGG TCACCGCCTT CCTCAAGTGG
1441 ATCGACAGGT CCATGAAAAC CAGGGGCTTG CCCAAGGCCA AGAGCCATGC CCCGGAGGTC
1501 ATAACGTCCT CTCCATTAAA GTGAGATCCC ACTCAAAAAA AAAAAAAAAA AAAAAAAAAA
```

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/058494

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/48 A61K31/437 A61K31/5377 A61P7/04
ADD.

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)
A61K A61P

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	NABIL K THALJI: "Biochemistry and Cell Biology", ORAL PRESENTATIONS II, , 27 July 2013 (2013-07-27), page 16, XP009177338, Retrieved from the Internet: URL:http://www.ucdenver.edu/academics/colleges/medicalschool/education/degree_programs/mstp/keystoneconference/Documents/2013_MDPhD_Conference_Program.pdf [retrieved on 2014-04-01] the whole document ----- -/--	1-41

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* 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	Date of mailing of the international search report
3 April 2014	22/04/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fayos, Cēcile

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2014/058494

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   - [ ] on paper
   - [X] in electronic form

   b. (time)

   - [X] in the international application as filed
   - [ ] together with the international application in electronic form
   - [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/058494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Nabil K Thalji et al.: "Zymogen-Like FXa Is An Effective Pro-Hemostatic To Reverse The Anticoagulant Effects Of Direct FXa Inhibitors", , 8 December 2013 (2013-12-08), XP002722746, Retrieved from the Internet: URL:https://ash.confex.com/ash/2013/webprogram/Paper63599.html [retrieved on 2014-04-01] the whole document	1-41
Y	"Combined Degree Retreat. August 1, 2012. Villanova University", Perelman School of Medicine , 1 August 2012 (2012-08-01), XP002722747, University of Pennsylvania Retrieved from the Internet: URL:https://www.med.upenn.edu/mstp/documents/RetreatAgenda2012.doc [retrieved on 2014-04-01] the whole document	1-41
X,P	WO 2013/049804 A1 (PHILADELPHIA CHILDREN HOSPITAL [US]) 4 April 2013 (2013-04-04) the whole document claims 1, 2-4, 14 page 8, lines 1-10	1-41
Y	LACRAMIOARA IVANCIU ET AL: "A zymogen-like factor Xa variant corrects the coagulation defect in hemophilia", NATURE BIOTECHNOLOGY., vol. 29, no. 11, 23 October 2011 (2011-10-23), pages 1028-1033, XP055111224, US ISSN: 1087-0156, DOI: 10.1038/nbt.1995 the whole document abstract	1-41
Y	ELISABETH PERZBORN ET AL: "The discovery and development of rivaroxaban, an oral, direct factor Xa inhibitor", NATURE REVIEWS. DRUG DISCOVERY, vol. 10, no. 1, 1 January 2011 (2011-01-01), pages 61-75, XP055111223, GB ISSN: 1474-1776, DOI: 10.1038/nrd3185 the whole document page 67, right-hand column, last paragraph - page 68, left-hand column, paragraph 1	1-41

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/058494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. W. BUNCE ET AL: "Zymogen-like factor Xa variants restore thrombin generation and effectively bypass the intrinsic pathway in vitro", BLOOD, vol. 117, no. 1, 6 January 2011 (2011-01-06), pages 290-298, XP055111227, US ISSN: 0006-4971, DOI: 10.1182/blood-2010-08-300756	26-35
Y	the whole document	1-41

X	PATRICIA QUADE-LYSSY, PETER MILANOV AND JÖRG SCHÜTTRUMPF: "Engineered Factor VII, Factor IX, and Factor X Variants for HemophiliaGene Therapy", J GENET SYNDR GENE THER, 2012, pages 1-7, XP002722748, ISSN: 2157-7412	26-35
Y	the whole document page 5, right-hand column, paragraph 1	1-41

X	WO 2007/059513 A2 (PHILADELPHIA CHILDREN HOSPITAL [US]; CAMIRE RODNEY M [US]) 24 May 2007 (2007-05-24) cited in the application	1,3,5,7, 8,11, 13-15, 17,19, 21,26-41
Y	the whole document	1-41

Y	WO 2011/008885 A1 (PORTOLA PHARM INC [US]; SINHA UMA [US]; LU GENMIN [US]; HUTCHALEELAHA) 20 January 2011 (2011-01-20) the whole document	1-41

Y	LU G ET AL: "Reconstructed recombinant factor Xa as an 5 antidote to reverse anticoagulation by factor Xa inhibitors", JOURNAL OF THROMBOSIS AND HAEMOSTASIS, vol. 7, no. suppl. 2, 1 July 2009 (2009-07-01), pages 309/OC-TH, XP009177312, BLACKWELL PUBLISHING, OXFORD, GB ISSN: 1538-7933 the whole document	1-41

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/058494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOLLENBACH S ET AL: "Bolus administration of PRT064445, a recombinant Factor Xa inhibitor antidote, reverses blood loss and PD markers in a rat model following enoxaparin induced anticoagulation", EUROPEAN HEART JOURNAL, vol. 33, no. Suppl, 1 January 2012 (2012-01-01), pages 309-310, XP009177324, OXFORD UNIVERSITY PRESS, GB ISSN: 0195-668X the whole document -----	1-41

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2014/058494

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		BR PI0618654 A2	06-09-2011
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		JP 2012533552 A	27-12-2012
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		WO 2011008885 A1	20-01-2011

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 104994868 A
(43)申请公布日 2015.10.21

(21)申请号 201480006917.0
(22)申请日 2014.01.23
(30)优先权数据
61/759,332 2013.01.31 US
(85)PCT国际申请进入国家阶段日
2015.07.31
(86)PCT国际申请的申请数据
PCT/IB2014/058494 2014.01.23
(87)PCT国际申请的公布数据
WO2014/118677 EN 2014.08.07
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(51)Int.Cl.
A61K 38/48(2006.01)
A61K 31/437(2006.01)
A61K 31/5377(2006.01)
A61P 7/04(2006.01)

权利要求书4页 说明书19页
序列表5页 附图18页

(54)发明名称

用于抵消因子 Xa 抑制的组合物和方法

(57)摘要

本公开提供了通过施用 FXa 的变体，用于抵消对象中的直接活化因子 X(FXa) 抑制剂的效应的组合物和方法。

图13A

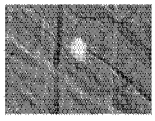


图13B

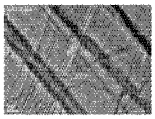
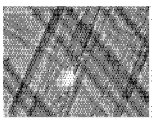


图13C



CN 104994868 A

1. 一种用于降低或预防用直接因子 Xa 抑制剂治疗的对象中的出血的方法，其包括给所述对象施用含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

2. 一种用于降低或预防用利伐沙班或阿哌沙班治疗的对象中的出血的方法，其包括给所述对象施用因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

3. 一种用于降低或预防用直接因子 Xa 抑制剂治疗的对象中的出血的药物组合物，其包含含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

4. 一种用于降低或预防用利伐沙班或阿哌沙班治疗的对象中的出血的药物组合物，其包含因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

5. 因子 Xa 变体在生产用于降低或预防用直接因子 Xa 抑制剂治疗的对象中的出血的药物中的用途，其中所述因子 Xa 变体含有选自下述的至少一种修饰：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

6. 因子 Xa 变体在生产用于降低或预防用利伐沙班或阿哌沙班治疗的对象中的出血的药物中的用途，其中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

7. 权利要求 1-6 中任一项的方法、组合物或用途，其中出血降低至少约 5％ -10％、10％ -15％、15％ -20％、20％ -25％、25％ -30％、30％ -35％、35％ -40％、40％ -45％、45％ -50％、50％ -55％、55％ -60％、60％ -65％、65％ -70％、70％ -75％、75％ -80％、80％ -85％、85％ -90％、90％ -95％、或 95％ -100％。

8. 一种用于增加有需要的对象中在直接因子 Xa 抑制剂存在下产生的凝血酶量的方法，其包括给所述对象施用含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

9. 一种用于增加有需要的对象中在利伐沙班或阿哌沙班存在下产生的凝血酶量的方法，其包括给所述对象施用因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

10. 一种用于增加有需要的对象中在利伐沙班或阿哌沙班存在下产生的凝血酶量的药物组合物，其包含因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

11. 一种用于增加有需要的对象中在直接因子 Xa 抑制剂存在下产生的凝血酶量的药

物组合物，其包含含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

12. 因子 Xa 变体在生产用于增加在利伐沙班或阿哌沙班存在下产生的凝血酶量的药物中的用途，其中所述因子 Xa 变体含有选自下述的至少一种修饰：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

13. 因子 Xa 变体在生产用于增加在直接因子 Xa 抑制剂存在下产生的凝血酶量的药物中的用途，其中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

14. 权利要求 8-13 中任一项的方法、组合物或用途，其中所产生的凝血酶量增加至少约 10％、20％、30％、40％、50％、60％、70％、80％、90％、95％、100％、1.5 倍、2 倍、3 倍、4 倍、5 倍、6 倍、7 倍、10 倍、15 倍、20 倍、25 倍、30 倍或 50 倍。

15. 一种用于减少有需要的对象中在直接因子 Xa 抑制剂存在下的凝血时间的方法，其包括给所述对象施用含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

16. 一种用于减少有需要的对象中在利伐沙班或阿哌沙班存在下的凝血时间的方法，其包括给所述对象施用因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

17. 一种用于减少有需要的对象中在直接因子 Xa 抑制剂存在下的凝血时间的药物组合物，其包含含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

18. 一种用于减少有需要的对象中在利伐沙班或阿哌沙班存在下的凝血时间的药物组合物，其包含因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

19. 因子 Xa 变体在生产用于减少有需要的对象中在直接因子 Xa 抑制剂存在下的凝血时间的药物中的用途，其中所述因子 Xa 变体含有选自下述的至少一种修饰：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

20. 因子 Xa 变体在生产用于减少有需要的对象中在利伐沙班或阿哌沙班存在下的凝血时间的药物中的用途，其中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

21. 权利要求 15-20 中任一项的方法、组合物或用途，其中凝血时间减少至少约

5％-10％、10％-15％、15％-20％、20％-25％、25％-30％、30％-35％、35％-40％、40％-45％、45％-50％、50％-55％、55％-60％、60％-65％、65％-70％、70％-75％、75％-80％、80％-85％、85％-90％、90％-95％、或95％-100％。

22. 权利要求15-20中任一项的方法、组合物或用途，其中所述凝血时间减少使用凝血酶原时间(PT)进行测量。

23. 权利要求22的方法，其中所述对象中的所述PT为约25秒、24秒、23秒、22秒、21秒、20秒、19秒、18秒、17秒、16秒、15秒、14秒、13秒、12秒、11秒或10秒。

24. 权利要求22的方法，其中所述对象中的国际标准化比率(INR)为约4.0、3.9、3.8、3.7、3.6、3.5、3.4、3.3、3.2、3.1、3.0、2.9、2.8、2.7、2.6、2.5、2.4、2.3、2.2、2.1、2.0、1.9、1.8、1.7、1.6、1.5、1.4、1.3、1.2、1.1、1.0、0.9、0.8或0.7。

25. 权利要求22、23或24中任一项的方法，其中PT在所述FXa变体施用后15分钟、20分钟、30分钟、40分钟、45分钟、50分钟、60分钟、75分钟或90分钟进行测定。

26. 一种包含因子Xa变体的药物组合物，所述因子Xa变体含有选自下述的至少一种修饰：

a)在对应于SEQ ID NO:1中的235的位置处的氨基酸由Thr、Leu、Phe、Asp或Gly取代；和

b)在对应于SEQ ID NO:1中的236的位置处的氨基酸由Leu、Ala或Gly取代，

其中在比直接因子Xa抑制剂的血浆浓度低至少100倍的血浆浓度下，所述因子Xa变体抵消所述直接因子Xa抑制剂的效应。

27. 一种包含因子Xa变体的药物组合物，所述因子Xa变体中在对应于SEQ ID NO:1中的235的位置处的氨基酸由Leu或Thr取代，并且在比利伐沙班或阿哌沙班的血浆浓度低至少100倍的血浆浓度下，所述因子Xa变体抵消所述利伐沙班或阿哌沙班的效应。

28. 前述权利要求中任一项的方法、组合物或用途，其中在比直接因子Xa抑制剂的血浆浓度低至少100倍的血浆浓度下，所述因子Xa变体抵消所述直接因子Xa抑制剂的效应。

29. 前述权利要求中任一项的方法、组合物或用途，其中所述因子Xa变体在计划手术前、在损伤后或在直接因子Xa抑制剂药物过量后施用。

30. 前述权利要求中任一项的方法、组合物或用途，其中因子Xa变体施用超过一次。

31. 前述权利要求中任一项的方法、组合物或用途，其中施用至少一种另外的促凝血剂。

32. 权利要求30的方法、组合物或用途，其中所述促凝血剂选自：不同的因子Xa变体、因子IX、因子XIa、因子XIIa、因子VIII、因子VIIa、FEIBA和凝血酶原复合物浓缩剂(PCC)。

33. 前述权利要求中任一项的方法、组合物或用途，其中所述直接FXa抑制剂的血浆浓度是超治疗量。

34. 前述权利要求中任一项的方法、组合物或用途，其中所述直接FXa抑制剂是利伐沙班，其中所述利伐沙班的血浆浓度为至少约100nM、200nM、300nM、400nM、500nM、600nM、700nM或800nM。

35. 前述权利要求中任一项的方法、组合物或用途，其中所述直接FXa抑制剂是阿哌沙班，其中所述阿哌沙班的血浆浓度为至少约50nM、100nM、150nM、200nM、250nM、300nM、350nM或400nM。

36. 一种用于在患有急性大出血的对象中对由于 FXa 抑制疗法的获得性凝血病实现紧急逆转的方法，其包括给所述对象施用含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

37. 一种用于在患有急性大出血的对象中对由于 FXa 抑制疗法的获得性凝血病实现紧急逆转的方法，其包括给所述对象施用因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

38. 一种用于在患有急性大出血的对象中对由于 FXa 抑制疗法的获得性凝血病实现紧急逆转的药物组合物，其包含含有选自下述的至少一种修饰的因子 Xa 变体：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

39. 一种用于在患有急性大出血的对象中对由于 FXa 抑制疗法的获得性凝血病实现紧急逆转的药物组合物，其包含因子 Xa 变体，所述因子 Xa 变体中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

40. 因子 Xa 变体在生产用于在患有急性大出血的对象中对由于 FXa 抑制疗法的获得性凝血病实现紧急逆转的药物中的用途，其中所述因子 Xa 变体含有选自下述的至少一种修饰：

a) 在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Thr、Leu、Phe、Asp 或 Gly 取代；和

b) 在对应于 SEQ ID NO:1 中的 236 的位置处的氨基酸由 Leu、Ala 或 Gly 取代。

41. 因子 Xa 变体在生产用于在患有急性大出血的对象中对由于 FXa 抑制疗法的获得性凝血病实现紧急逆转的药物中的用途，其中在对应于 SEQ ID NO:1 中的 235 的位置处的氨基酸由 Leu 或 Thr 取代。

用于抵消因子 Xa 抑制的组合物和方法

[0001] 相关申请的交叉引用

[0002] 本申请要求于 2013 年 1 月 31 日提交的美国临时申请号 61/759,332 的利益，所述美国临时申请的内容整体援引加入本文。

技术领域

[0003] 根据 37CFR § 1.821，以计算机可读形式 (CRF) 经由 EFS-Web 作为文件名 PC072006_SEQLIST_ST25.txt 同时提交的序列表援引加入本文。序列表的电子拷贝于 2013 年 12 月 18 日创建，具有 7 千字节的文件大小。

背景技术

[0004] 药理学抗凝是患有血栓前状况的患者的治疗主体。过去五十年，唯一可用的经口抗凝血药是华法林，维生素 K 环氧化物还原酶 (VKOR) 的抑制剂，其再利用氧化的维生素 K。华法林具有许多缺点，包括无法预测的药物代谢动力学，其需要频繁监控凝血参数和剂量调整。然而，在紧急出血或需要急诊手术的情况下，存在允许快速和完全逆转的解毒药。

[0005] 经口直接 FXa 抑制剂是新出现的抗凝血药，当与标准治疗例如华法林相比较时，其具有简化患有血栓前疾病的给药方案和止血监控的潜力。尽管这些药物具有许多超过华法林的优点，但这些新型抗凝血药没有完全有效的逆转试剂。

[0006] 然而，由于害怕难控制的出血，对其效应的特异性对抗措施的缺乏是关键的未满足的临床需要，其可以限制这些试剂的广泛采用。

发明内容

[0007] 通过提供用于抵消直接活化因子 X(FXa) 抑制剂的效应的组合物和方法，申请人已解决该关键的未满足的临床需要。

[0008] 根据一些实施方案，本公开提供了通过施用包含因子 Xa 变体的组合物，用于降低或预防用直接因子 Xa(FXa) 抑制剂治疗的对象中的出血的方法，所述因子 Xa 变体含有至少一种修饰，包括在位置 16 处（使用胰凝乳蛋白酶编号系统）使用 Thr、Leu、Phe、Asp 或 Gly 取代野生型氨基酸，或者在位置 17 处（使用胰凝乳蛋白酶编号系统）使用 Leu、Ala 或 Gly 取代野生型氨基酸。在某些实施方案中，用包含 FXa 变体的组合物治疗导致出血降低至少 50％。根据某些实施方案，直接因子 Xa 抑制剂包括利伐沙班或阿哌沙班。在一些实施方案中，直接 FXa 抑制剂的血浆浓度通常为治疗量或超治疗量。例如，在一些实施方案中，利伐沙班的血浆浓度可以是约 500nM 或更高，并且阿哌沙班的血浆浓度可以是约 250nM 或更高。根据某些实施方案，FXa 变体含有取代 I16L。在一些实施方案中，在比因子 Xa 抑制剂的血浆浓度低至少 100 倍的血浆浓度下，FXa 变体能够抵消直接因子 Xa 抑制剂的效应。在一些实施方案中，包含 FXa 变体的组合物在计划的手术前、在损伤后、或者在用直接 FXa 抑制剂的有意或意外药物过量后施用。在一些实施方案中，在用 FXa 变体的第一个剂量后，使用止血测定监控对象中的止血，并且如果通过预定时间未获得足够的止血，则施用 FXa 变体的

至少一次第二个剂量，以实现充分止血。根据一些实施方案，预定时间是施用FXa变体的第一个剂量后约15分钟、30分钟、45分钟或60分钟。其他时间也是可能的。在一些其他实施方案中，除FXa变体之外还施用至少第二种促凝血剂，包括例如不同的FXa变体、因子IX、因子XIa、因子XIIa、因子VIII、因子VIIa、FEIBA或凝血酶原复合物浓缩剂(PCC)。

[0009] 根据一些实施方案，本公开提供了通过施用包含因子Xa变体的组合物，用于增加用直接因子Xa(FXa)抑制剂治疗的对象中，响应外源或固有凝血途径的活化而产生的凝血酶量的方法，所述因子Xa变体含有至少一种修饰，包括在位置16处（使用胰凝乳蛋白酶编号系统）使用Thr、Leu、Phe、Asp或Gly取代野生型氨基酸，或者在位置17处（使用胰凝乳蛋白酶编号系统）使用Leu、Ala或Gly取代野生型氨基酸。根据某些实施方案，直接因子Xa抑制剂包括利伐沙班或阿哌沙班。在一些实施方案中，直接FXa抑制剂的血浆浓度通常为治疗量或超治疗量。例如，在一些实施方案中，利伐沙班的血浆浓度可以是约500nM或更高，并且阿哌沙班的血浆浓度可以是约250nM或更高。根据某些实施方案，FXa变体含有取代I16L。根据某些实施方案，所产生的凝血酶量增加约5％、10％、15％、20％、30％、40％、50％、60％、70％、80％、90％、100％、150％、200％或更多。在一些实施方案中，FXa变体能够在比因子Xa抑制剂的血浆浓度低至少100倍的血浆浓度下抵消直接因子Xa抑制剂的效应。在一些实施方案中，包含FXa变体的组合物在计划的手术前、在损伤后、或者在用直接FXa抑制剂的有意或意外药物过量后施用。在一些实施方案中，在用FXa变体的第一个剂量后，使用止血测定监控对象中的止血，并且如果通过预定时间未获得足够的止血，则施用FXa变体的至少一次第二个剂量，以实现充分止血。根据一些实施方案，预定时间是施用FXa变体的第一个剂量后约15分钟、30分钟、45分钟或60分钟。其他时间也是可能的。在一些其他实施方案中，除FXa变体之外还施用至少第二种促凝血剂，包括例如不同的FXa变体、因子IX、因子XIa、因子XIIa、因子VIII、因子VIIa、FEIBA或凝血酶原复合物浓缩剂(PCC)。

[0010] 根据一些实施方案，本公开提供了通过施用包含因子Xa变体的组合物，用于减少用直接因子Xa(FXa)抑制剂治疗的对象中的凝血时间（如例如使用PT或INR、或一些其他测定测量的）的方法，所述因子Xa变体含有至少一种修饰，包括在位置16处（使用胰凝乳蛋白酶编号系统）使用Thr、Leu、Phe、Asp或Gly取代野生型氨基酸，或者在位置17处（使用胰凝乳蛋白酶编号系统）使用Leu、Ala或Gly取代野生型氨基酸。根据某些实施方案，直接因子Xa抑制剂包括利伐沙班或阿哌沙班。在一些实施方案中，直接FXa抑制剂的血浆浓度通常为治疗量或超治疗量。例如，在一些实施方案中，利伐沙班的血浆浓度可以是约500nM或更高，并且阿哌沙班的血浆浓度可以是约250nM或更高。根据某些实施方案，FXa变体含有取代I16L。根据某些实施方案，凝血时间减少约5％、10％、15％、20％、30％、40％、50％、60％、70％、80％、90％或更多。在一些实施方案中，FXa变体能够在比因子Xa抑制剂的血浆浓度低至少100倍的血浆浓度下抵消直接因子Xa抑制剂的效应。在一些实施方案中，包含FXa变体的组合物在计划的手术前、在损伤后、或者在用直接FXa抑制剂的有意或意外药物过量后施用。在一些实施方案中，在用FXa变体的第一个剂量后，使用止血测定监控对象中的止血，并且如果通过预定时间未获得足够的止血，则施用FXa变体的至少一次第二个剂量，以实现充分止血。根据一些实施方案，预定时间是施用FXa变体的第一个剂量后约15分钟、30分钟、45分钟或60分钟。其他时间也是可能的。在一些其他实施方案中，

除FXa变体之外还施用至少第二种促凝血剂，包括例如不同的FXa变体、因子IX、因子XIa、因子XIIa、因子VIII、因子VIIa、FEIBA或凝血酶原复合物浓缩剂(PCC)。

附图说明

[0011] 图1A-B显示了利伐沙班对游离wt-FXa或FXa^{I16L}的抑制。A)wt-FXa(2nM)或B)FXa^{I16L}(6nM)对肽基底物(SpecXa;200uM)水解的初速度在增加浓度的利伐沙班下进行测定。Ki值在每个图上给出。

[0012] 图2A-B显示了在凝血酶原酶中装配的wt-FXa或$FXaI^{16L}$的利伐沙班抑制。在PCPS(20uM)和FVa(40nM)的存在下，A)wt-FXa(2nM)或B)$FXaI^{16L}$(6nM)对肽基底物(SpecXa;200uM)水解的初速度在增加浓度的利伐沙班下进行测定。

[0013] 图3显示了不同浓度的FXa^{I16L}对逆转利伐沙班的凝血酶生成效应的作用。

[0014] 图4A-D显示了FXa^{I16L}对逆转利伐沙班的效应的作用。在增加浓度的FXa^{I16L}的存在和不存在下，正常人血浆与500nM利伐沙班一起温育。在数据分析后，标绘生成的峰凝血酶(A和C)和总凝血酶(ETP;B和D)。

[0015] 图5A-B显示了FXa^{I16L}逆转高剂量利伐沙班的效应。在增加浓度的FXa^{I16L}的存在和不存在下，正常人血浆与7.5uM利伐沙班一起温育。在数据分析后，标绘生成的峰凝血酶(A)和总凝血酶(ETP;B)。

[0016] 图6A-B显示了FXa^{I16L}或FXa^{I16T}逆转250nM阿哌沙班的效应。在增加浓度的FXa^{I16L}或FXa^{I16T}的存在和不存在下，正常人血浆与250nM阿哌沙班一起温育。在数据分析后，标绘生成的峰凝血酶(A)和总凝血酶(ETP;B)。

[0017] 图7A-B显示了FXa^{I16L}或FXa^{I16T}逆转高剂量阿哌沙班的效应。在增加浓度的FXa^{I16L}或FXa^{I16T}的存在和不存在下，正常人血浆与2.0uM阿哌沙班一起温育。在数据分析后，标绘生成的峰凝血酶(A)和总凝血酶(ETP;B)。

[0018] 图8A-B显示了FXa^{I16L}校正在利伐沙班的存在下的全血凝固。全血血栓弹力图用于评价FXa^{I16L}逆转典型(A)和高(B)剂量利伐沙班效应的能力。

[0019] 图9A-B显示了FXa^{I16L}校正在阿哌沙班的存在下的全血凝固。全血血栓弹力图用于评价FXa^{I16L}逆转典型(A)和高(B)剂量阿哌沙班效应的能力。

[0020] 图10A-B显示了FXa^{I16L}在凝血酶生成测定中抵消利伐沙班。图10A显示了利伐沙班的剂量应答，并且图10B显示了在利伐沙班的存在下，FXa^{I16L}的剂量应答。

[0021] 图11显示了FXa^{I16L}在小鼠尾部修剪出血模型中抵消利伐沙班。

[0022] 图12证实了施用于小鼠的利伐沙班延迟使用ROTEM测量的全血的凝固时间，并且用FXa^{I16L}的施用剂量应答性抵消利伐沙班的效应。

[0023] 图13显示了施用于小鼠的利伐沙班阻止在通过激光引起的提睾肌中的血管损伤部位处的血块形成，并且FXa^{I16L}的施用抵消利伐沙班的效应。使用活体显微镜检查以及针对纤维蛋白和血小板的荧光标记的抗体，显现血块形成。图13A显示了在未经处理的小鼠中的血块形成。图13B显示了利伐沙班延迟且降低血小板累积且阻止纤维蛋白沉积。相比之下，图13C显示了在施用利伐沙班和FXa^{I16L}的小鼠中，在损伤部位处发生血块形成。

[0024] 图14是野生型人因子X前蛋白的氨基酸序列(SEQ ID NO:1)。信号肽对应于氨基酸1-23。前肽对应于氨基酸24-40。活化因子X(FXa)的成熟轻链对应于氨基酸41-179。

活化 FXa 的成熟重链（在活化肽去除后）对应于氨基酸 235-488。活化肽 (AP) 对应于氨基酸 183-234。

[0025] 图 15 是编码野生型人因子 X 前蛋白的 cDNA 的核苷酸序列 (SEQ ID NO:2)。编码序列对应于核苷酸 58-1524。

具体实施方式

[0026] 本公开提供了用于抵消有此需要的对象中的直接 FXa 抑制剂的抗凝效应的组合物和方法。申请人已发现某些 FXa 变体以剂量依赖性方式快速且完全抵消直接 FXa 抑制剂的效应。更具体而言，申请人已发现在以治疗浓度且甚至是超治疗浓度的 FXa 抑制剂的存在下，相对少量的 FXa 变体在体内恢复正常凝血活性。通过提供快速和有效的直接 FXa 抑制剂的抗凝效应的解毒药，申请人的公开因此促成实现这些有利的抗凝血药的承诺。

[0027] 凝血因子 X(FX) 是酶原，其在通过固有因子 IX/ 因子 VIII 或外源途径（组织因子 / 因子 VIIa) 活化后，变成 FXa，其为凝血酶原酶的蛋白酶部分。在 Arg-Ile 易断裂键的蛋白酶解切割后，释放活化肽 (AP)，酶原中一系列充分确定的结构变化驱动至成熟活性丝氨酸蛋白酶的活化过程（参见 Toso 等人，(2008)J.Biol.Chem.283,18627-18635 ;Bunce 等人，(2011)Blood 117,290-298 ;和 Ivanciu 等人，(2011)Nat.Biotechnol.29,1028-1033，整体援引加入本文）。成熟 FXa 具有轻链和含有催化结构域的重链。成熟 FXa 在凝血酶原酶复合物形成（其包括活化辅因子，因子 Va(FVa) 的结合）后变成活性丝氨酸蛋白酶。

[0028] 已开发了多种形式的 FX，其在活化切割后获得酶原样 FXa 变体。即，一旦被切割，所得到的 FXa 变体就具有弱活性位点功能，并且对通过循环抑制剂（即抗凝血酶 III 和 TFPI) 的失活更有抵抗力。FXa 变体因此在血浆中具有比野生型 FXa 更长的半衰期。FXa 变体结合 FVa、脂质膜和钙，以形成完全活性的凝血酶原酶复合物，其有效活化凝血酶原。

[0029] 酶原样 FXa 变体以酶原样构象循环，并且看起来不是血栓形成的（参见 Toso 等人，(2008)J.Biol.Chem.283,18627-18635 和 Ivanciu 等人，(2011)Nat.Biotechnol.29,1028-1033，整体援引加入本文）。此类 FXa 变体的例子在国际专利公开 WO2007/059513 中得到描述，所述国际专利公开整体援引加入本文。

[0030] 凝血酶是胰蛋白酶样酶，其属于具有胰凝乳蛋白酶样折叠的蛋白酶的 S1 肽酶家族。凝血蛋白酶含有催化结构域，其与彼此及消化的祖先丝氨酸蛋白酶是高度同源的。结构同源性 / 相同性如此之大 (>70％)，使得凝血酶（包括因子 Xa) 的催化结构域中的残基根据胰凝乳蛋白酶原中的相应残基进行编号。（胰凝乳蛋白酶编号系统 ;参见 Bajaj 和 Birktoft，Methods Enzymol.1993 ;222:96-128，表 2，以及 Bode W，Mayr I，Bauman Y 等人 The refined 1.9 Å crystal structure of human alpha-thrombin:interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Trp insertion segment.EMBO J 1989 ;8(11):3467-3475，所述两个参考文献均整体援引加入本文）。相应地，氨基酸在本文中可以根据胰凝乳蛋白酶编号系统提及，所述胰凝乳蛋白酶编号系统是本领域技术人员众所周知的。

[0031] 根据本公开，FXa 变体可以是包含氨基酸取代的 FXa 蛋白质，在体内或在体外与野生型 FXa 蛋白质相比较，所述氨基酸取代使得变体更酶原样。本公开的 FXa 变体在凝血酶原酶形成后基本上恢复野生型 FXa 活性。可用于本公开的方法中的 FXa 变体的例子是包含

选自下述的修饰的变体：根据胰凝乳蛋白酶编号系统，a) 在位置 16 处的 Ile 是 Thr、Leu、Phe、Asp 或 Gly，以及 b) 在位置 17 处的 Val 是 Leu、Ala 或 Gly。胰凝乳蛋白酶编号系统中的氨基酸 16 和 17 分别在 SEQ ID NO:1（人因子 X 前蛋白）的氨基酸 235 和 236 处存在。在某些实施方案中，FXa 变体是 FXa^{I16L} 和 FXa^{I16T}（本文使用的用于 FXa 变体的命名法叙述在根据胰凝乳蛋白酶编号系统的编号位置处的原始氨基酸，随后为取代氨基酸）。FXa 变体可以是任何哺乳动物 FXa 的变体。然而，特别感兴趣的是人 FXa 的 FXa 变体。

[0032] 在某些实施方案中，活化成本公开的变体 FXa 的 FX 变体可以通过插入非天然细胞内蛋白酶解切割位点进一步修饰。在非限制性例子中，为了在哺乳动物细胞中表达“活化的”酶原样 FXa 变体，非天然细胞内蛋白酶解切割位点可以插入在变体 FX 酶原的 SEQ ID NO:1 的位置 234（胰凝乳蛋白酶编号系统中的位置 15）处的 Arg 和在对应于 SEQ ID NO:1 的位置 235（胰凝乳蛋白酶编号系统中的位置 16）的位置处的氨基酸之间。在某些实施方案中，非天然细胞内蛋白酶切割位点是 Arg-Lys-Arg 或 Arg-Lys-Arg-Arg-Lys-Arg(SEQ ID NO:3)。这些切割位点由细胞内的蛋白酶 (PACE/ 弗林蛋白酶样酶）有效识别且被去除。该切割可以导致经加工的变体 FXa，其中分子的成熟重链现在在对应于 SEQ ID NO:1 的位置 235（胰凝乳蛋白酶编号系统中的位置 16）的位置处的氨基酸处开始。在所述位置处的该切割位点的引入允许 FX 至 FXa 的细胞内转换。

[0033] 在某些实施方案中，FX 变体活化肽 (AP) 的整个氨基酸序列（即 SEQ ID NO:1 的氨基酸 183-234）替换为非天然细胞内蛋白酶切割位点。根据某些实施方案，非天然细胞内蛋白酶切割位点是 Arg-Lys-Arg 或 Arg-Lys-Arg-Arg-Lys-Arg(SEQ ID NO:3)。如上所述，该修饰允许由细胞表达的 FX 变体的细胞内切割。细胞内切割将 FX 变体转换为活化的酶原样 FXa 变体，其随后被细胞分泌用于后续纯化。这种方法避免细胞外切割的需要，否则例如在分离蛋白质后或仅在血液凝固前活化变体凝血因子需要所述细胞外切割。

[0034] 在某些实施方案中，本公开的 FXa 变体衍生自包含天然野生型人信号序列和 / 或前肽序列的 FX 变体前蛋白。在其他实施方案中，来自非人物种的 FX 信号序列和 / 或前肽可以用于代替相应的天然氨基酸序列。此外，在另外其他实施方案中，来自其他人或非人分泌蛋白质的信号序列和 / 或前肽序列可以用于代替相应的天然氨基酸序列。

[0035] 在示例性实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 235 处的氨基酸（野生型序列中的异亮氨酸）由选自下述的不同氨基酸取代：苏氨酸 (Thr)、亮氨酸 (Leu)、苯丙氨酸 (Phe)、天冬氨酸 (Asp) 或甘氨酸 (Gly)。这些取代可以分别使用命名法 I235T、I235L、I235F、I235D 和 I235G 进行书写，其中第一个字母是异亮氨酸的单字母代码，并且最后一个字母是取代到野生型序列内的氨基酸的单字母代码。因为 SEQ ID NO:1 的位置 235 等价于胰凝乳蛋白酶编号系统的位置 16，所以相同取代可以书写为 I16T、I16L、I16F、I16D 和 I16G。在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 235 处的氨基酸由 Thr 取代（即 I235T 或 I16T）。在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 235 处的氨基酸由 Leu 取代（即 I235L 或 I16L）。在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 235 处的氨基酸由 Phe 取代（即 I235F 或 I16F）。在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 235 处的氨基酸由 Asp 取代（即 I235D 或 I16D）。

在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 235 处的氨基酸由 Gly 取代（即 I235G 或 I16G）。

[0036] 根据另一个示例性实施方案，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 236 处的氨基酸（野生型序列中的缬氨酸）由选自下述的不同氨基酸取代：亮氨酸 (Leu)、丙氨酸 (Ala) 或甘氨酸 (Gly)。这些取代可以分别使用命名法 V236L、V236A 和 V236G 进行书写，其中第一个字母是缬氨酸的单字母代码，并且最后一个字母是取代到野生型序列内的氨基酸的单字母代码。因为 SEQ ID NO:1 的位置 236 等价于胰凝乳蛋白酶编号系统的位置 17，所以相同取代可以书写为 V17L、V17A 和 V17G。在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 236 处的氨基酸由 Leu 取代（即 V236L 或 V17L）。在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 236 处的氨基酸由 Ala 取代（即 V236A 或 V17A）。在一个实施方案中，FXa 变体包含 SEQ ID NO:1 的氨基酸 41-179 和氨基酸 235-488，其中在位置 236 处的氨基酸由 Gly 取代（即 V236G 或 V17G）。

[0037] 在其他实施方案中，本公开的 FXa 变体包括前述段落中所述的那些具体变体，可以包括蛋白质的轻链和 / 或成熟重链的不同同种型 (isoform)。FXa 变体成熟重链的非限制性示例性同种型包括成熟重链的 α 和 β 版本。Jesty 等人，J Biol Chem. 1975Jun 25；250(12):4497-504，援引加入本文。本公开的组合物可以包括 FXa 变体蛋白质，在其中呈现 α 和 β 成熟重链同种型中一个或另一个或两者。

[0038] 根据另外其他实施方案，FXa 变体蛋白质的同种型包括前述段落中所述的那些具体变体，可以包括这样的同种型，其中可变数目的氨基酸（例如 1、2、3、4、5、6 个或更多个氨基酸）从蛋白质的轻链和 / 或成熟重链的羧基末端缺失或添加。

[0039] 根据某些实施方案，本公开的 FXa 变体包括与 SEQ ID NO:1 中的野生型 FXa 的氨基酸序列相比较，具有某一最小程度的同源性或序列相同性的蛋白质。因此，例如，FXa 变体包括含有轻链和成熟重链的蛋白质，其与 SEQ ID NO:1 中的野生型 FXa 轻链和成熟重链具有至少 60％、70％、80％、85％、90％、95％、98％或 99％序列同源性或相同性，其中此类 FXa 变体还包括在对应于 SEQ ID NO:1 的位置 235 的氨基酸位置处由 Thr、Leu、Phe、Asp 或 Gly 的取代，或者在对应于 SEQ ID NO:1 的位置 236 的氨基酸位置处由 Leu、Ala 或 Gly 的取代，并且进一步地其中此类 FXa 变体是酶原的，直至并入凝血酶原酶复合物内。在 SEQ ID NO:1 的氨基酸序列中，野生型 FXa 轻链序列对应于氨基酸 41-179，并且野生型 FXa 成熟重链序列对应于氨基酸 235-488。氨基酸序列同源性或相同性百分比可以使用软件容易地进行测定，所述软件例如在美国国家生物技术信息中心 (National Center for Biotechnology Information) 网站 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) 处可获得的 Protein BLAST。

[0040] 根据其他非限制性实施方案，本公开的 FXa 变体还可以包括含有一种或多种翻译后修饰的 FXa 变体，所述翻译后修饰包括但不限于一个或多个 O 联或 N 联碳水化合物基团或者可变数目的 γ 羧基谷氨酸 (Gla) 残基。本公开的 FXa 变体可以进一步包括化学修饰的 FXa 变体蛋白质。在本公开的方法中有用的其他 FXa 变体也是可能的。

[0041] 如本文使用的，术语 FXa^{I16x} 指活化的因子 X 的变体，其中对应于 SEQ ID NO:1 中的位置 235（对应于胰凝乳蛋白酶编号系统中的位置 16）的氨基酸从野生型序列中的氨基酸

（异亮氨酸）变成指定为“x”的不同氨基酸。在一些非限制性示例性实施方案中，氨基酸“x”可以是苏氨酸 (Thr 或 T)、亮氨酸 (Leu 或 L)、苯丙氨酸 (Phe 或 F)、天冬氨酸 (Asp 或 D) 或甘氨酸 (Gly 或 G)。

[0042] 如本文使用的，术语 FXa^{V17y} 指活化的因子 X 的变体，其中对应于 SEQ ID NO:1 中的位置 236（对应于胰凝乳蛋白酶编号系统中的位置 17）的氨基酸从野生型序列中的氨基酸（缬氨酸）变成指定为“y”的不同氨基酸。在一些非限制性示例性实施方案中，氨基酸“y”可以是亮氨酸 (Leu 或 L)、丙氨酸 (Ala 或 A) 或甘氨酸 (Gly 或 G)。

[0043] 术语 FXa^{I16x} 和 FXa^{V17y} 不受 SEQ ID NO:1 中所示的蛋白质序列限制。相反，这些术语另外包括本文描述的多种同种型和同源蛋白质，具有在胰凝乳蛋白酶编号系统中的位置 16 和 7 处的指定取代突变，所述蛋白质表现为酶原，直至掺入凝血酶原酶复合物内。

[0044] 本公开的 FXa 变体可以通过用于表达蛋白质的任何技术产生。

[0045] “分离的蛋白质”、“分离的多肽”或“分离的变体”是这样的蛋白质、多肽或变体，通过其起源或衍生来源，其 (1) 不与在其天然状态伴随它的天然结合的组分结合，(2) 不含来自相同物种的其他蛋白质，(3) 通过来自不同物种的细胞表达，或 (4) 在自然界中不存在。因此，化学合成的或在不同于它天然源于其的细胞的细胞系统中合成的多肽将是与其天然结合组分“分离的”。通过使用本领域众所周知的蛋白质纯化技术分离，蛋白质还可以致使基本上不含天然结合的组分。

[0046] 当样品的至少约 60-75％显示出单一种类的多肽时，蛋白质或多肽是“基本上纯的”、“基本上同质的”或“基本上纯化的”。多肽或蛋白质可以是单体或多聚的。基本上纯的多肽或蛋白质通常包含约 50％、60％、70％、80％或 90％ W/W 的蛋白质样品，更通常约 95％，并且可以是超过 99％纯的。蛋白质纯度或同质性可以通过本领域众所周知的许多方法进行指示，所述方法例如蛋白质样品的聚丙烯酰胺凝胶电泳，随后基于用本领域众所周知的染剂的凝胶染色来显现单一多肽条带。出于某些目的，更高的分辨率可以通过使用 HPLC 或本领域众所周知的用于纯化的其他方法来提供。

[0047] 本公开的方法可用于抵消直接 FXa 抑制剂。直接 FXa 抑制剂是与 FXa 直接结合且选择性结合 FXa 超过其他蛋白酶的抑制剂。直接 FXa 抑制剂是就凝血酶原而言的 FXa 的非竞争性抑制剂。它们结合底物结合裂缝且就小肽底物而言竞争性抑制 FXa，所述小肽底物也结合该区域。它们以高皮摩尔亲和力抑制 FXa 且在血浆中是高度蛋白质结合的。直接 FXa 抑制剂的例子是利伐沙班、阿哌沙班、贝曲西班、达瑞沙班、依度沙班和奥米沙班。在某些实施方案中，直接 FXa 抑制剂选自利伐沙班和阿哌沙班。

[0048] 根据本公开，FXa 变体可以用于抵消直接 FXa 抑制剂，其结合 FXa 或结合已形成凝血酶原酶的 FXa。直接 FXa 抑制剂可以需要或不需要 FXa 的辅因子用于抑制。根据本公开的方法，将 FXa 变体例如 FXa^{I16L} 和 FXa^{I16T} 施用于其血液含有直接 FXa 抑制剂的对象。

[0049] 本公开涵盖 FXa 变体抵消直接 FXa 抑制剂的用途，包括但不限于合成抑制剂、小分子抑制剂、经口可用抑制剂或可逆抑制剂。FXa 抑制剂可以是这些特点的任何组合，例如经口可用、合成、可逆的小分子抑制剂。在某些实施方案中，直接 FXa 抑制剂可以选自利伐沙班、阿哌沙班、贝曲西班、达瑞沙班、依度沙班和奥米沙班（参见 Perzborn 等人，Nat Rev Drug Discov.2011Jan ;10(1):61-75 ;Turpie,Arterioscler Thromb Vasc Biol.2007Jun ;27(6):1238-47 ;Pinto 等人，Expert Opin.Ther.Patents 22:645-661(2012) ;Pinto, 等

人，J.Med.Chem.50:5339-5356(2007)，所述参考文献各自援引加入本文）。在某些实施方案中，直接 FXa 抑制剂选自利伐沙班或阿哌沙班。

[0050] 在一些实施方案中，本公开的 FXa 变体可以施用于对象，以逆转直接 FXa 抑制剂的效应，其中此类抑制剂以治疗浓度存在。在其他实施方案中，本公开的 FXa 变体可以施用于对象，以逆转直接 FXa 抑制剂的效应，其中此类抑制剂以超治疗浓度存在。超治疗浓度是高于通常视为安全地实现特定对象或对象类别中的抗凝所需的浓度。直接 FXa 抑制剂的超治疗浓度可以起因于意外或有意药物过量。直接 FXa 抑制剂的超治疗浓度还可以起因于特定对象中出乎意料的效应，例如出乎意料地对这些药物的高敏感性，或出乎意料的缓慢清除速率，由于例如药物相互作用或其他因素。何种浓度为特定对象或对象类别中的直接 FXa 抑制剂的治疗浓度或超治疗浓度的测定在本领域普通技术人员的知识内。

[0051] 根据本公开，FXa 变体用于抵消一种或多种直接 FXa 抑制剂，其选择性结合 FXa 超过其他胰蛋白酶样蛋白酶至少 5 倍、至少 6 倍、至少 7 倍、至少 10 倍、至少 15 倍、至少 20 倍、至少 25 倍、至少 30 倍、至少 50 倍、至少 100 倍、至少 500 倍、至少 1,000 倍、至少 5,000 倍或至少 10,000 倍。

[0052] 直接 FXa 抑制剂可以以约 $2x10^{-7}M$ 或更少的 K_i 结合 FXa 变体。“K_i”指特定抑制剂 - 靶相互作用的抑制剂常数，其是产生半数最大抑制所需的浓度。可以通过使用本领域已知的方法测定 K_i。本公开因此考虑抵消直接 FXa 抑制剂，其以下述 K_i 结合不含凝血酶原酶复合物的 FXa 变体：约 $2x10^{-8}M$ 或更少、约 $1x10^{-8}M$ 或更少、约 $9x10^{-9}M$ 或更少、约 $8x10^{-9}M$ 或更少、约 $7x10^{-9}M$ 或更少、约 $6x10^{-9}M$ 或更少、约 $5x10^{-9}M$ 或更少、约 $4x10^{-9}M$ 或更少、约 $3x10^{-9}M$ 或更少、约 $2x10^{-9}M$ 或更少、约 $1x10^{-9}M$ 或更少、约 $9x10^{-10}M$ 或更少、约 $8x10^{-10}M$ 或更少、约 $7x10^{-10}M$ 或更少、约 $6x10^{-10}M$ 或更少、约 $5x10^{-10}M$ 或更少、约 $4x10^{-10}M$ 或更少、约 $3x10^{-10}M$ 或更少、约 $2x10^{-10}M$ 或更少、约 $1x10^{-10}M$ 或更少、约 $9x10^{-11}M$ 或更少、约 $8x10^{-11}M$ 或更少、约 $7x10^{-11}M$ 或更少、约 $6x10^{-11}M$ 或更少、约 $5x10^{-11}M$ 或更少、约 $4x10^{-11}M$ 或更少、约 $3x10^{-11}M$ 或更少、约 $2x10^{-11}M$ 或更少、约 $1x10^{-11}M$ 或更少、约 $9x10^{-12}M$ 或更少、约 $8x10^{-12}M$ 或更少、约 $7x10^{-12}M$ 或更少、约 $6x10^{-12}M$ 或更少、约 $5x10^{-12}M$ 或更少、约 $4x10^{-12}M$ 或更少、约 $3x10^{-12}M$ 或更少、约 $2x10^{-12}M$ 或更少、或约 $1x10^{-12}M$ 或更少。根据本公开的方法待由 FXa 变体抵消的直接 FXa 抑制剂可以与野生型 FXa 结合，其 K_i 比它结合 FXa 变体小至少 1.5 倍、至少 2 倍、至少 3 倍、至少 4 倍、至少 5 倍、至少 6 倍、至少 7 倍、至少 10 倍、至少 15 倍、至少 20 倍、至少 25 倍、至少 30 倍、或至少 50 倍。直接 FXa 抑制剂可以结合野生型 FXa，其 K_i 比不含凝血酶原酶复合物的 FXa 变体的 K_i 小至少 20％、至少 30％、至少 40％、至少 50％、至少 60％、至少 70％、至少 80％、至少 90％、至少 95％、或至少 99％。直接 FXa 抑制剂可以结合包含野生型 FXa 的凝血酶原酶复合物，其 K_i 与它结合包含 FXa 变体的凝血酶原酶复合物大约相同。

[0053] 在一个方面，本公开提供了通过施用 FXa 变体抵消对象中的直接 FXa 抑制剂效应的方法，所述对象正在出血（内部或外部）或处于出血的危险中（例如在计划手术的过程中）。在一些实施方案中，直接 FXa 抑制剂可以以治疗浓度或更高浓度（即，超治疗浓度）存在于对象中。在一些实施方案中，治疗浓度可以是敏感个体中的药物过量。本公开的方法因此可用于提供对直接 FXa 抑制剂的药物过量的解毒药。在多个实施方案中，治疗对象可以是人或兽医对象。

[0054] 基于过度降低的凝血能力的症状或体征的存在，可以检测直接抑制剂药物过量。

非限制性例子包括胃肠道出血的证据，包括黑柏油样便、血便和呕血。其他例子包括鼻出血，以及对来自微小切口和擦伤的瘀血或出血的趋势或严重性增加。

[0055] 在临床背景下，直接抑制剂药物过量可以直接地或通过下述进行检测：测量对象血液凝固的能力，且检测与预期抗凝程度的偏差。血液凝固潜力可以以本领域普通技术人员熟悉的方式进行测量。例如，当对象的凝血酶原时间过度延长时，可以怀疑药物过量。在某些实施方案中，当表示为国际标准化比率 (INR) 的凝血酶原时间测量为大于约 1.0、1.5、2.0、2.5、3.0、3.5、4.0、4.5、5.0、5.5、6.0、6.5、7.0、7.5、8.0、8.5、9.0、9.5、10、12、14、16、18、20 或更大时，证实药物过量。

[0056] 每当需要抵消直接 FXa 抑制剂的效应时，包括但不限于在计划手术前、导致外部或内部出血的损伤后或者直接 FXa 抑制剂药物过量后，可以施用 FXa 变体。根据本公开，当需要抵消效应时，例如在计划手术前、导致外部或内部出血的损伤后或者直接 FXa 抑制剂药物过量后，FXa 变体可以施用至少约 12 小时、至少约 6 小时、至少约 3 小时、至少约 2 小时、至少约 1 小时、至少约 30 分钟、至少约 10 分钟、或至少约 5 分钟。

[0057] 根据另一个实施方案，本公开提供了施用 FXa 变体以实现患有急性大出血的对象中由于 FXa 抑制疗法的获得性凝血病的紧急逆转的方法。在一些实施方案中，对象是成人患者。在其他实施方案中，对象是儿科人患者。

[0058] 在一些实施方案中，急性大出血由创伤引起。在其他实施方案中，急性大出血在手术或其他类型的干预操作期间发生。示例性非限制性干预操作包括切口、引流、血管手术、阑尾切除术、疝切开术或疝修补术、腹部手术、胆囊切除术、环钻术（钻孔）、腰椎穿刺、心脏起搏器插入、髋部骨折手术及其他。在另外其他实施方案中，急性大出血可以是没有明显原因的自发性出血。

[0059] 无限制地，急性大出血的部位包括胃肠道出血、皮下或肌内出血、膀胱出血、关节积血、硬膜下血肿、鼻出血、腹膜出血、子宫出血及其他部位的出血。

[0060] 用本公开的 FXa 变体的有效治疗可以逆转直接 FXa 抑制剂的效应。此类效应通过 FXa 变体的成功逆转可以以多种方式进行测定，并且可以使用不同测定、方法或终末点进行测量或监控。

[0061] 在一些实施方案中，使用对来自用 FXa 变体治疗的对象的血液或血浆进行的测试或测定，监控用 FXa 变体治疗逆转直接 FXa 抑制剂的效应。血样可以在用 FXa 变体治疗后的预定时间取自对象。随后对由其制备的血液或血浆实施一种或多种测试，以测定某些止血药效学参数是否已正常化，尽管存在直接 FXa 抑制剂。如果发现正常化，则对象无需进一步用 FXa 变体治疗。然而，如果未发现正常化，则可能需要依照本公开的方法用 FXa 变体的进一步治疗，以逆转直接 FXa 抑制剂的效应。用于监控用 FXa 变体治疗的有效性的测试包括这样的测试，其直接或间接测量凝血的能力或者测量直接 FXa 抑制剂的活性。非限制性示例性测试包括凝血酶原时间或相关国际标准化比率、凝血酶原酶诱导的凝血时间测定、血栓弹力描记术 (thromboelastometry)、血栓弹力图、生色抗 FXa 测定、凝血酶生成测定、凝血酶原片段 1+2 水平、凝血酶 - 抗凝血酶 III 复合物水平、活化部分凝血活酶时间和部分凝血活酶时间。其他测试在本领域普通技术人员的知识内也是可能的。

[0062] 根据一些实施方案，通过施用 FXa 变体逆转对象中的直接 FXa 抑制剂的效应降低对象中的出血。在一些实施方案中，与不存在用 FXa 变体治疗相比较，在直接 FXa 抑制剂

的存在下，用 FXa 变体治疗使对象中的出血降低至少 10％、20％、30％、40％、50％、60％、70％、80％、90％、95％或 99％。在其他实施方案中，用 FXa 变体治疗使对象中的出血降低约 5％ -10％、10％ -15％、15％ -20％、20％ -25％、25％ -30％、30％ -35％、35％ -40％、40％ -45％、45％ -50％、50％ -55％、55％ -60％、60％ -65％、65％ -70％、70％ -75％、75％ -80％、80％ -85％、85％ -90％、90％ -95％、或 95％ -100％。

[0063] 根据一些实施方案，通过施用 FXa 变体逆转对象中的直接 FXa 抑制剂的效应降低对象中的直接 FXa 抑制剂的活性。在一些实施方案中，与不存在用 FXa 变体治疗相比较，在直接 FXa 抑制剂的存在下，用 FXa 变体治疗使对象中的直接 FXa 抑制剂的活性降低至少 10％、20％、30％、40％、50％、60％、70％、80％、90％、95％或 99％。在其他实施方案中，用 FXa 变体治疗使对象中的直接 FXa 抑制剂的活性降低约 5％ -10％、10％ -15％、15％ -20％、20％ -25％、25％ -30％、30％ -35％、35％ -40％、40％ -45％、45％ -50％、50％ -55％、55％ -60％、60％ -65％、65％ -70％、70％ -75％、75％ -80％、80％ -85％、85％ -90％、90％ -95％、或 95％ -100％。

[0064] 使用生色抗 FXa 测定，例如 Asmis 等人，Thromb Res.，129:492-498(2012)，或 Barrett 等人，Thromb Haemost.104:1263-71(2010) 中所述的那种（所述参考文献各自援引加入本文），可以监控直接 FXa 抑制剂的活性。

[0065] 根据一些实施方案，通过施用 FXa 变体逆转对象中的直接 FXa 抑制剂的效应增加对象的血液或血浆中产生的凝血酶量。在一些实施方案中，与不存在 FXa 变体的情况相比较，在直接 FXa 抑制剂的存在下，用 FXa 变体治疗使对象中的凝血酶生产增加至少 10％、20％、30％、40％、50％、60％、70％、80％、90％、95％、100％、1.5 倍、2 倍、3 倍、4 倍、5 倍、6 倍、7 倍、10 倍、15 倍、20 倍、25 倍、30 倍、至少 50 倍或更多。使用凝血酶生成测定 (TGA) 或本领域普通技术人员熟悉的其他技术，可以测定对象的血液或血浆中的凝血酶产生。

[0066] 根据一些实施方案，通过施用 FXa 变体逆转对象中的直接 FXa 抑制剂的效应增加对象的凝血。在一些实施方案中，与不存在 FXa 变体的情况相比较，在直接 FXa 抑制剂的存在下，用 FXa 变体治疗使对象中的凝血增加至少 10％、20％、30％、40％、50％、60％、70％、80％、90％、95％、100％、1.5 倍、2 倍、3 倍、4 倍、5 倍、6 倍、7 倍、10 倍、15 倍、20 倍、25 倍、30 倍、至少 50 倍或更多。

[0067] 根据一些实施方案，通过施用 FXa 变体逆转对象中的直接 FXa 抑制剂的效应降低对象中的凝血时间。在一些实施方案中，与不存在用 FXa 变体治疗相比较，在直接 FXa 抑制剂的存在下，用 FXa 变体治疗使对象中的凝血时间降低至少 10％、20％、30％、40％、50％、60％、70％、80％、90％、95％或 99％。在其他实施方案中，用 FXa 变体治疗使对象中的凝血时间降低约 5％ -10％、10％ -15％、15％ -20％、20％ -25％、25％ -30％、30％ -35％、35％ -40％、40％ -45％、45％ -50％、50％ -55％、55％ -60％、60％ -65％、65％ -70％、70％ -75％、75％ -80％、80％ -85％、85％ -90％、90％ -95％、或 95％ -100％。

[0068] 根据一些实施方案，凝血时间通过测量对象的凝血酶原时间 (PT) 进行测定，所述凝血酶原时间随着止血恢复而减少。PT 是在添加组织因子后血清凝固花费的时间量。PT 因此测量外源凝血系统支持凝血的能力。PT 可以取决于实验室用于运行测试的特定试剂，但正常 PT 为约 11 至 13 秒。凝血时间还可以使用国际标准化比率 (INR) 进行表示，所述国际标准化比率消除在凝血时间测量中的实验室间可变性。使用 INR，0.8 至 1.1 的比率指示

正常凝血。在FXa变体施用于需要逆转直接FXa抑制剂的效应的对象后，PT或INR可以在预定时间进行测定。

[0069] 在一些实施方案中，用FXa变体治疗以逆转直接FXa抑制剂的效应使对象的PT降低约25秒、24秒、23秒、22秒、21秒、20秒、19秒、18秒、17秒、16秒、15秒、14秒、13秒、12秒、11秒、10秒或更少。在其他实施方案中，用FXa变体治疗使对象的INR降低至约4.0、3.9、3.8、3.7、3.6、3.5、3.4、3.3、3.2、3.1、3.0、2.9、2.8、2.7、2.6、2.5、2.4、2.3、2.2、2.1、2.0、1.9、1.8、1.7、1.6、1.5、1.4、1.3、1.2、1.1、1.0、0.9、0.8、0.7或更少。根据其他实施方案，用FXa变体治疗使对象中的PT或INR降低约5％-10％、10％-15％、15％-20％、20％-25％、25％-30％、30％-35％、35％-40％、40％-45％、45％-50％、50％-55％、55％-60％、60％-65％、65％-70％、70％-75％、75％-80％、80％-85％、85％-90％、90％-95％、或95％-100％。

[0070] 凝血酶原时间可以在FXa变体施用后的预定时间进行测量。因此，在一些非限制性实施方案中，PT在FXa施用后15分钟、20分钟、30分钟、40分钟、45分钟、50分钟、60分钟或更久进行测量。其他时间根据本领域普通技术人员的知识也是可能的。

[0071] 凝血时间还可以使用一步凝血酶原酶诱导的凝血时间(PiCT)测定进行测量，如Graff等人，Monitoring effects of direct FXa-inhibitors with a new one-step prothrombinase-induced clotting time(PiCT)assay:comparative in vitro investigation with heparin，enoxaparin，fondaparinux and DX 9065a，Int J Clin Pharmacol Ther.，45:237-43(2007)和Harder等人，Monitoring direct FXa-inhibitors and fondaparinux by prothrombinase-induced Clotting Time(PiCT):relation to FXa-activity and influence of assay modifications，Thromb Res.，123:396-403(2008)中所述，所述参考文献各自援引加入本文。

[0072] 在另外其他实施方案中，血栓弹力描记术或血栓弹力图方法可以用于分析血块形成或凝血时间。

[0073] 根据一些实施方案，通过施用FXa变体逆转对象中的直接FXa抑制剂的效应增加对象的血液或血浆中的凝血酶原片段1□+□2(PF1□+□2)水平。在一些实施方案中，与不存在FXa变体的情况相比较，在直接FXa抑制剂的存在下，用FXa变体治疗使对象中的PF1□+□2增加至少10％、20％、30％、40％、50％、60％、70％、80％、90％、95％、100％、1.5倍、2倍、3倍、4倍、5倍、6倍、7倍、10倍、15倍、20倍、25倍、30倍、至少50倍或更多。

[0074] 根据一些实施方案，通过施用FXa变体逆转对象中的直接FXa抑制剂的效应增加对象的血液或血浆中的凝血酶-抗凝血酶III复合物(TAT)水平。在一些实施方案中，与不存在FXa变体的情况相比较，在直接FXa抑制剂的存在下，用FXa变体治疗使对象中的TAT增加至少10％、20％、30％、40％、50％、60％、70％、80％、90％、95％、100％、1.5倍、2倍、3倍、4倍、5倍、6倍、7倍、10倍、15倍、20倍、25倍、30倍、至少50倍或更多。

[0075] 根据一些实施方案，通过施用FXa变体逆转对象中的直接FXa抑制剂的效应降低对象中的活化部分凝血活酶时间(aPTT)。在一些实施方案中，与不存在用FXa变体治疗相比较，在直接FXa抑制剂的存在下，用FXa变体治疗使对象中的活化部分凝血活酶时间(aPTT)降低至少10％、20％、30％、40％、50％、60％、70％、80％、90％、95％或99％。在其他实施方案中，用FXa变体治疗使对象中的aPTT降低约5％-10％、10％-15％、15％-20％、

20％-25％、25％-30％、30％-35％、35％-40％、40％-45％、45％-50％、50％-55％、55％-60％、60％-65％、65％-70％、70％-75％、75％-80％、80％-85％、85％-90％、90％-95％、或95％-100％。

[0076] 根据一些实施方案，通过施用FXa变体逆转对象中的直接FXa抑制剂的效应降低对象中的部分凝血活酶时间(PTT)。在一些实施方案中，与不存在用FXa变体治疗相比较，在直接FXa抑制剂的存在下，用FXa变体治疗使对象中的部分凝血活酶时间(PTT)降低至少10％、20％、30％、40％、50％、60％、70％、80％、90％、95％或99％。在其他实施方案中，用FXa变体治疗使对象中的PTT降低约5％-10％、10％-15％、15％-20％、20％-25％、25％-30％、30％-35％、35％-40％、40％-45％、45％-50％、50％-55％、55％-60％、60％-65％、65％-70％、70％-75％、75％-80％、80％-85％、85％-90％、90％-95％、或95％-100％。

[0077] 在其他实施方案中，临床终末点可以依赖测定止血在用FXa变体治疗以逆转直接FXa抑制剂效应的对象中是否已充分恢复。例如，当对象呈现急性出血时，当在用FXa变体治疗后发生现有出血的及时停止时，临床止血功效可以评分为“极佳的”；当出血停止存在1-2小时延迟时，评分为“良好的”；当出血停止存在>2小时延迟时，评分为“有问题的”；并且当不存在对出血的作用时，评分为“无”。当用FXa变体的治疗测定为小于良好时，则可以施用另外剂量的FXa变体，以实现足够的止血。在进一步例子中，当对象经历干预操作时，当在操作期间获得正常止血时，临床止血功效可以评分为“极佳的”；当如通过失血的数量或质量（例如轻微渗出）判断的，操作内止血轻度异常时，评分为“良好的”；当如通过失血的数量或质量（例如可控制的出血）判断的，操作内止血中度异常时，评分为“有问题的”；并且当如通过失血的数量或质量（例如严重难治性出血）判断的，操作内止血重度异常时，评分为“无”。

[0078] 直接FXa抑制剂的治疗有效剂量依赖医学领域熟练从业者众所周知的众多因素。利伐沙班的典型治疗血浆浓度为约500nM。然而，根据本公开，可以施用FXa变体以抵消更低或更高浓度的抑制剂。在待用FXa变体治疗的对象中的利伐沙班的血浆浓度可以低于或高于典型治疗浓度，例如约100nM、约200nM、约300nM、约400nM、约500nM、约600nM、约700nM、约800nM、约900nM或约1,000nM。

[0079] 阿哌沙班的典型治疗血浆浓度为约250nM。在某些实施方案中，FXa变体施用于具有下述血浆浓度的阿哌沙班的对象：约100nM、约200nM、约300nM、约400nM、约500nM、约600nM、约700nM、约800nM、约900nM或约1,000nM。

[0080] 同样地，根据本公开，在药物过量的情况下，例如当抑制剂的血浆浓度比典型治疗血浆浓度高至少20％、至少30％、至少40％、至少50％、至少60％、至少70％、至少80％、至少90％、至少95％、至少99％、或至少1.5倍、至少2倍、至少3倍、至少4倍、至少5倍、至少6倍、至少7倍、至少10倍、至少15倍、至少20倍、至少25倍、至少30倍、或至少50倍时，FXa变体可以用于抵消直接FXa抑制剂。

[0081] 在比直接FXa抑制剂的血浆浓度低的血浆浓度下，FXa变体令人惊讶地有效抵消直接FXa抑制剂。根据本公开，FXa变体在下述变体与抑制剂的血浆浓度比下抵消直接FXa抑制剂的效应：约1-10、约1-25、约1-50、约1-100、约1-250、约1-500、约1-1,000、约1-2,500、约1-5,000或约1-10,000。在某些实施方案中，在比直接FXa抑制剂的血浆浓度

低至少 10 倍、至少 25 倍、至少 50 倍、至少 100 倍、至少 250 倍、至少 500 倍、至少 1,000 倍、至少 2,500 倍、至少 5,000 倍、或至少 10,000 倍的血浆浓度下，FXa 变体抵消直接 FXa 抑制剂的效应。

[0082] 在其他实施方案中，通过将直接抑制剂的血浆浓度乘以范围为约 $0.1x10^{-4}$至约 $1000x10^{-4}$、约 $4x10^{-4}$至约 $40x10^{-4}$、约 $20x10^{-4}$至约 $200x10^{-4}$、或其他范围的换算因子，来计算足以逆转直接 FXa 抑制剂效应的 FXa 变体的血浆浓度。在另外其他实施方案中，换算因子可以为约 $0.1x10^{-4}$、$0.5x10^{-4}$、$1x10^{-4}$、$2x10^{-4}$、$3x10^{-4}$、$4x10^{-4}$、$5x10^{-4}$、$6x10^{-4}$、$7x10^{-4}$、$8x10^{-4}$、$9x10^{-4}$、$10x10^{-4}$、$11x10^{-4}$、$12x10^{-4}$、$13x10^{-4}$、$14x10^{-4}$、$15x10^{-4}$、$16x10^{-4}$、$17x10^{-4}$、$18x10^{-4}$、$19x10^{-4}$、$20x10^{-4}$、$21x10^{-4}$、$22x10^{-4}$、$23x10^{-4}$、$24x10^{-4}$、$25x10^{-4}$、$26x10^{-4}$、$27x10^{-4}$、$28x10^{-4}$、$29x10^{-4}$、$30x10^{-4}$、$31x10^{-4}$、$32x10^{-4}$、$33x10^{-4}$、$34x10^{-4}$、$35x10^{-4}$、$36x10^{-4}$、$37x10^{-4}$、$38x10^{-4}$、$39x10^{-4}$、$40x10^{-4}$、$45x10^{-4}$、$50x10^{-4}$、$55x10^{-4}$、$60x10^{-4}$、$65x10^{-4}$、$70x10^{-4}$、$75x10^{-4}$、$80x10^{-4}$、$85x10^{-4}$、$90x10^{-4}$、$95x10^{-4}$、$100x10^{-4}$、$110x10^{-4}$、$120x10^{-4}$、$130x10^{-4}$、$140x10^{-4}$、$150x10^{-4}$、$160x10^{-4}$、$170x10^{-4}$、$180x10^{-4}$、$190x10^{-4}$、$200x10^{-4}$、$250x10^{-4}$、$300x10^{-4}$、$350x10^{-4}$、$400x10^{-4}$、$450x10^{-4}$、$500x10^{-4}$、$550x10^{-4}$、$600x10^{-4}$、$650x10^{-4}$、$700x10^{-4}$、$750x10^{-4}$、$800x10^{-4}$、$850x10^{-4}$、$900x10^{-4}$、$950x10^{-4}$、或 $1000x10^{-4}$，以及这些数目之间的范围。根据技术人员的知识，例如通过放射性免疫测定 (RIA) 或其他方法，可以测量 FXa 直接抑制剂的血浆浓度。

[0083] 实现足以逆转直接 FXa 抑制剂的药物过量的 FXa 变体的靶血浆浓度在本领域普通技术人员的知识内。在非限制性例子中，有关药物代谢动力学参数，例如对象血浆容量或其他参数的估计量，可以基于对象性别、高度和重量或其他因素进行制备，并且用于计算需要施用多少 FXa 变体以实现靶浓度。在施用 FXa 变体后，血浆浓度可以根据本领域普通技术人员的知识进行监控，并且该信息用于维持任何所需范围内的浓度。

[0084] 本公开的组合物和方法包括“治疗有效量”或“预防有效量”的 FXa 变体。“治疗有效量”指在所需剂量和时间段下有效实现所需治疗结果的量。FXa 变体的治疗有效量可以根据诸如下述因素而改变：疾病状态，个体的年龄、性别和重量，以及 FXa 变体在个体中引发所需应答的能力。治疗有效量也是其中 FXa 变体的任何毒性或有害效应由治疗有利效应超过的量。“预防有效量”指在所需剂量和时间段下有效实现所需预防结果的量。例如，剂量可以在计划手术前给予。

[0085] 剂量方案可以进行调整，以提供最佳所需应答（例如治疗或预防应答）。例如，可以施用单次推注，可以在一段时间内施用几个分份剂量，或剂量可以如通过治疗情况的紧迫指示的按比例降低或增加。尤其有利的是以剂量单位形式配制肠胃外组合物，以易于施用和剂量一致性。如本文使用的剂量单位形式指物理上不连续的单位，适合作为用于待治疗的哺乳动物对象的单位剂量；每个单位含有计算为与所需药学载体结合产生所需疗效的活性化合物的预定数量。本公开的剂量单位形式的规格通过下述指出且直接取决于下述：(a)FXa 变体的独特特征和待实现的特定治疗或预防效应，和 (b) 在用于个体治疗的此类 FXa 变体的配制领域中固有的局限性。

[0086] 在某些实施方案中，所施用的 FXa 变体的治疗或预防有效量为约 0.0001-50mg/kg、约 0.001-50mg/kg、约 0.001-5mg/kg、约 0.001-0.5mg/kg、约 0.001-0.05mg/kg、约 0.01-5mg/kg 或约 0.01-0.5mg/kg。

[0087] 在某些实施方案中，本公开的 FXa 变体的治疗或预防有效血清浓度为约

0.0003-300nM、约 0.003-300nM、约 0.03-300nM、约 0.003-30nM、约 0.03-30nM 或 约 0.3-3nM。例如在血液或血浆中的 FXa 变体浓度可以通过本领域已知的任何方法进行测量。

[0088] 应当指出剂量值可以随 FXa 抑制剂浓度而改变。应进一步理解对于任何特定对象，根据个体需要和施用或监督组合物施用的个人的专业判断，具体剂量方案应在一段时间内进行调整，并且本文所述的剂量方案仅是示例性的，并且不预期限制要求保护的组合物的范围或实践。

[0089] 本公开的另一个方面提供了包含 FXa 变体或包含此类 FXa 变体的组合物的试剂盒。除 FXa 变体或组合物之外，试剂盒还可以包括诊断试剂或另外的治疗试剂。试剂盒还可以包括治疗方法的使用说明书，以及包装材料例如但不限于冰、干冰、泡沫聚苯乙烯、泡沫、塑料、玻璃纸、收缩包装、气泡包装、卡纸板和淀粉花生。在一个实施方案中，试剂盒包括 FXa 变体或包含其和一种或多种治疗试剂的组合物，所述治疗试剂可以用于本文描述的方法中。

[0090] FXa 变体可以例如在包含其的组合物中一次或多次施用于对象，直至恢复足够的止血或者一种或多种直接 FXa 抑制剂不再有效。当使用多重施用时，它们可以每小时、每天或以任何其他适当间隔包括例如多重日剂量进行施用。多重剂量可以在时间表上施用，例如每 10 分钟、每 15 分钟、每 20 分钟、每 30 分钟、每小时、每两小时、每三小时、每四小时、每天三次、每天两次、每天一次、每两天一次、每三天一次和每周一次。FXa 变体可以例如经由微型泵连续施用。FXa 变体可以例如经由肠胃外途径（例如静脉内、皮下、腹膜内或肌内）施用。FXa 变体一般作为如下所述的药物组合物的一部分进行施用。

[0091] 在另一个实施方案中，FXa 变体可以与另一种促凝血剂共施用，所述另一种促凝血剂包括另一种 FXa 变体、因子 IX、因子 XIa、因子 XIIa、因子 VIII、因子 VIIa、FEIBA 和凝血酶原复合物浓缩剂 (PCC)。

[0092] 本公开的 FXa 变体与另外的治疗试剂的共施用（组合疗法）涵盖施用包含 FXa 变体的药物组合物和另外的治疗试剂，以及施用两种或更多种分开的药物组合物，一种即包含 FXa 变体并且另一种或多种包含一种或多种另外的治疗试剂。共施用或组合疗法进一步包括同时或顺次或两者施用 FXa 变体和一种或多种另外的治疗试剂。例如，FXa 变体可以每三天施用一次，而另外的治疗试剂每天施用一次，与 FXa 变体相同或不同时间。FXa 变体可以在用另外的治疗试剂治疗前或后进行施用。类似地，本公开的 FXa 变体的施用可以是治疗方案的一部分，所述治疗方案包括其他治疗模式包括手术。可以施用组合疗法以预防状况的复发。组合疗法可以从每小时多次施用到每周多次。施用可以在时间表上施用，例如每 10 分钟、每 15 分钟、每 20 分钟、每 30 分钟、每小时、每两小时、每三小时、每四小时、每天三次、每天两次、每天一次、每两天一次、每三天一次、每周一次，或可以例如经由微型泵连续施用。组合疗法可以例如经由肠胃外途径（例如静脉内、皮下、腹膜内或肌内）施用。

[0093] 在进一步方面，本公开提供了包含 FXa 变体的组合物用于抵消对象中的直接 FXa 抑制剂。组合物可以包含药学可接受的载体、媒介物或生理学相容的其他成分。此类载体、媒介物及其他成分的非限制性例子包括溶剂（例如水、乙醇、盐水、磷酸盐缓冲盐水）、去污剂、表面活性剂、分散介质、包衣、抗菌剂或抗真菌剂、等渗剂、吸收延迟剂、糖（例如蔗糖、右旋糖、乳糖）、多元醇（例如甘油、甘露醇、山梨糖醇）、盐（例如氯化钠、氯化钾）、湿润剂、乳化剂、防腐剂、缓冲剂以及能够增强 FXa 变体的稳定性或有效性的试剂。

[0094] 用于根据本公开使用的组合物可以采取施用于对象的任何合适形式，例如液体溶液（例如可注射和可输注溶液）。组合物可以例如在小瓶或预填充注射器中，以准备施用于对象的预混合形式提供。此类形式不需要在施用前用稀释剂重构。可替代地，组合物可以以需要在施用前用稀释剂（例如无菌水或盐水）重构的冻干形式提供。如果是后者，则稀释剂可以在分开容器中与冻干产物一起提供。根据本领域普通技术人员的知识，组合物可以配制用于在冷藏或室温下贮存。组合物的形式至少部分取决于预期施用模式。在某些实施方案中，施用模式是肠胃外的，包括例如静脉内、皮下、腹膜内或肌内施用。

[0095] 治疗组合物通常在制造和贮存条件下必须是无菌且稳定的。组合物可以配制为溶液、微乳剂、分散体、在脂质体中或其他适合高药物浓度的有序结构。无菌可注射溶液可以通过下述进行制备：根据需要，掺入在适当溶剂中以所需量的FXa变体与上文列举的成分之一或组合，随后为无菌过滤。一般地，分散体通过将活性化合物掺入无菌媒介物内进行制备，所述无菌媒介物含有基本分散介质以及来自上文列举那些的所需其他成分。在用于制备无菌可注射溶液的无菌粉末的情况下，优选制备方法是真空干燥和冷冻干燥，其获得来自其先前无菌过滤溶液的活性成分加上任何另外所需成分的粉末。溶液的适当流动性可以通过下述得到维持：例如通过使用包衣例如卵磷脂，在分散体的情况下通过维持所需粒子大小和通过使用表面活性剂。可注射组合物的延长吸收可以通过在组合物中使用延迟吸收的试剂来实现，所述试剂例如单硬脂酸盐和明胶。

[0096] 本公开进一步考虑本文的组合物中的任一种均可施用于用直接FXa抑制剂治疗的对象。

[0097] 应当理解本文描述的例子和实施方案仅用于举例说明性目的，并且根据其的多种修饰或变化对于本领域技术人员将是显而易见的，并且应包括在本发明的真正范围内，并且可以无需背离本发明的真正范围而作出。

[0098] 实施例

[0099] 实施例1-FXa^{I16L}针对利伐沙班的敏感性

[0100] 为了测试FXa^{I16L}针对利伐沙班的敏感性，建立抑制测定。利伐沙班是野生型FXa的有效抑制剂，显示出0.582nM的抑制常数(K_i)（图1A）。由于FXa^{I16L}的酶原样性质，利伐沙班以降低～15倍的亲和力与该变体结合(K_i＝9.3nM)（图1B）。相比之下，当变体在凝血酶原酶复合物（即，在添加FVa和磷脂囊泡后）中装配时，利伐沙班的K_i几乎恢复至与野生型酶(wt FXa，K_i＝2.67nM（图2A）；FXa^{I16L}，K_i＝3.4nM（图2B）可比较的值。

[0101] 实施例2-FXa变体抵消利伐沙班和阿哌沙班

[0102] 凝血酶生成测定(TGA)用于评价酶原样FXa变体是否可以在更生理学的环境中逆转直接FXa抑制剂的效应。TGA测量在凝血起始后的一段时间内在血浆中的凝血酶产生，并且如先前所述（参见Bunce等人，(2011)Blood 117，290-298，整体援引加入本文）进行。在500nM利伐沙班的存在或不存在下，正常人血浆中的凝血酶生成在37℃下测量90分钟。为了评估FXa^{I16L}是否可以逆转利伐沙班的效应，将渐增量的FXa^{I16L}加入含有500nM利伐沙班的血浆中。凝血酶生成用2.0pM组织因子/4uM磷脂以及$CaCl_2$和凝血酶荧光底物起始。

[0103] 数据证实与不存在利伐沙班的血浆相比较，在500nM利伐沙班的存在下，血浆的凝血酶生成谱基本上降低。相比之下，从0.03到1nM的渐增浓度的FXa^{I16L}恢复凝血酶生成（图3）。这些数据显示在纳摩尔和亚纳摩尔范围内的出乎意料低浓度的FXa^{I16L}可以逆转抑

制剂的效应。在500nM利伐沙班（典型的治疗血浆浓度）的存在下，FXa^{I16L}的剂量应答分析显示在这些条件下在1-3nM FXa^{I16L}之间，凝血酶生成的峰高（图4A和C）和产生的总凝血酶(ETP)（图4B和D）基本上达到最大值，并且完全恢复至正常水平。进一步实验显示即使在高浓度利伐沙班(7.5μM；超治疗的）的存在下，FXa^{I16L}在相对低剂量（≤3.0nM）下在恢复生成的峰凝血酶（图5A）以及总凝血酶（图5B）方面仍是非常有效的。

[0104] 还进行相似实验，以评估FXa酶原样变体是否可以逆转另一种直接FXa抑制剂阿哌沙班的效应。在这些实验中，还比较FXa^{I16L}与另一种酶原样FXa变体FXa^{I16T}的有效性。FXa^{I16T}类似于FXa^{I16L}，然而，当在凝血酶原酶复合物中装配时，与FXa^{I16L}相比较，它具有固有更低的活性，具有更长的血浆半衰期，并且具有降低～3-5倍的活性。与利伐沙班数据一致，在250nM阿哌沙班（典型的治疗血浆浓度）的存在下，FXa^{I16L}可以以剂量依赖性方式恢复生成的峰凝血酶（图6A）和总凝血酶（图6B），其看起来在1-3nM FXa^{I16L}之间达到最大值。FXa^{I16T}也有效逆转阿哌沙班的效应；然而，看起来需要更高浓度的该变体，以完全恢复凝血酶生成（图6）。即使在高浓度阿哌沙班(2μM)的存在下，两种变体仍是有效的。然而，在这些条件下，看起来需要更高浓度的两种变体，以完全恢复凝血酶生成（图7A和B）。

[0105] 实施例3-FXa^{I16L}抵消全血中的利伐沙班和阿哌沙班

[0106] 全血血栓弹力描记术用于评价FXa^{I16L}变体逆转直接FXa抑制剂在全血中的效应的能力。在这种系统中，从健康志愿者中抽取血液。弃去前2mL血液，并且将以后5mL血液收集到真空采血管(BD，Franklin Lakes，NJ)内。玉米胰蛋白酶抑制剂和柠檬酸钠在血样收集前存在于收集管中，以实现血液中0.105M柠檬酸盐和25μg/mL玉米胰蛋白酶抑制剂(Haematologic Technologies，Burlington，VT)的最终浓度。对于每个供体分析两组反应。初始反应在血液收集后5分钟起始。第二个反应在第一个反应起始后1小时起始（收集后1小时5分钟）。

[0107] 使用Thromboelastometry ROTEM® delta(Tem International GmbH，Munich，德国）分析血液。对于反应：(1)将6μL媒介物、蛋白质和/或抑制剂加入空杯中，将(2)20μl 0.2M $CaCl_2$（最终浓度11.6mM），和(3)20μl Innovin（反应中的最终浓度1:10,000；组织因子来源）加入杯中。将如上所述收集的全血加入反应中(300μL)，并且允许记录进行大约30-60分钟。使用制造商的软件(Rotem Gamma Software版本1.1.1)分析收集的数据。

[0108] 使用旋转血栓弹力描记术(ROTEM)，检查在利伐沙班或阿哌沙班的存在下，FXa^{I16L}加速全血血块形成的能力。单独的且以两种不同浓度的两种直接FXa抑制剂对全血血块形成具有重大作用：在低剂量（治疗浓度）下，全血血块形成被部分消除（图8A和图9A），而在高剂量（超治疗浓度）下，全血血块形成几乎完全被消除（图8B和图9B）。利伐沙班或阿哌沙班对全血血块形成的作用可以被FXa^{I16L}逆转。在500nM利伐沙班或250nM阿哌沙班，0.3nM FXa^{I16L}可以完全或几乎完全恢复全血凝固（图8A和图9A）。当使用更高浓度的直接FXa抑制剂时（～2uM），0.3nM FXa^{I16L}部分恢复全血凝固，并且3nM FXa^{I16L}完全恢复全血凝固（图8B和图9B）。这些数据证实在治疗和超治疗浓度的抑制剂下，在基于血浆和全血的凝血测定中，FXa酶原样变体可以有效逆转利伐沙班或阿哌沙班的抗凝效应。

[0109] 当两种试剂均体内施用时，通过测试FXa^{I16L}是否可以抵消利伐沙班的抗凝效应，这些研究的结果得到证实和扩展。在这些实验中，C57BL/6小鼠经由尾静脉输注利伐沙班

(1mg/kg) 或缓冲液。随后准备小鼠，以暴露颈静脉和腔静脉。大约 10 分钟后，FXa^{I16L}(1 或 2mg/kg) 通过直接注射到颈静脉内进行输注。注射后五分钟，血液经由腔静脉收集到柠檬酸盐和玉米胰蛋白酶抑制剂内。随后使用稀释组织因子 (Innovin，1:42,000 稀释度)，通过 ROTEM 分析收集的血液。来自仅施用缓冲液的全血到约 2 分钟时凝固 (图 12)。1mg/kg 利伐沙班的施用将凝血时间基本上延长到约 10 分钟 (图 12)。在利伐沙班的存在下，FXa^{I16L} 的进一步施用以剂量依赖性方式缩短凝血时间 (图 12)。

[0110] 实施例 4 - FXa^{I16L}在凝血酶生成测定中抵消利伐沙班

[0111] 使用自动校准血栓图像仪 (CAT) 系统 (Thrombinoscope BV，Maastricht，荷兰)，在凝血酶生成测定 (TGA) 中检查 FXa^{I16L}对逆转血浆中的利伐沙班的作用。正常人血浆得自 George King Biomedical(Overland Park，KS)。在反应中，在反应一式两份的 Immulon 2HB 圆底 96 孔板中，将 20μL 含有 4μM 磷脂和 1pM 组织因子的 PPP-Reagent LOW 加入 70μL 合并的含柠檬酸盐的正常人血浆 (用 250nM 利伐沙班 (在治疗血浆浓度范围内) 处理)。紧在反应起始前，将 10μL 媒介物或 FXa^{I16L}以范围为 0.03125nM-0.5nM FXa^{I16L}的最终浓度加入血浆中，获得 120μL 总反应体积。通过添加 20μL 含有氯化钙和荧光底物的 FluCa 缓冲液来起始反应。血浆反应的荧光在 37℃下以 20 秒间隔在 Fluoroskan Ascent 荧光计上进行读数，并且与参考凝血酶校准物反应的那些相比较，以测定凝血酶浓度。使用 CAT 在 37℃下连续监控荧光信号 (FU) 的强度。使用 Thromboscope 软件 (Thrombinoscope BV 版本)，分析凝血酶生成曲线 (nM 凝血酶相对于时间)，以提取滞后时间、峰高、到峰的时间和代表内源凝血酶潜力 (ETP) 的曲线下面积。

[0112] 用体外利伐沙班处理 (5-200nM) 观察到正常人血浆中的凝血酶生成的剂量依赖性抑制 (图 10A)。利伐沙班导致与峰凝血酶减少和 ETP 减少偶联的滞后时间增加。向利伐沙班 (250nM) 抑制的人血浆添加 FXa^{I16L}导致凝血酶抑制的剂量依赖性逆转 (图 10B)：峰凝血酶生成得到回复，滞后期更短，并且 ETP 增加。在低剂量的 0.03125nM FXa^{I16L}下，凝血酶生成恢复至与媒介物处理的正常人血浆可比较的水平。

[0113] 实施例 5 - FXa^{I16L}在小鼠尾部修剪出血模型中抵消利伐沙班

[0114] 在正常小鼠中的急性出血模型中，评价 FXa^{I16L}在体内克服利伐沙班的效应的能力。结果证实酶原样 FXa 变体可以逆转直接 FXa 抑制剂的抗凝效应。

[0115] 为了确定延长出血的利伐沙班剂量，雄性 C57Bl/6 小鼠 (The Jackson Laboratory，Bar Harbor，ME) 接受以 10、25 或 50mg/kg 剂量的利伐沙班的单次静脉内注射。三十分钟后，将小鼠用异氟烷麻醉且置于加热平台上，并且在尾部切割前小鼠的体温维持在 37℃下。将尾部在 37℃下浸入 50mL 预温的磷酸盐缓冲盐水 (PBS) 中 2 分钟。作出 3mm 尾部切割，并且将血液收集到 PBS 内共 10 分钟时期。通过收集到 PBS 内的血液的血红蛋白含量来测定出血量的定量评价。将管离心以收集红细胞，重悬浮于 5mL 裂解缓冲液 (8.3g/L NH_4Cl、1.0g/L $KHCO_3$和 0.037g/L EDTA) 中，并且样品的吸光度在 575nm 下进行测量。使用标准曲线将吸光度值转换为总失血量 (μL)。在尾部切割后，利伐沙班的施用导致剂量依赖性的失血增加 (图 11)。

[0116] 在该模型中，50mg/kg 利伐沙班的剂量导致在尾部横切后的失血增加。小鼠用 50mg/kg 利伐沙班进行给药，并且 30 分钟后，在尾部切割前，在 37℃下将 50 或 200ug/kg FXa^{I16L}静脉内给药。随后将小鼠用异氟烷麻醉且置于加热平台上，并且在尾部切割前小鼠

的体温维持在37℃下。将尾部在37℃下浸入50mL预温的磷酸盐缓冲盐水(PBS)中2分钟。作出3mm尾部切割,并且将血液收集到PBS内共10分钟时期,并且如所述的通过血红蛋白含量来测定出血量的评价。在该模型中,止血FXa^{I16L}变体的施用减少由利伐沙班诱导的过度失血(图11)。

[0117] 实施例6- 使用活体显微镜检查证实的,FXa^{I16L}在小鼠出血模型中抵消利伐沙班

[0118] 如使用活体显微镜检查显现的,证实在激光诱导的损伤后,利伐沙班抑制小鼠提睾肌的微循环中的血栓形成。FXa^{I16L}的进一步施用可以抵消利伐沙班在该系统中的抗凝效应。

[0119] 使用标准技术,使小鼠的提睾肌暴露且使用活体显微镜检查显现。随后使用激光诱导肌肉中的血管损伤。在损伤后,使用不同荧光标记的抗体显现血块形成,所述抗体特异性识别纤维蛋白和血小板。凝血通过来自两种类型抗体的荧光信号的存在得到指示。

[0120] 在激光损伤后,未经处理的小鼠在损伤部位处快速形成凝块,所述凝块稳定几分钟(图13A)。在视频帧中,可见与荧光信号一致的凝块,所述荧光信号与针对纤维蛋白和血小板的抗体相关(淡灰色区域重叠暗灰色区域)。然而,将1mg/kg利伐沙班施用于小鼠延迟在损伤部位处的血小板累积,并且消除任何纤维蛋白征兆(图13B)。在视频帧中,如通过暗灰色区域指示的,仅可见降低程度的血小板,这反映与抗血小板抗体相关的荧光信号的存在。相比之下,当小鼠施用1mg/kg利伐沙班随后为0.5mg/kg FXa^{I16L}时,在损伤部位处快速形成凝块(图13C)。在视频帧中,凝块通过荧光信号的特征性模式指示,所述荧光信号与针对血小板和纤维蛋白的抗体相关。

[0121] 除非本文另有定义,否则与本公开结合使用的科学和技术术语应具有本领域普通技术人员通常理解的含义。进一步地,除非上下文另有要求,否则单数术语应包括复数,并且复数术语应包括单数。一般地,与下述结合使用的命名法和技术是本领域众所周知的和通常使用的那些:本文描述的细胞和组织培养、分子生物学、免疫学、微生物学、遗传学以及蛋白质和核酸化学和杂交。

[0122] 除非另有说明,否则本公开的方法和技术一般根据本领域众所周知的常规方法且如多种一般和更具体的参考文献中所述的进行,所述参考文献在本说明书自始至终被引用且讨论。参见例如援引加入本文的Sambrook J.&Russell D..Molecular Cloning:A Laboratory Manual,3rd ed.,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,N.Y.(2000);Ausubel等人,Short Protocols in Molecular Biology:A Compendium of Methods from Current Protocols in Molecular Biology,Wiley,John&Sons,Inc.(2002);Harlow和Lane Using Antibodies:A Laboratory Manual,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,N.Y.(1998);和Coligan等人,Short Protocols in Protein Science,Wiley,John&Sons,Inc.(2003)。酶促反应和纯化技术根据制造商的说明书进行,如本领域通常完成的或如本文描述的。与下述结合使用的命名法以及实验室操作和技术是本领域众所周知的和通常使用的那些:本文描述的分析化学、合成有机化学、以及医学和药物化学。

[0123] 本文引用的所有出版物、专利、专利申请或其他文件为了所有目的在此整体引入作为参考,其程度与每个个别出版物、专利、专利申请或其他文件个别指示为了所有目的引入作为参考相同。

[0124] 本说明书和权利要求自始至终，单词“包含”或其变化应理解为暗示所述整数或整数组的包括，但不排除任何其他整数或整数组。

[0001]

序列表

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费城儿童医院

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ggctgtgccc gtaaggggaa gtacgggatc tacaccaagg tcaccgcctt cctcaagtgg 1440

atcgacaggt ccatgaaaac caggggcttg cccaaggcca agagccatgc cccggaggtc 1500

ataacgtcct ctccattaaa gtgagatccc actcaaaaaa aaaaaaaaaa aaaaaaaaaa 1560

<210> 3
<211> 6
<212> PRT
<213> Artificial Sequence

<220>

[0005]

<223> Proteolytic Cleavage Site

<400> 3

Arg Lys Arg Arg Lys Arg
1 5

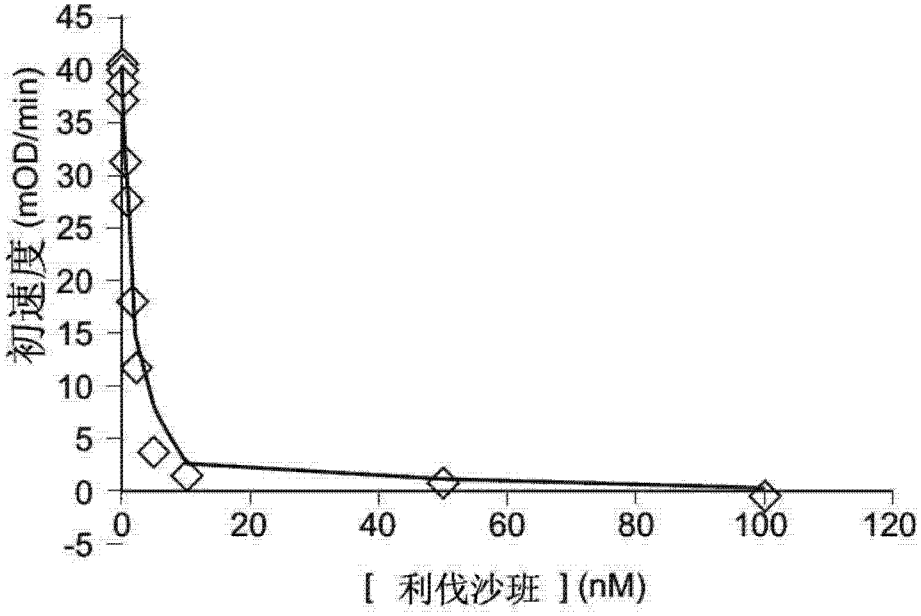
初速度 (mOD/min)
45
40
35
30
25
20
15
10
5
0
-5
0
20
40
60
80
100
120
[利伐沙班] (nM)

图 1A

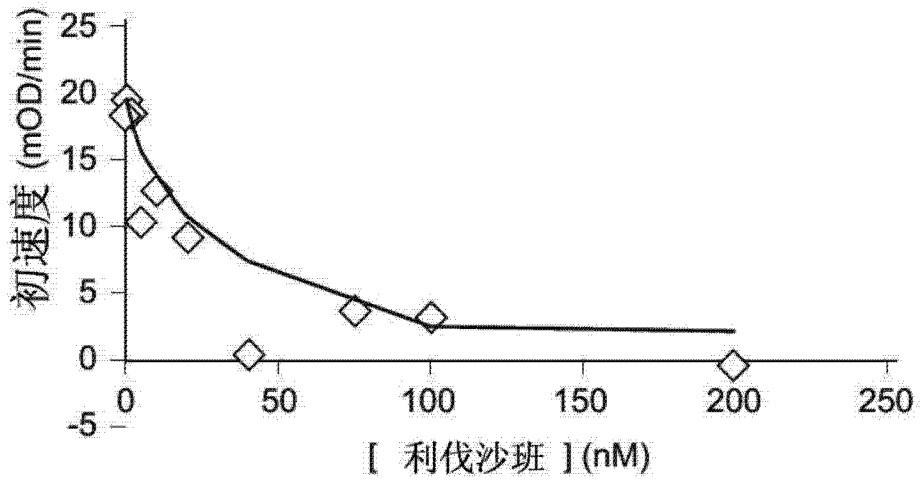
初速度 (mOD/min)
25
20
15
10
5
0
-5
0
50
100
150
200
250
[利伐沙班] (nM)

图 1B

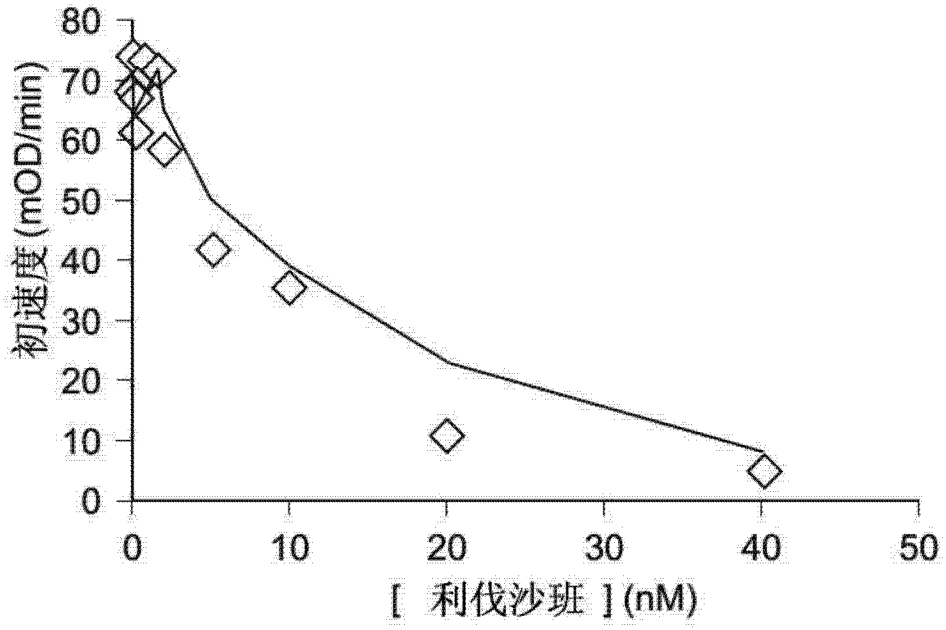
初速度 (mOD/min)
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
[利伐沙班] (nM)

图 2A

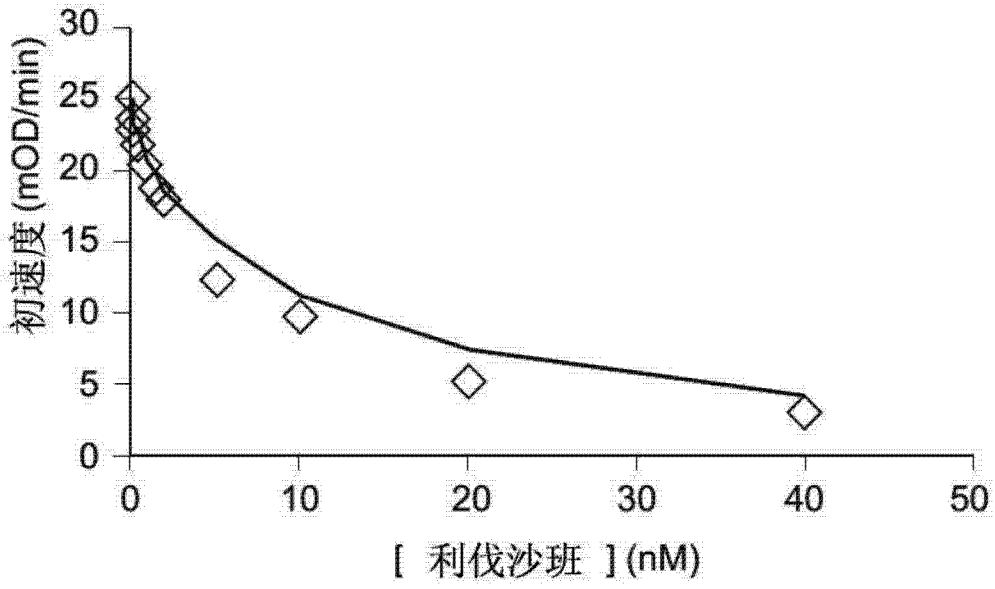
初速度 (mOD/min)
30
25
20
15
10
5
0
0
10
20
30
40
50
[利伐沙班] (nM)

图 2B

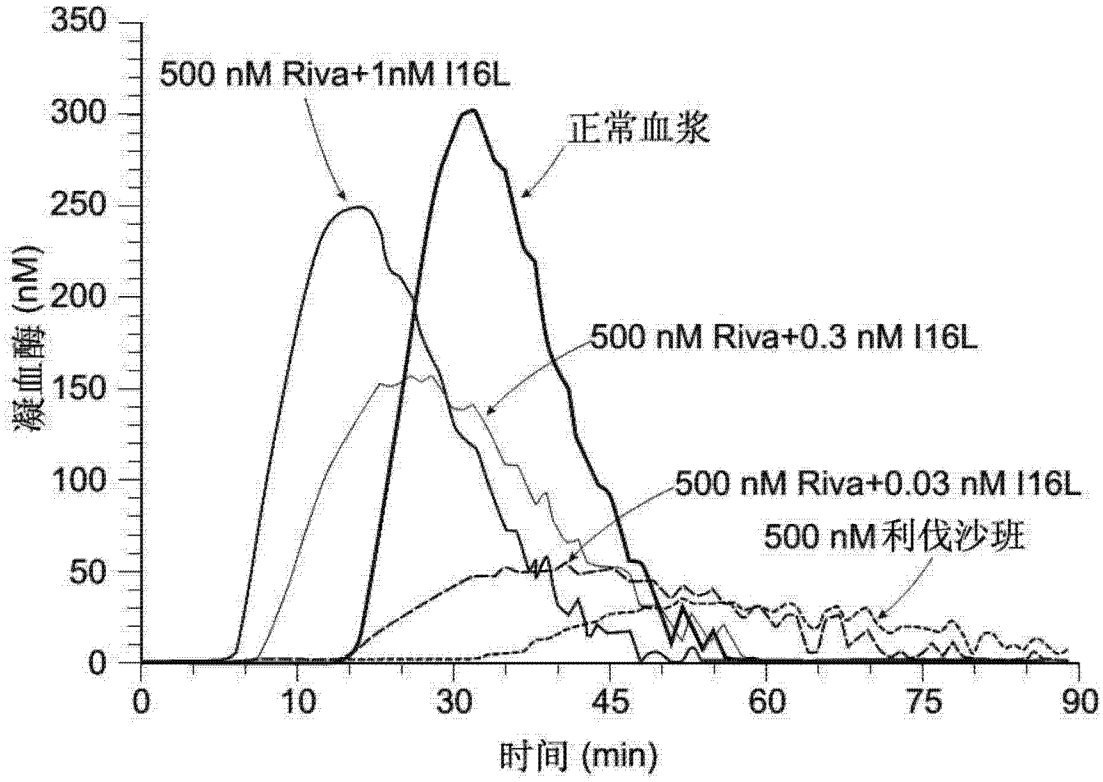
500 nM Riva+1nM I16L
正常血浆
500 nM Riva+0.3 nM I16L
500 nM Riva+0.03 nM I16L
500 nM利伐沙班
凝血酶 (nM)
时间 (min)
0
50
100
150
200
250
300
350
0
10
30
45
60
75
90

图 3

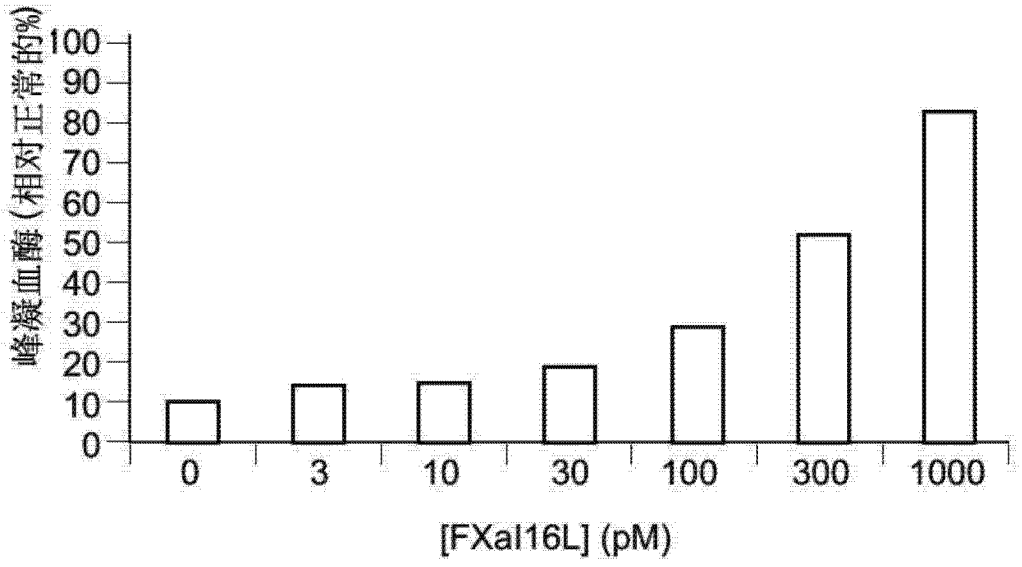
峰凝血酶 (相对正常的%)
[FXaI16L] (pM)
0
10
20
30
40
50
60
70
80
90
100
0
3
10
30
100
300
1000

图 4A

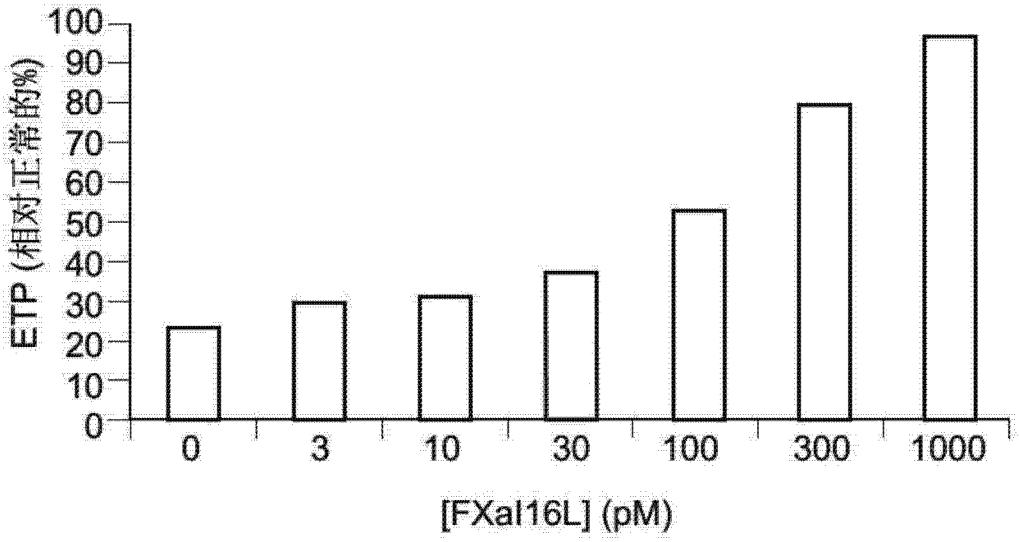
ETP (相对正常的%)
100
90
80
70
60
50
40
30
20
10
0
0
3
10
30
100
300
1000
[FXaI16L] (pM)

图 4B

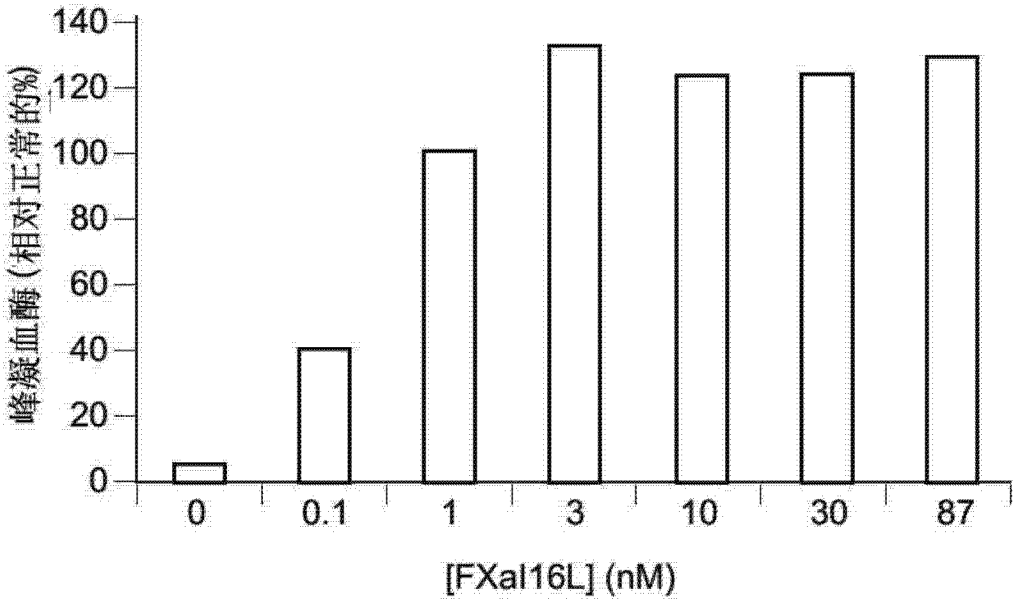
峰凝血酶 (相对正常的%)
140
120
100
80
60
40
20
0
0
0.1
1
3
10
30
87
[FXaI16L] (nM)

图 4C

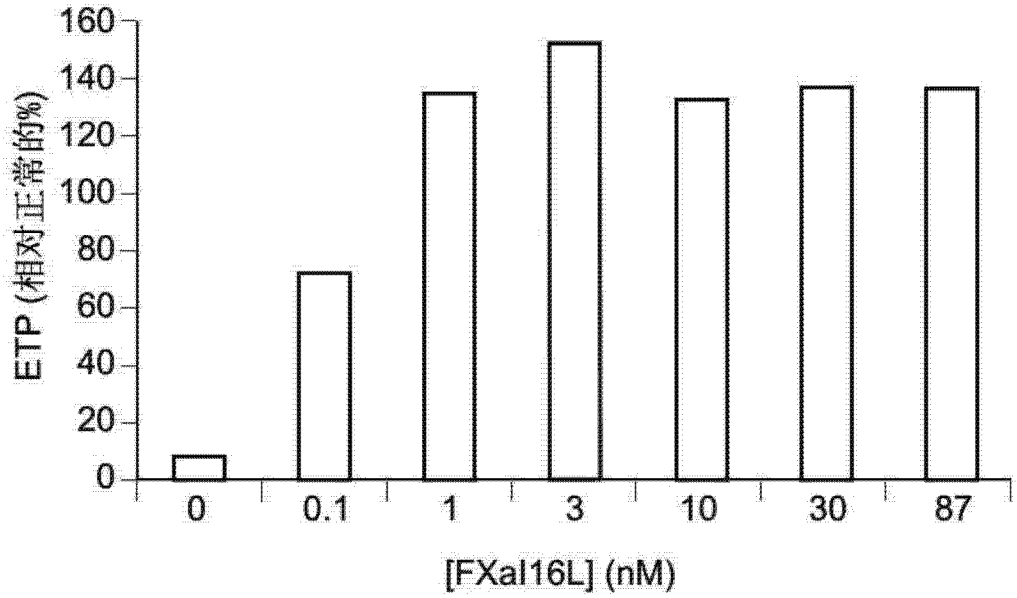
160
140
120
100
80
60
40
20
0
ETP (相对正常的%)
0
0.1
1
3
10
30
87
[FXaI16L] (nM)

图 4D

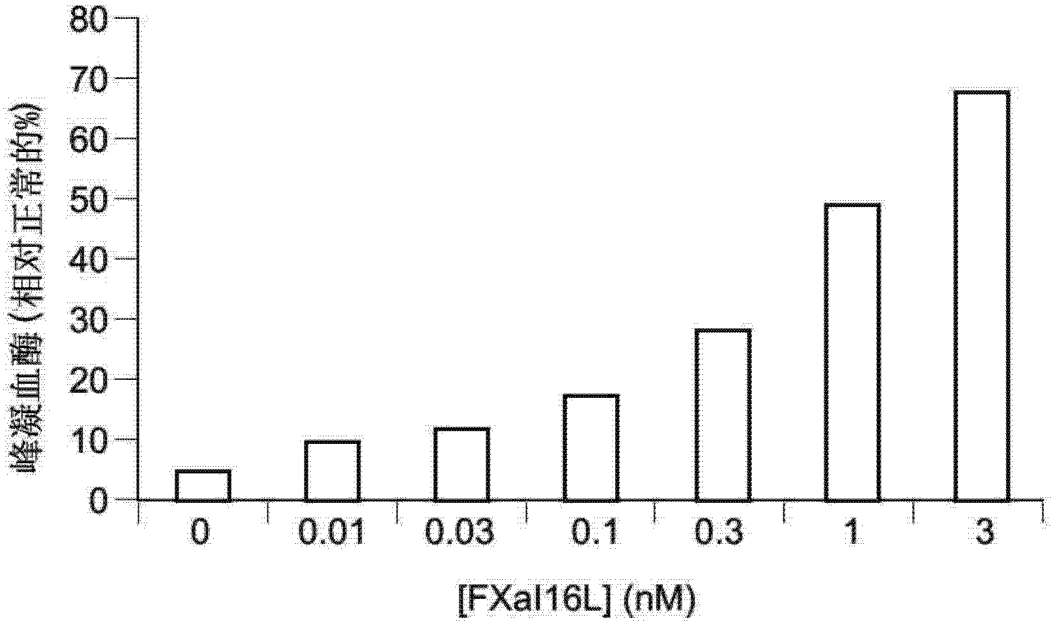
80
70
60
50
40
30
20
10
0
峰凝血酶(相对正常的%)
0
0.01
0.03
0.1
0.3
1
3
[FXaI16L] (nM)

图 5A

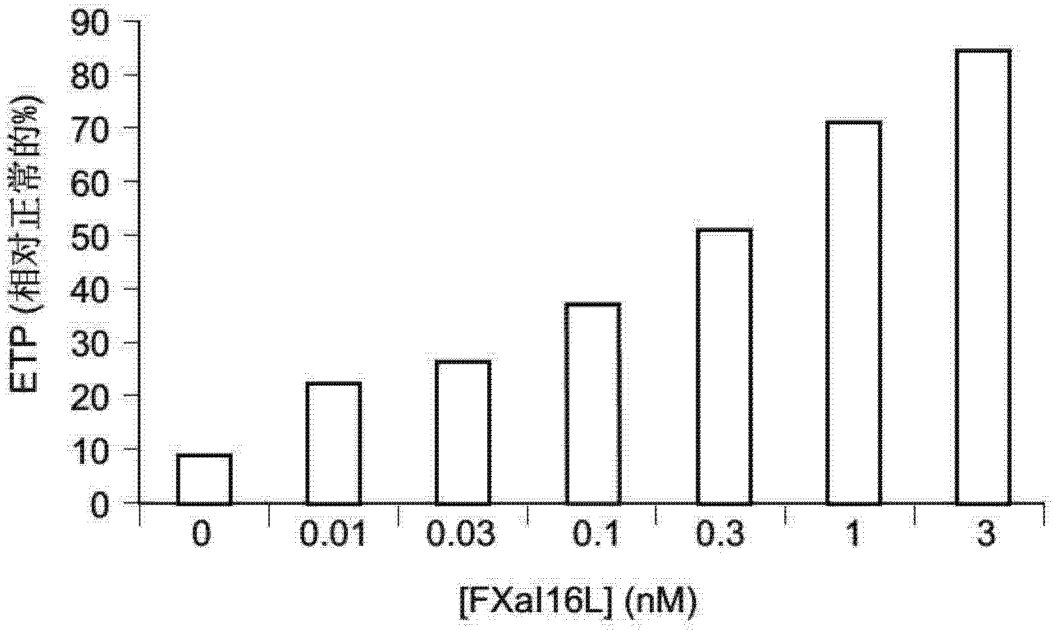
ETP (相对正常的%)
90
80
70
60
50
40
30
20
10
0
0
0.01
0.03
0.1
0.3
1
3
[FXaI16L] (nM)

图 5B

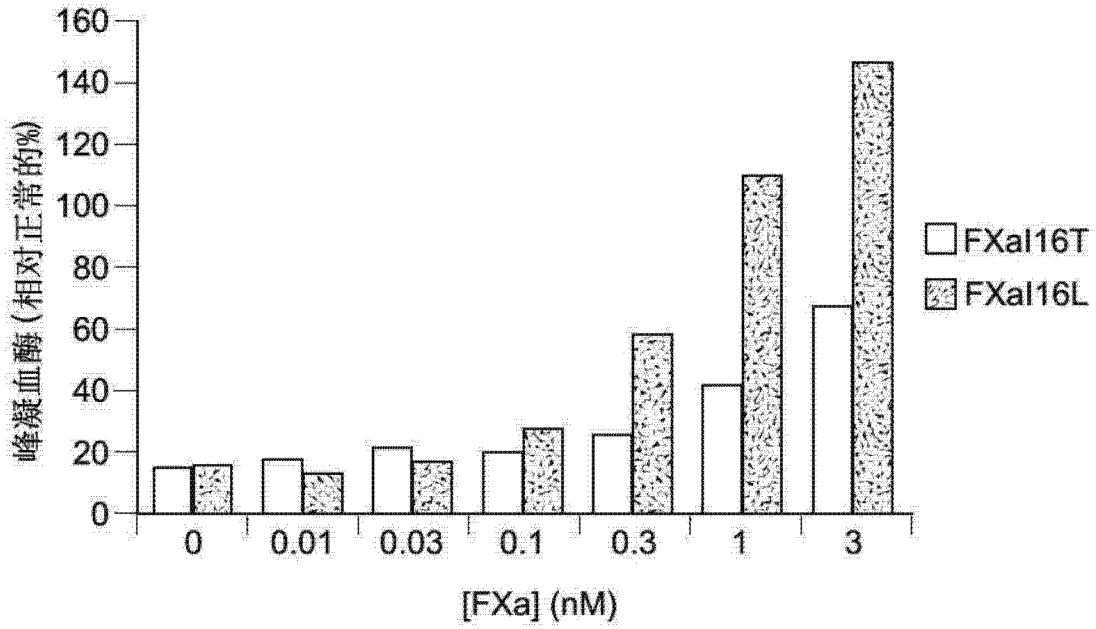
峰凝血酶 (相对正常的%)
160
140
120
100
80
60
40
20
0
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXaI16T
FXaI16L

图 6A

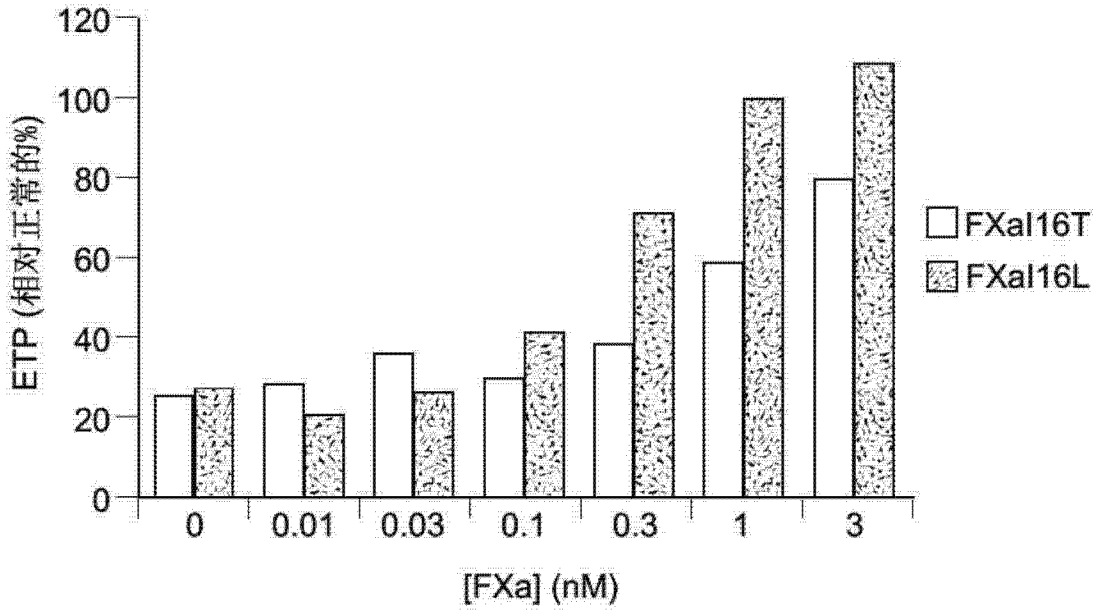
120
100
80
60
40
20
0
ETP (相对正常的%)
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXaI16T
FXaI16L

图 6B

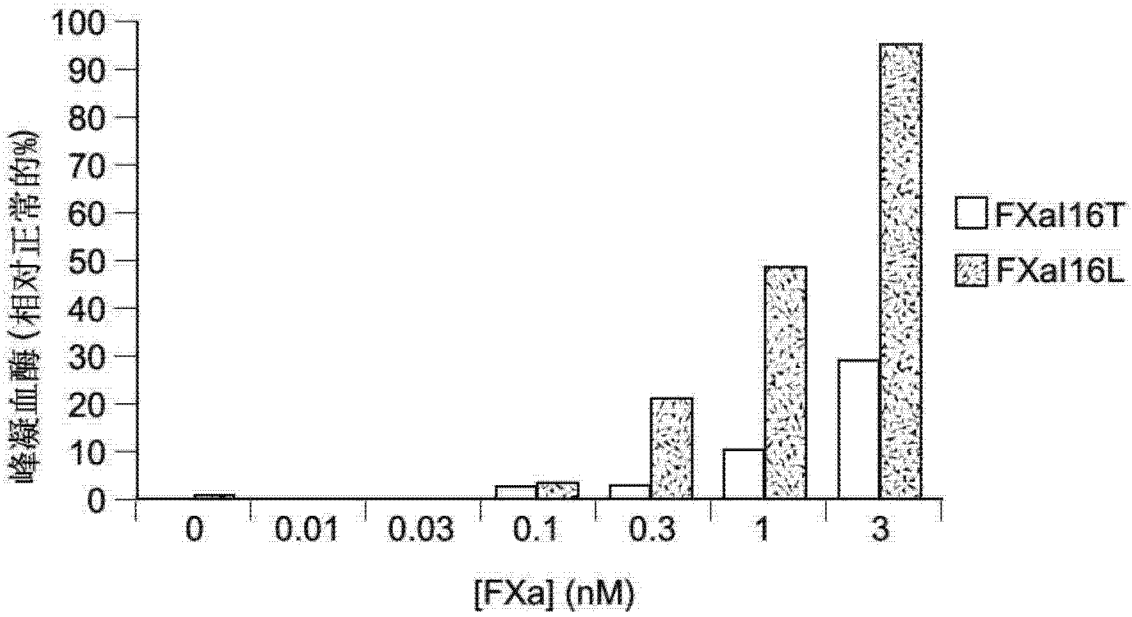
100
90
80
70
60
50
40
30
20
10
0
峰凝血酶(相对正常的%)
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXaI16T
FXaI16L

图 7A

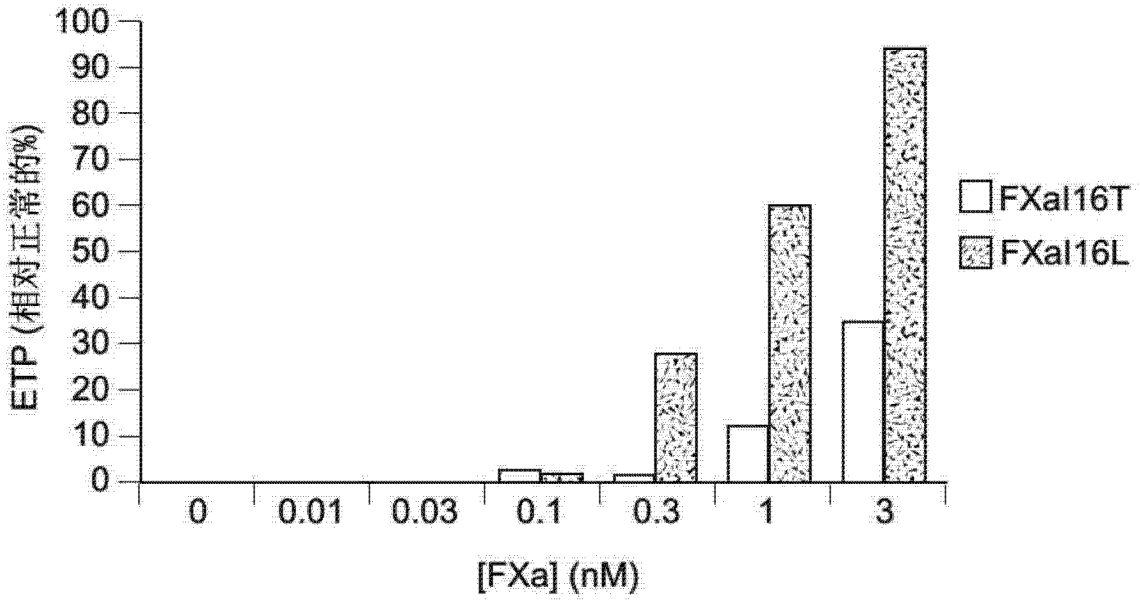
100
90
80
70
60
50
40
30
20
10
0
ETP (相对正常的%)
0
0.01
0.03
0.1
0.3
1
3
[FXa] (nM)
FXaI16T
FXaI16L

图 7B

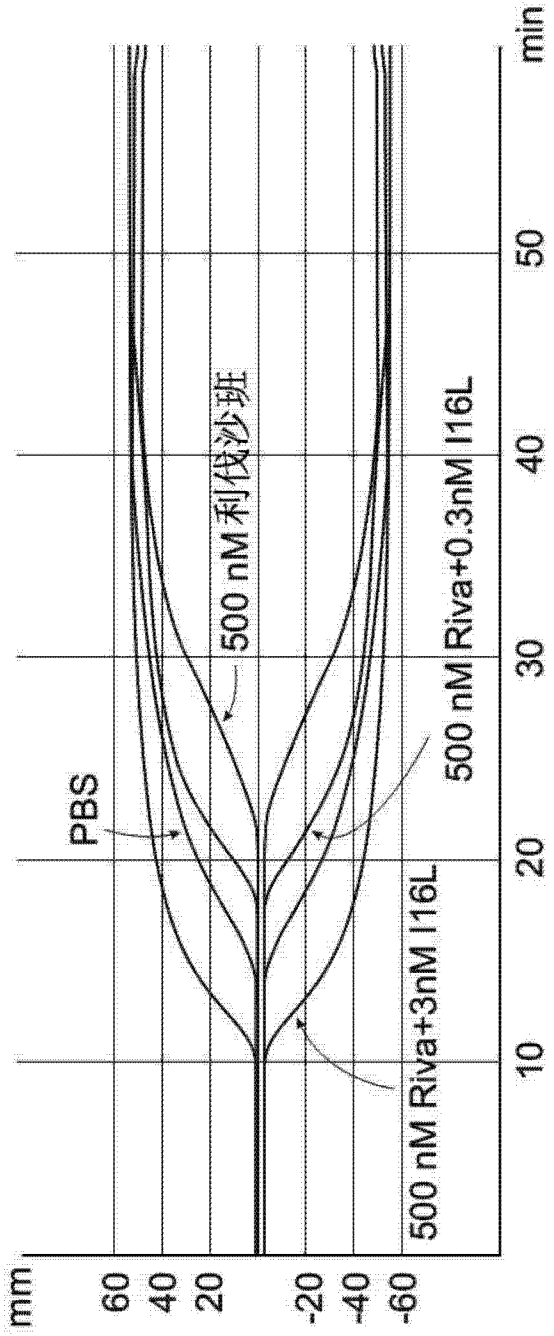
mm
60
40
20
-20
-40
-60
10
20
30
40
50
min
PBS
500 nM 利伐沙班
500 nM Riva+0.3nM I16L
500 nM Riva+3nM I16L

图 8A

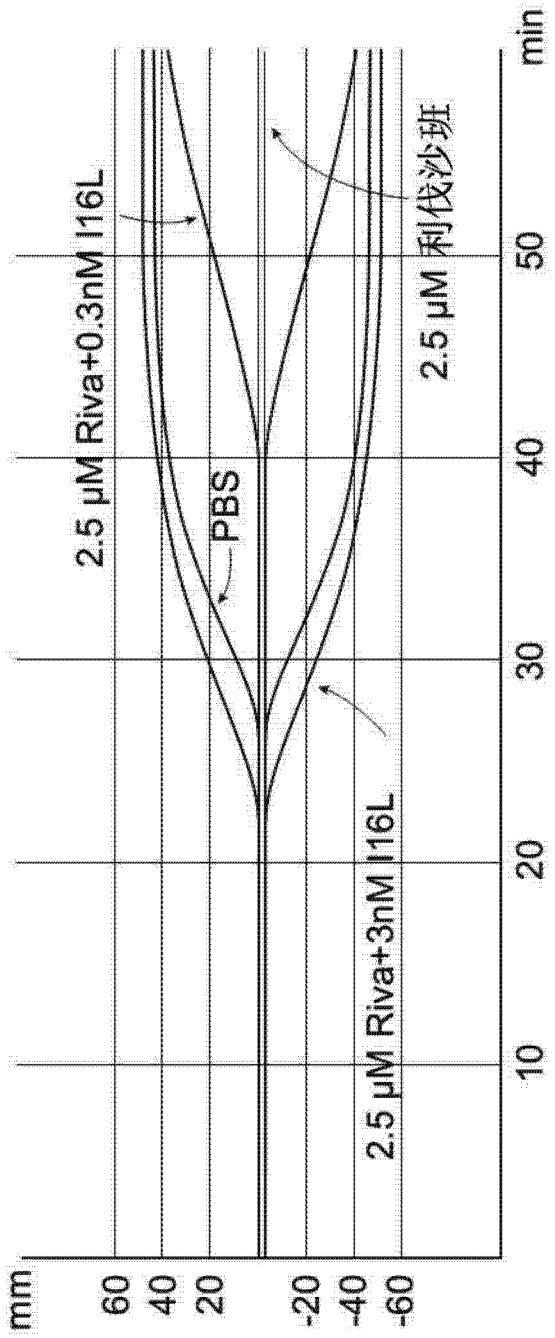
mm
60
40
20
-20
-40
-60
10
20
30
40
50
min
2.5 μM Riva+0.3nM I16L
PBS
2.5 μM 利伐沙班
2.5 μM Riva+3nM I16L

图 8B

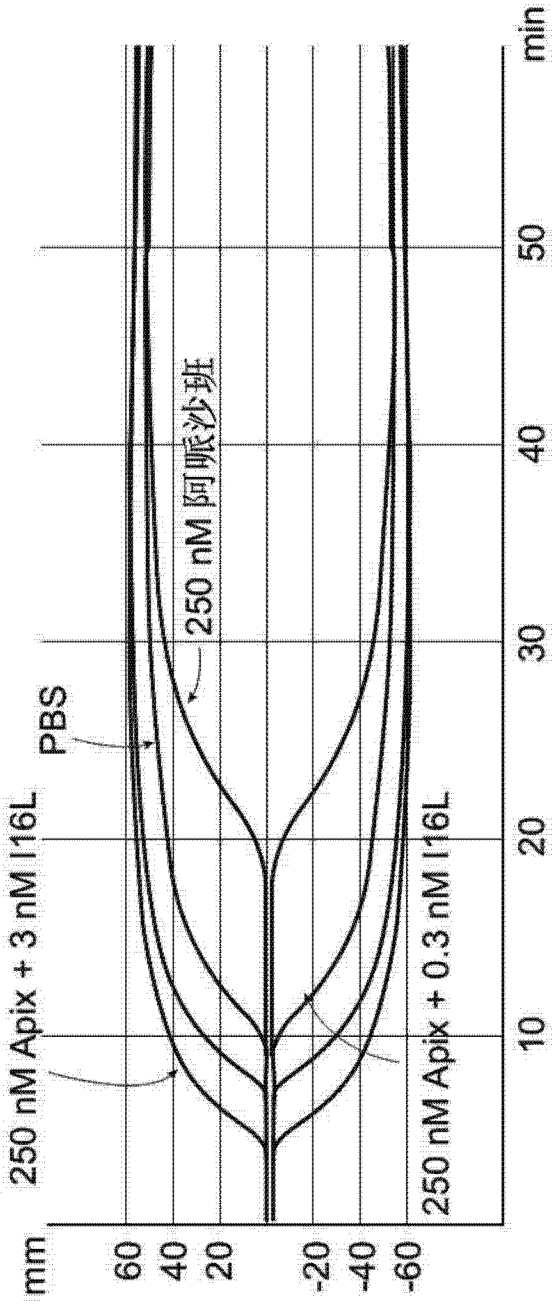
250 nM Apix + 3 nM I16L
PBS
250 nM 阿哌沙班
250 nM Apix + 0.3 nM I16L
mm
60
40
20
-20
-40
-60
10
20
30
40
50
min

图 9A

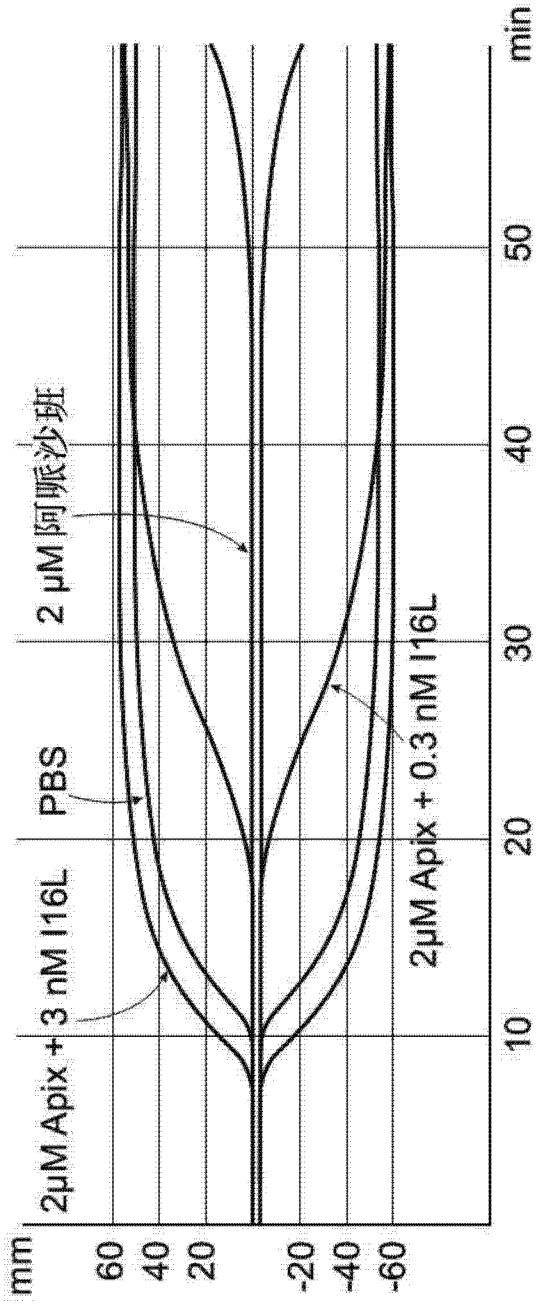
2μM Apix + 3 nM I16L
PBS
2 μM 阿哌沙班
2μM Apix + 0.3 nM I16L
mm
60
40
20
-20
-40
-60
10
20
30
40
50
min

图 9B

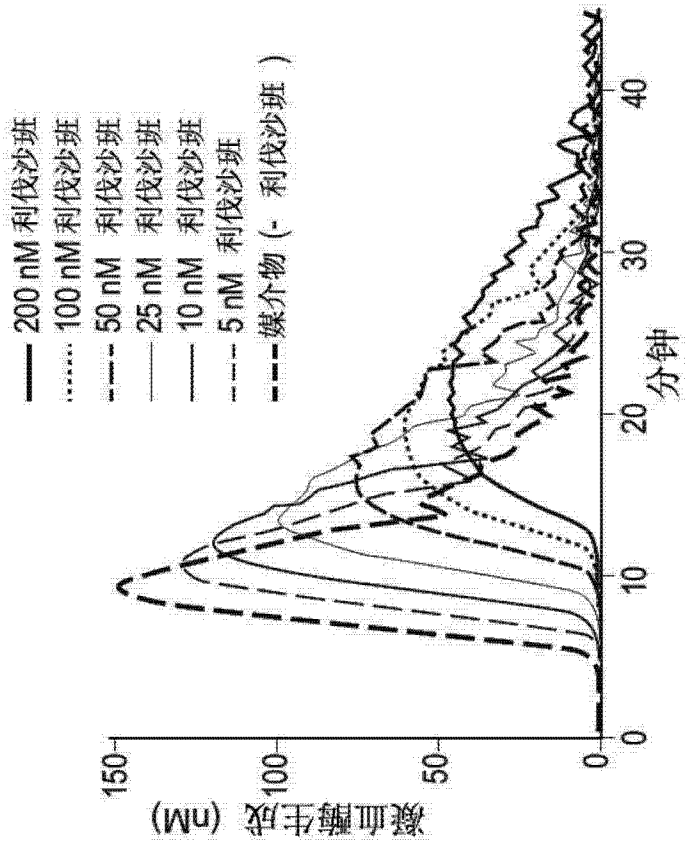
200 nM 利伐沙班
100 nM 利伐沙班
50 nM 利伐沙班
25 nM 利伐沙班
10 nM 利伐沙班
5 nM 利伐沙班
媒介物（- 利伐沙班）
凝血酶生成 (nM)
150
100
50
0
0
10
20
30
40
分钟

图 10A

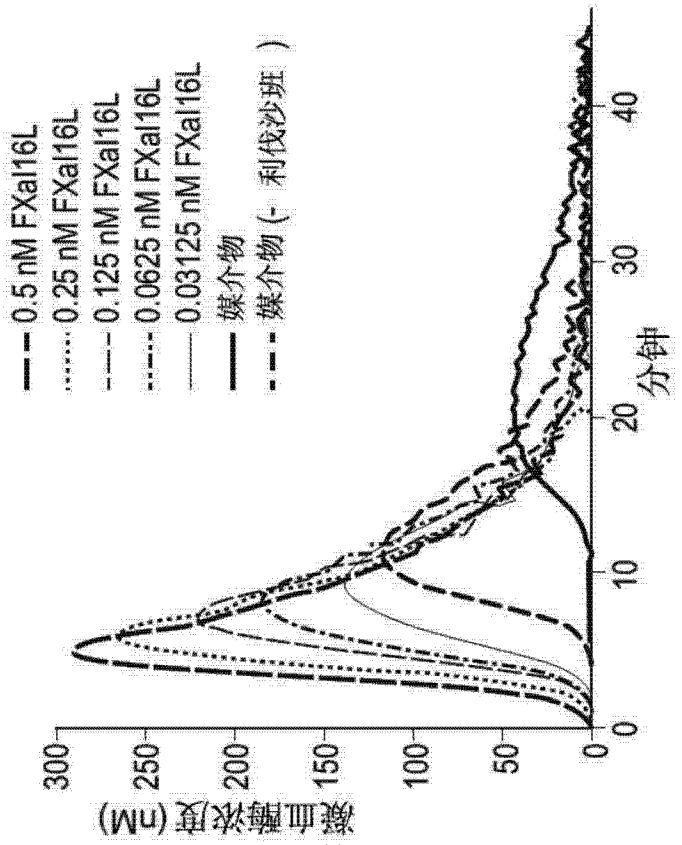
0.5 nM FXal16L
0.25 nM FXal16L
0.125 nM FXal16L
0.0625 nM FXal16L
0.03125 nM FXal16L
媒介物
媒介物 (- 利伐沙班)
凝血酶浓度 (nM)
300
250
200
150
100
50
0
0
10
20
30
40
分钟

图 10B

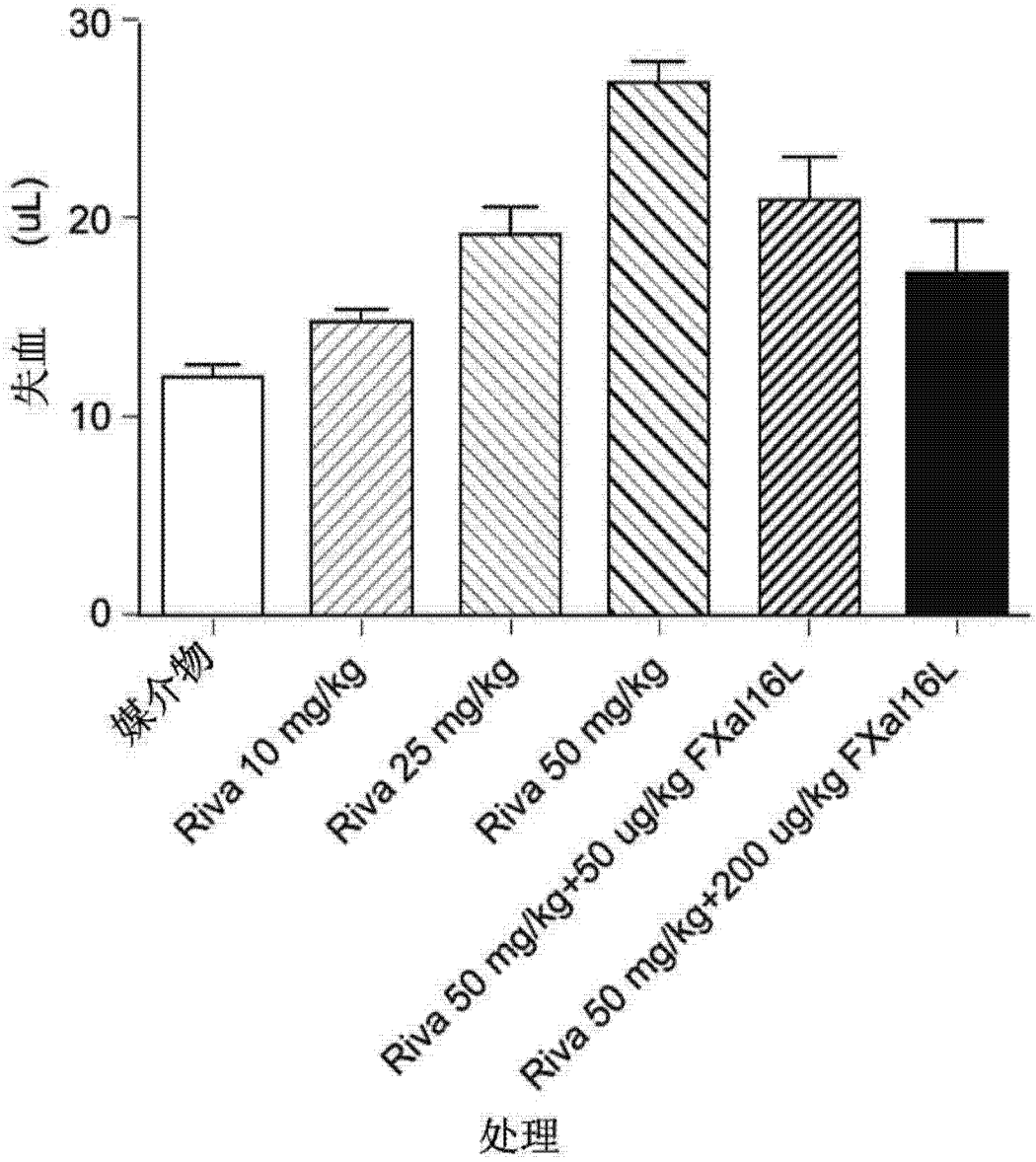
30
20
10
0
失血 (uL)
媒介物
Riva 10 mg/kg
Riva 25 mg/kg
Riva 50 mg/kg
Riva 50 mg/kg+50 ug/kg FXaI16L
Riva 50 mg/kg+200 ug/kg FXaI16L
处理

图 11

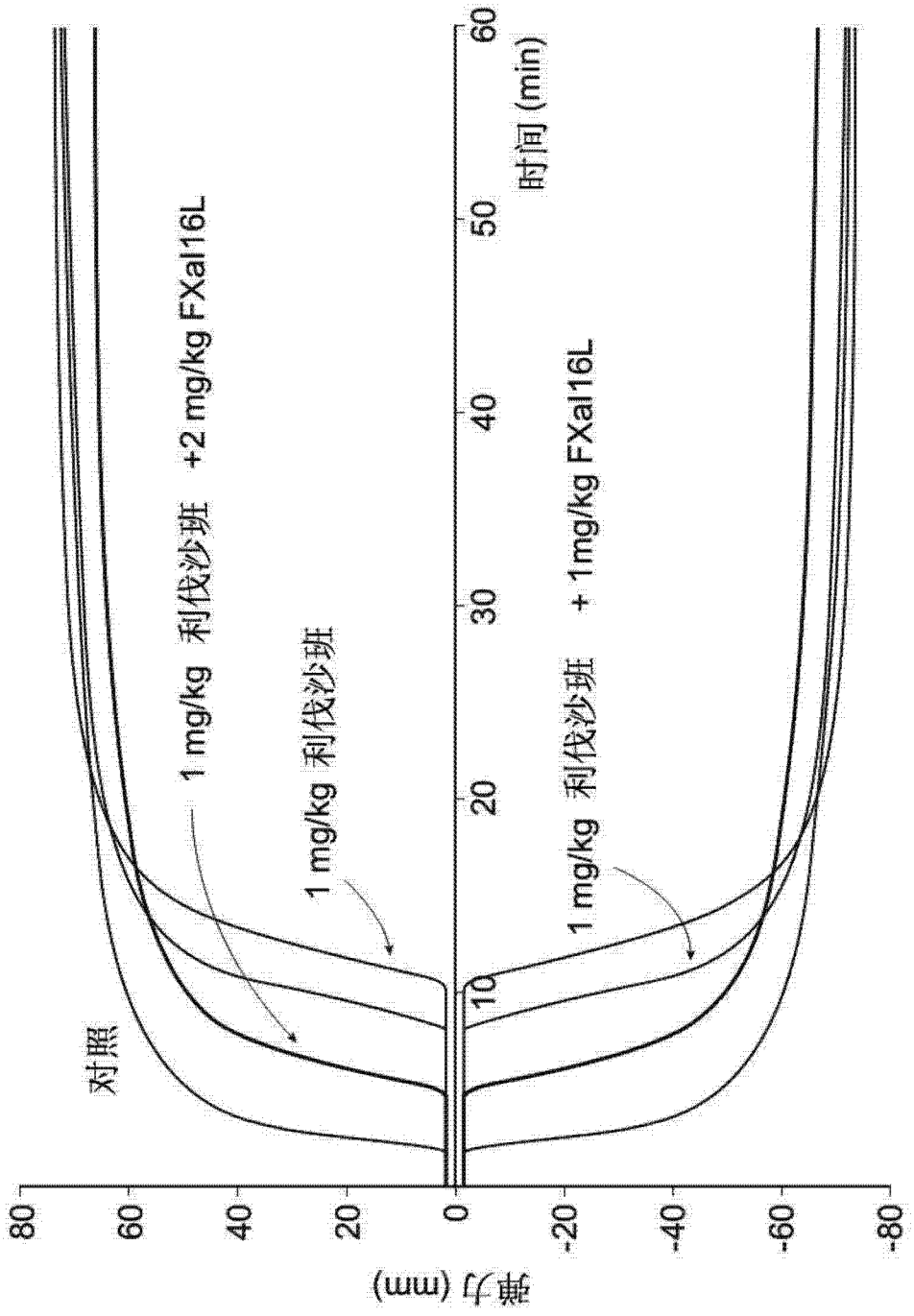
对照
1 mg/kg 利伐沙班 +2 mg/kg FXal16L
1 mg/kg 利伐沙班
1 mg/kg 利伐沙班 + 1mg/kg FXal16L
时间 (min)
弹力 (mm)
80
60
40
20
0
-20
-40
-60
-80
10
20
30
40
50
60

图 12

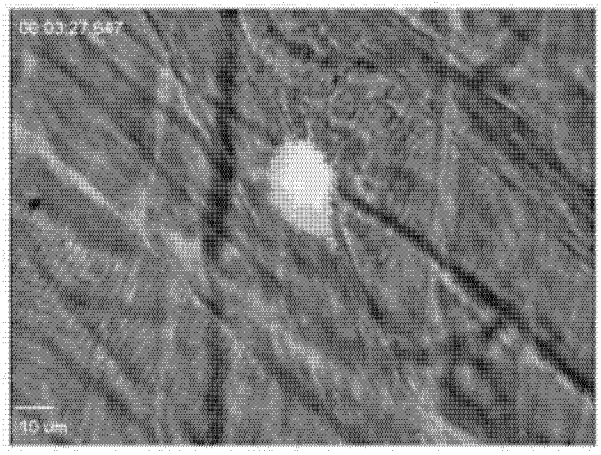

图 13A

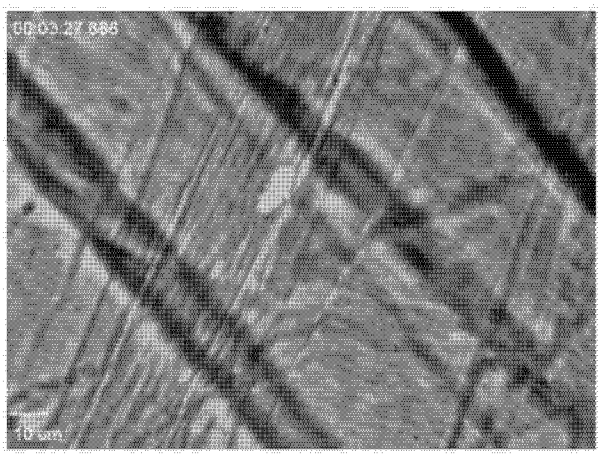

图 13B

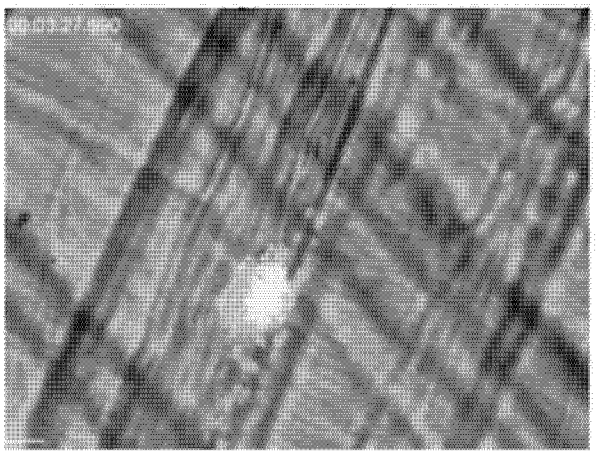

图 13C

人因子X前蛋白 (SEQ ID NO:1)

```
  1 MGRPLHLVLL SASLAGLLLL GESLFIRREQ ANNILARVTR ANSFLEEMKK GHLERECMEE
 61 TCSYEEAREV FEDSDKTNEF WNKYKDGDQC ETSPCQNQGK CKDGLGEYTC TCLEGFEGKN
121 CELFTRKLCS LDNGDCDQFC HEEQNSVVCS CARGYTLADN GKACIPTGPY PCGKQTLERR
181 KRSVAQATSS SGEAPDSITW KPYDAADLDP TENPFDLLDF NQTQPERGDN NLTRIVGGQE
241 CKDGECPWQA LLINEENEGF CGGTILSEFY ILTAAHCLYQ AKRFKVRVGD RNTEQEEGGE
301 AVHEVEVVIK HNRFTKETYD FDIAVLRLKT PITFRMNVAP ACLPERDWAE STLMTQKTGI
361 VSGFGRTHEK GRQSTRLKML EVPYVDRNSC KLSSSFIITQ NMFCAGYDTK QEDACQGDSG
421 GPHVTRFKDT YFVTGIVSWG EGCARKGKYG IYTKVTAFLK WIDRSMKTRG LPKAKSHAPE
481 VITSSPLK
```

图 14

人因子X前蛋白cDNA (SEQ ID NO:2)

```
   1 GACTTTGCTC CAGCAGCCTG TCCCAGTGAG GACAGGGACA CAGTACTCGG CCACACCATG
  61 GGGCGCCCAC TGCACCTCGT CCTGCTCAGT GCCTCCCTGG CTGGCCTCCT GCTGCTCGGG
 121 GAAAGTCTGT TCATCCGCAG GGAGCAGGCC AACAACATCC TGGCGAGGGT CACGAGGGCC
 181 AATTCCTTTC TTGAAGAGAT GAAGAAAGGA CACCTCGAAA GAGAGTGCAT GGAAGAGACC
 241 TGCTCATACG AAGAGGCCCG CGAGGTCTTT GAGGACAGCG ACAAGACGAA TGAATTCTGG
 301 AATAAATACA AAGATGGCGA CCAGTGTGAG ACCAGTCCTT GCCAGAACCA GGGCAAATGT
 361 AAAGACGGCC TCGGGGAATA CACCTGCACC TGTTTAGAAG GATTCGAAGG CAAAAACTGT
 421 GAATTATTCA CACGGAAGCT CTGCAGCCTG GACAACGGGG ACTGTGACCA GTTCTGCCAC
 481 GAGGAACAGA ACTCTGTGGT GTGCTCCTGC GCCCGCGGGT ACACCCTGGC TGACAACGGC
 541 AAGGCCTGCA TTCCCACAGG GCCCTACCCC TGTGGGAAAC AGACCCTGGA ACGCAGGAAG
 601 AGGTCAGTGG CCCAGGCCAC CAGCAGCAGC GGGGAGGCCC CTGACAGCAT CACATGGAAG
 661 CCATATGATG CAGCCGACCT GGACCCCACC GAGAACCCCT TCGACCTGCT TGACTTCAAC
 721 CAGACGCAGC CTGAGAGGGG CGACAACAAC CTCACCAGGA TCGTGGGAGG CCAGGAATGC
 781 AAGGACGGGG AGTGTCCCTG GCAGGCCCTG CTCATCAATG AGGAAAACGA GGGTTTCTGT
```

图 15

```
 841 GGTGGAACCA TTCTGAGCGA GTTCTACATC CTAACGGCAG CCCACTGTCT CTACCAAGCC
 901 AAGAGATTCA AGGTGAGGGT AGGGGACCGG AACACGGAGC AGGAGGAGGG CGGTGAGGCG
 961 GTGCACGAGG TGGAGGTGGT CATCAAGCAC AACCGGTTCA CAAAGGAGAC CTATGACTTC
1021 GACATCGCCG TGCTCCGGCT CAAGACCCCC ATCACCTTCC GCATGAACGT GGCGCCTGCC
1081 TGCCTCCCCG AGCGTGACTG GGCCGAGTCC ACGCTGATGA CGCAGAAGAC GGGGATTGTG
1141 AGCGGCTTCG GGCGCACCCA CGAGAAGGGC CGGCAGTCCA CCAGGCTCAA GATGCTGGAG
1201 GTGCCCTACG TGGACCGCAA CAGCTGCAAG CTGTCCAGCA GCTTCATCAT CACCCAGAAC
1261 ATGTTCTGTG CCGGCTACGA CACCAAGCAG GAGGATGCCT GCCAGGGGGA CAGCGGGGGC
1321 CCGCACGTCA CCCGCTTCAA GGACACCTAC TTCGTGACAG GCATCGTCAG CTGGGGAGAG
1381 GGCTGTGCCC GTAAGGGGAA GTACGGGATC TACACCAAGG TCACCGCCTT CCTCAAGTGG
1441 ATCGACAGGT CCATGAAAAC CAGGGGCTTG CCCAAGGCCA AGAGCCATGC CCCGGAGGTC
1501 ATAACGTCCT CTCCATTAAA GTGAGATCCC ACTCAAAAAA AAAAAAAAAA AAAAAAAAAA
```

图 15 续

Abstract

The disclosure provides compositions and methods for counteracting the effects of direct activated Factor X (FXa) inhibitors in a subject by administering a variant of FXa.