

Title: Prohemostatic proteins for the treatment of bleeding

FIELD OF THE INVENTION

- 5 The present invention is in the field of medical treatment. In particular, the invention is in the field of treating, preventing or ameliorating bleeding complications resulting from a modulated hemostatic response.

STATE OF THE ART

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Millions of patients worldwide require anticoagulant drugs for the prophylactic management of stroke in atrial fibrillation or prevention and treatment of venous thrombosis. Prophylaxis is traditionally centered around the coumarin-based oral anticoagulant Vitamin K Antagonists (VKA's) such as Warfarin, Acenocoumarol and Phenprocoumon, which block the synthesis of vitamine K-dependent blood coagulation factors. Further anticoagulant drugs include target specific anticoagulants such as dabigatran, that inhibit the enzyme thrombin which is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin. Efficacious reversal of the anticoagulant effect, with a so-called antidote, is a prerequisite for safe drug usage. This is particularly important considering that, alone in the Netherlands, annually over 10,000 patients treated with anticoagulants suffer from an adverse severe bleeding event, including up to 2,000 fatalities (Adriaansen H., et al: "Samenvatting Medische Jaarverslagen van de Federatie van Nederlandse Trombosediensten", 2011; 1-44).

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Currently available anticoagulant-antidote pairs to prevent over-anticoagulation are heparin-protamine and warfarin-vitamin K. Prothrombin complex concentrates (PCC) containing vitamin K-dependent coagulation factors II, IX, X (3-factor PCC) or II, VII, IX, X (4-factor PCC) and varying amounts of proteins C and S have been indicated for the reversal of warfarin-related effects (see, for example, Frumkin, Ann Emerg Med, 2013, 62: 616-626). Fresh frozen plasma and recombinant factor VIIa (rfVIIa) have also been used as non-specific antidotes in

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patients under low molecular weight heparin treatment, suffering from major trauma or severe hemorrhage (Lauritzen et al., 2005. Blood 106: Abstract 2149, 607A-608A). Also reported are protamine fragments (US Patent No. 6,624,141) and small synthetic peptides (US Patent No. 6,200,955) as heparin or low
5 molecular weight heparin antidotes; and thrombin muteins (US Patent No. 6,060,300) as antidotes for thrombin inhibitors. Prothrombin intermediates and derivatives have been reported as antidotes to hirudin and other thrombin inhibitors (US Patent Nos. 5,817,309 and 6,086,871). Despite the absence of solid clinical data, dabigatran-associated severe bleeding is preferably treated with the
10 non-specific reversal agent activated prothrombin complex concentrate (APCC) (Siegal et al., 2014. Blood 123: 1152-1158).

Newly developed direct factor Xa (FXa) inhibitors (DFXIs), such as rivaroxaban, apixaban and edoxaban, are anticoagulants and may largely replace the classic
15 VKA's in the near future because of their rapid therapeutic effectiveness, ease of dosing and lack of monitoring requirements due to fewer drug and food interactions and predictable pharmacokinetics. DFXIs are small compound inhibitors that have been specifically designed to tightly bind to and halt the activity of blood coagulation FXa. Coagulation FXa is an essential serine protease
20 which normally circulates as a ~60 kDa inactive precursor (zymogen) coagulation factor X (FX) in blood, but is converted upon vascular damage to its active protease form in a complex series of protein activation steps, collectively known as the blood coagulation cascade. Central to this system is the formation of the cofactor-protease complex known as the prothrombinase complex that consists of
25 coagulation FXa in association with the cofactor factor Va (FVa), which assemble exclusively on a negatively charged phospholipid membrane and convert inactive prothrombin into the active serine protease thrombin.

A major drawback to the use of the DFXIs is the absence of a specific and
30 adequate reversal strategy to prevent and stop potential life-threatening bleeding complications associated with its anticoagulant therapy.

Since DFXIs inhibit both free and prothrombinase bound coagulation FXa (European Medicines Agency, 2008. CHMP assessment report for Xarelto, Procedure No. EMEA/H/C/000944. Doc.Ref.: EMEA/543519/2008), effective restoration of normal hemostasis would therefore require either full replacement of circulating coagulation FXa or effective removal of inhibitory compounds from blood.

Currently, there are no specific reversal strategies to prevent and stop potential life-threatening bleeding complications associated with DFXI therapy available.

Next to life-supporting and surgical therapies, non-specific reversal therapy using 3- and 4-factor PCC may be considered based on limited evidence (Siegal et al., 2014. Blood 123: 1152-1158; Levi et al., 2014. J Thrombosis Haemostasis, Published online 8 May 2014; doi: 10.1111/jth.12599). A reversal strategy specific for DFXI-associated bleeding is in development, which is based on a catalytically inactive form of recombinant FXa (andexanet alpha) that serves as a decoy for DFXIs by binding and thereby trapping circulating DFXIs, thereby enabling endogenous coagulation FXa to normally participate in coagulation (Lu et al., 2013. Nature Medicine 19: 446). A downside to this approach is that high doses of andexanet alpha need to be administered since stoichiometric concentrations are required to attain inhibition (400mg IV bolus in phase III trial; Portola News Release March 19, 2014). Furthermore, since the half life of DFXI partially depends on renal clearance, the amount of decoy FXa required to trap all circulating inhibitory molecules may even be higher in case of renal failure. This reversal strategy does not provide a fast and direct procoagulant response, as the response is dependent on the generation of free, endogenous coagulation FXa.

At this moment, a direct, adequate reversal strategy to prevent and stop potential life-threatening bleeding complications associated with DFXI anticoagulant therapy is not available.

In one aspect, there is provided a recombinant protein comprising a coagulation Factor Xa polypeptide, said polypeptide having an alteration in a region of amino

acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320, preferably between His-311 and Asp-320 of SEQ ID NO: 1; wherein the alteration is an insertion of at least one amino acid residue, which altered protein has a decreased sensitivity to inhibition by a direct Factor Xa inhibitor.

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In another aspect, there is provided a nucleic acid molecule comprising a DNA sequence that encodes the protein as described herein.

10 In yet another aspect, there is provided an expression vector comprising the nucleic acid molecule as described herein.

In yet another aspect, there is provided a host cell comprising the nucleic acid molecule as described herein or the expression vector as described herein.

15 In yet another aspect, there is provided a pharmaceutical composition comprising the protein as described herein, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

20 Also disclosed is a method of completely or partially reversing an anti-coagulant effect of a coagulation inhibitor in a subject, said method comprising administering to said subject a therapeutically effective amount of the protein as described herein or the pharmaceutical composition as described herein.

25 In yet another aspect, there is provided use of the protein as described herein, for the manufacture of a medicament for completely or partially reversing an anti-coagulant effect of a direct Factor Xa inhibitor in a subject.

30 In yet another aspect, the present invention provides use of the altered recombinant protein described herein, wherein the alteration is an insertion of 1-50 amino acid residues, or of the pharmaceutical composition described herein, comprising an altered recombinant protein having an insertion of 1-50 amino acid residues, for the manufacture of a medicament for completely or partially reversing an anti-coagulant effect of a direct Factor Xa inhibitor in a subject.

35 **Detailed Description**

The present invention solves this problem by providing, as an adequate reversal strategy to prevent and stop potential life-threatening bleeding complications

associated with DFXI anticoagulant therapy, a recombinant protein comprising, or consisting of, a mammalian, preferably primate, more preferably human, coagulation FXa polypeptide, said polypeptide having an alteration in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1, preferably between Glu-297 and Asp-320 of SEQ ID NO:1, more preferably between Val-305 and Asp-320 of SEQ ID NO:1 and most preferably between His-311 and Asp-320 or between His-311 and Tyr-319 of SEQ ID NO: 1, wherein the alteration is an insertion and/or replacement and/or deletion of at least one amino acid residue, preferably an insertion of at least one amino acid residue. For clarification purposes, the amino acid residue numbering is based on the human coagulation FX amino acid sequence as provided in SEQ ID NO: 1.

It was found that a catalytically active human coagulation FXa, with an altered amino acid composition at a region between the Gly and Asp corresponding to Gly-289 and Asp-320 of SEQ ID NO: 1, participates in the coagulation cascade as a procoagulant, whereby said factor has a decreased sensitivity to inhibition by DFXIs, compared to a coagulation FXa not having said altered amino acid composition. The present invention provides therefore a procoagulant antidote that does not depend on the generation of free, endogenous coagulation FXa and offers a fast and direct reversal strategy to prevent and stop complications associated with DFXI anticoagulant therapy.

The amino acid sequence of human coagulation FX is provided in SEQ ID NO: 1 and can be found in GENBANK® under "AAH46125.1" at <http://www.ncbi.nlm.nih.gov/protein/AAH46125.1>. The amino acid residue numbering in this sequence is based on the human coagulation FX sequence. Coagulation FX with the sequence listed in SEQ ID NO: 1 is a precursor containing a prepro-leader sequence (amino acid residues 1 to 40 of SEQ ID NO: 1), followed by sequences corresponding to a coagulation FX light chain (amino acid residues 41 to 179 of SEQ ID NO: 1), a RKR triplet (amino acid residues 180 to 182 of SEQ ID NO: 1) which is removed during secretion of coagulation FX,

and a coagulation FX heavy chain (amino acid residues 183 to 488 of SEQ ID NO: 1) containing the activation peptide (AP) (amino acid residues 183 to 234 of SEQ ID NO: 1) and the catalytic serine protease domain (amino acid residues 235 to 488 of SEQ ID NO: 1).

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Maturation of human coagulation FX involves *inter alia* proteolytic cleavage and post-translational modification in the Golgi apparatus. The mature FX protein is a two-chain molecule, composed of a light and a heavy chain that are linked by a disulfide bond (Uprichard et al., 2002. Blood Reviews 16: 97-110). Mature human coagulation FX is activated by cleavage of a peptide bond on the heavy chain between Arg-234 and Ile-235 of SEQ ID NO: 1, thereby releasing a 52-residue activation peptide from the heavy chain of coagulation FX. The resulting disulfide-linked light chain and truncated heavy chain constitute an activated FXa polypeptide.

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The amino acid sequence of the light chain of human coagulation FXa is provided in SEQ ID NO: 2. The amino acid sequence of the heavy chain of human coagulation FXa is provided in SEQ ID NO: 3.

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The term “recombinant”, as used herein, refers to a protein that is produced using recombinant DNA techniques known to the person skilled in the art. A recombinant coagulation FX or FXa polypeptide is also indicated as rFX or rFXa. A recombinant protein preferably is not identical to a native protein, for example because the amino acid composition differs and/or because of a difference in posttranslational modification such as glycosylation.

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The term “alteration”, as used herein, refers to an insertion and/or replacement and/or deletion of at least one amino acid residue. Said alteration preferably is an insertion of at least one amino acid.

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The phrase “recombinant protein comprising a coagulation FXa polypeptide”, as used herein, is meant to encompass a protein that comprises a recombinant

coagulation FXa polypeptide, preferably of mammalian, more preferably primate, and most preferably of human origin. The phrase includes, for example, a recombinant mammalian precursor protein, such as human coagulation FX, that is processed and/or activated into a mammalian coagulation rFXa polypeptide.

5 Thus, a protein of the invention is preferably a recombinant mammalian, preferably primate, more preferably human coagulation FX, having an insertion and/or replacement and/or deletion, preferably an insertion, of at least one amino acid residue in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1, preferably
10 between Glu-297 and Asp-320 of SEQ ID NO:1, more preferably between Val-305 and Asp-320 of SEQ ID NO:1 and most preferably between His-311 and Asp-320 of SEQ ID NO: 1. In addition, said phrase includes a protein that comprises one or more additional amino acid sequences, besides the coagulation rFXa polypeptide, for example an amino acid sequence that constitutes a tag, for
15 example a FLAG tag as described in EP0150126, and/or one or more other identification peptides.

In one embodiment, therefore, a recombinant protein comprising a coagulation FXa polypeptide according to the invention is a coagulation factor X polypeptide,
20 said polypeptide having an alteration in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320, preferably between His-311 and Asp-320 of SEQ ID NO: 1; wherein the alteration is an insertion of at least one amino acid residue.

25 The term “coagulation FX”, as used herein, refers to an inactive coagulation FX precursor protein. The skilled person knows that coagulation FX is also referred to as preproprotein FX. As is used herein, a coagulation FX comprises a coagulation FXa polypeptide.

30 The term “mature coagulation FX”, as used herein, refers to an inactive coagulation FX protein that is composed of a light and a heavy chain that are linked by a disulfide bond. This FX protein is also referred to as proprotein FX, or

zymogen FX. As is used herein, a mature coagulation FX comprises a coagulation FXa polypeptide.

A protein of the invention preferably comprises, or is, a mammalian, preferably primate, more preferably human or humanized, coagulation FXa polypeptide, having an insertion and/or replacement and/or deletion, preferably an insertion, of at least one amino acid residue in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1.

The term “humanized”, as is used herein, refers to the replacement or humanization of preferably exterior amino acid residues of a protein of one species for amino acid residues that are present in a human homologue of the protein so that the proteins of the first species will not be immunogenic, or are less immunogenic, when applied to a human. The replacement of exterior residues preferably has little, or no, effect on the interior domains, or on the interdomain contacts between light and heavy chains. A protein of the invention of non-human origin, preferably mammalian origin, more preferably primate origin, is preferably humanized in order to reduce the immunogenicity of said protein in a human.

A non-human protein of the invention preferably comprises a humanized mammalian, more preferably a humanized primate, coagulation FXa polypeptide, as the risk of an antigenic response upon administration in the human body is expected to be lower as compared to a protein of the invention comprising a non-humanized coagulation FXa polypeptide.

In the context of humanizing proteins, attention can be paid to the process of humanizing that is applicable to antibodies. This process makes use of the available sequence data for human antibody variable domains compiled by Kabat et al. (1987) Sequences of Proteins of Immunological Interest, 4th ed., Bethesda, Md., National Institutes of Health, updates to this database, and other accessible

U.S. and foreign databases (both nucleic acid and protein). Non-limiting examples of the methods used to generate humanized antibodies include EP 519596; U.S. Patent No. 6,797,492; and described in Padlan et al., 1991. Mol Immunol 28: 489-498. Further exemplifying the process of humanization of non-
5 human proteins, Sarkar et al., 2012, Journal of Lipids, Article ID 610937, p. 1-13 described that Paraoxonase-1 was successfully humanized by altering the surface of the enzyme to reflect the human sequence.

The term “coagulation FXa polypeptide” refers to the catalytically active form of a
10 coagulation FX. Said coagulation FXa polypeptide is obtained by cleavage of the activation peptide from the heavy chain of a mature coagulation FX. A coagulation FXa polypeptide activates prothrombin and as a result promotes coagulation. In the context of the invention, a protein is a coagulation FXa polypeptide if it is a procoagulant serine protease and if the full-length amino
15 acid sequence of said protein comprises stretches of, or single, amino acid residues that correspond to stretches of, or single, amino acid residues that are conserved between coagulation FX factors of different species, as is indicated in figure 8. For example, a procoagulant serine protease comprising a polypeptide that contains stretches of amino acid residues that correspond to amino acid
20 residues Cys-246 to Ala-250, Phe-260 to Leu-266 and/or Asp-413 to His-423 of SEQ ID NO: 1, is assumed to be a coagulation FXa polypeptide. Said coagulation FXa polypeptide is preferably obtained by local and/or topical application of a recombinant protein according to the invention. Methods to determine whether a protein is a serine protease are known in the art and include sequence
25 comparison and use of a protease detection kit, for example of Sigma-Aldrich.

The term “mammalian coagulation FXa polypeptide”, as used herein, refers to a coagulation FXa polypeptide that is endogenously present in a mammal, preferably a primate, more preferably a human.

30 The term “coagulation inhibitor”, as used herein, refers to an anti-coagulation agent. The term “coagulation inhibitor” includes, but is not limited to (i) agents,

such as heparin, that stimulate the activity of antithrombin, (ii) coumarin-based oral anticoagulant vitamin K antagonists, such as warfarin, acenocoumarol and phenprocoumon and (iii) DFXIs.

5 The term “DFXI”, as used herein, refers to direct FXa inhibitors, for example oral direct FXa inhibitors. DFXIs are small compound inhibitors that bind to and halt the activity of coagulation FXa. The group of DFXIs includes, but is not limited to, rivaroxaban (5-chloro-N-[[[(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-5-oxazolidinyl]methyl]-2-thiophenecarboxamide), apixaban (1-(4methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-
10 c]pyridine-3-carboxamide), edoxaban (N'-(5-chloropyridin-2-yl)-N-[(1S,2R,4S)-4-(dimethylcarbamoyl)-2-[(5-methyl-6,7-dihydro-4H-[1,3]thiazolo[5,4-c]pyridine-2-carbonyl)amino]cyclohexyl]oxamide;4-methylbenzenesulfonic acid), betrixaban (N-(5-chloropyridin-2-yl)-2-[[4-(N,N-dimethylcarbamimidoyl)benzoyl]amino]-5-
15 methoxybenzamide), darexaban (N-[2-[[4-(Hexahydro-4-methyl-1H-1,4-diazepin-1-yl)benzoyl]amino]-3-hydroxyphenyl]-4-methoxybenzamide), otamixaban (methyl (2R,3R)-2-[(3-carbamimidoylphenyl)methyl]-3-[[4-(1-oxidopyridin-1-ium-4-yl)benzoyl]amino]butanoate), eribaxaban (2R,4R)-1-N-(4-chlorophenyl)-2-N-[2-fluoro-4-(2-oxopyridin-1-yl)phenyl]-4-methoxypyrrolidine-1,2-dicarboxamide),
20 letaxaban (1-[1-[(2S)-3-(6-chloronaphthalen-2-yl)sulfonyl-2-hydroxypropanoyl]piperidin-4-yl]-1,3-diazinan-2-one, LY517717 (N-[2-[4-(1-methylpiperidin-4-yl)piperazin-1-yl]-2-oxo-1-phenylethyl]-1H-indole-6-carboxamide) and 813893 (N-cyclohexyl-N-[2-[(4-methyl-1,3-thiazol-2-yl)amino]-2-oxoethyl]furan-2-carboxamide). The terms “DOAC” (direct oral anticoagulant)
25 and “DFXI” are used interchangeable herein.

The term “homologous”, as used herein, refers to amino acid sequence identity between two amino acid sequences, expressed as a percentage of the total length of the two amino acid sequences. Sequence identity is determined by comparing
30 the identity of individual amino acid residues of an amino acid sequence to the corresponding amino acid residue in another amino acid sequence.

The term “region”, as used herein, refers to a stretch of amino acid residues that is bordered by two amino acid residues. The numbering of amino acid residues as applied herein is based on the amino acid sequence of SEQ ID NO: 1.

5 The term “insertion” or “inserted”, as used herein, refers to the addition of amino acid residues in a specific region of a native coagulation FXa polypeptide, thereby increasing the number of amino acid residues in said region, compared to the number of amino acid residues in that region of the native coagulation factor FXa polypeptide.

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The term “replacement” or “replaced”, as used herein, refers to the substitution of one or more amino acid residues in a specific region, or at a specific site, of a coagulation factor Xa polypeptide, thereby altering the amino acid sequence, but not the number of amino acid residues in said region. A replacement is the
15 consequence of the deletion of an amino acid residue followed by the insertion of a different amino acid residue at the same position.

The term “deletion” or “deleted”, as used herein, refers to deleting one or more amino acid residues in a specific region, or at a specific site, of a coagulation
20 factor Xa polypeptide, thereby reducing the number of amino acid residues in said region of said polypeptide.

The term “native coagulation FXa polypeptide”, as used herein, refers to an endogenous coagulation FXa polypeptide that naturally occurs in an animal,
25 preferably in a mammal, more preferably in a primate, more preferably in a human.

The term “amino acid composition”, as used herein, refers to the amino acid sequence and length of a stretch of amino acid residues, wherein the length is
30 determined by the number of amino acid residues in that stretch.

The insertion, replacement and/or deletion, preferably insertion, of one or more amino acid residues can be performed using recombinant DNA techniques that are well known to the person skilled in the art. For example, the person skilled in the art can use synthetic DNA, PCR technology and molecular cloning to obtain recombinant DNA constructs having a DNA sequence encoding a protein of the present invention. Suitable methods and means are described in Green and Sambrook, "Molecular Cloning: A Laboratory Manual", CSHL Press, 2012.

The phrase "corresponding to the region of amino acid residues between", for example with regard to the region of amino acid residues corresponding to the region of amino acid residues between His-311 and Asp-320 of SEQ ID NO: 1", is used herein to indicate that the residue number of the conserved His and Asp residues of another coagulation FXa corresponding to the His-311 and Asp-320 of SEQ ID NO: 1, may differ from the residue number attributed to said His and Asp residue in SEQ ID NO: 1 (see figure 8). Differences in amino acid residue number can for example be the result of a different way of numbering amino acid residues. Also, a difference in amino acid residue number can be the result of a difference in length of a coagulation FXa polypeptide as compared to the length of the human coagulation FXa polypeptide that is indicated in figure 8. Similarly, the amino acid residues Gly-289, Glu-297, Val-305 and Tyr-319, of SEQ ID NO: 1, are conserved between coagulation FXa polypeptides of different species (see figures 1 and 8). It is therefore possible to identify amino acid residues that correspond to said amino acid residues in another coagulation FXa polypeptide. The person skilled in the art will therefore understand that the amino acid residue numbering as applied herein is not limiting for the invention, but is only applied for clarity purposes.

The skilled person will know how to identify a region of amino acid residues that corresponds to the region of amino acid residues between said conserved amino acid residues of SEQ ID NO: 1 that border a region as described herein. When the amino acid residues 289-322 of SEQ ID NO: 1 are aligned with the corresponding amino acid residues in coagulation FXa polypeptides of different species, it is to

be concluded that the amino acid residues at positions 289, 297, 305, 311, 313, 314, 318, 319, 320, and 322 of SEQ ID NO:1 are conserved, though not identical, in coagulation FXa polypeptides of different species, especially in mammals, wherein Asp-322 of SEQ ID NO: 1 is a highly conserved catalytic residue (Asp-102 in chymotrypsinogen numbering; Bode et al., 1989. EMBO Journal 8: 3467-3475; Messier et al., 1996. Blood Coagulation and Fibrinolysis 7: 5-14 and figures 1 and 8).

Due to the highly conserved nature of the region of amino acid residues in and around Gly-289 and Asp-320 of SEQ ID NO: 1, or in and around the corresponding Gly and Asp residues in a non-human coagulation FXa, the person skilled in the art is able to identify a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1. The same general principle applies to other amino acid residues that border a region as described herein. In other words, the conserved nature of specific amino acid residues will give the skilled person an unambiguous pointer as to which amino acid residues constitute a region.

A person skilled in the art will understand that the present invention relates to the amino acid composition of a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320, most preferably between His-311 and Asp-320 of SEQ ID NO: 1. Therefore, the person skilled in the art will understand that the amino acid sequence of the remainder of a protein of the invention can vary, under the condition that said protein remains a, or is activated into a, procoagulant FXa polypeptide with decreased sensitivity to DFXIs. Said remainder of a protein of the invention may thus vary as it for example varies between coagulation FX, or coagulation FXa, polypeptides of different species.

The number of amino acid residues in a region corresponding to the region between Gly-289 and Asp-320, preferably between His-311 and Asp-320 of SEQ ID NO: 1 is conserved between coagulation FX proteins of different species,

especially between species belonging to the group of mammals or to the group of primates. This region is also present in zymogen FX protein and FXa polypeptide. Hence, the number of amino acid residues is also conserved in zymogen FX protein and FXa polypeptide and in the corresponding region of zymogen FX protein and FXa polypeptide. Said conserved number of amino acid residues in a region of amino acid residues corresponding to the region between Gly-289 and Asp-320 of SEQ ID NO: 1 is thirty, not including Gly-289 and Asp-320. Said conserved number of amino acid residues in a region of amino acid residues corresponding to the region between His-311 and Asp-320 of SEQ ID NO: 1 is eight, not including His-311 and Asp-320.

It was found that the insertion and/or replacement and/or deletion, preferably the insertion, of at least one amino acid residue in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320, preferably between His-311 and Asp-320 of SEQ ID NO: 1 in a protein of the invention, yields a catalytically active coagulation FXa with decreased sensitivity to inhibition by DFXIs.

Tyr-319 of human FXa has been demonstrated to be a DFXI-coordinating residue (Roehrig et al., 2005. J Med Chem 48: 5900–5908; Pinto et al., 2007. J Med Chem 50: 5339–5356), while Asp-322 of SEQ ID NO: 1 is present in the catalytic serine protease site (Messier et al., 1996. Blood Coagulation and Fibrinolysis 7: 5-14). Without being bound by theory, it is possible that the close proximity of an altered amino acid residue, such as an insertion of at least one amino acid residue, in the region between Gly-289 and Asp-320 to the DFXI-coordinating residue Tyr-319 of SEQ ID NO: 1 – or the corresponding tyrosine– and/or the close proximity to the catalytic domain is responsible for the decreased sensitivity for a DFXI. It is found that the region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1 in a protein of the invention can be altered in amino acid residue number and in amino acid sequence, thereby generating a catalytically active coagulation FXa with decreased sensitivity for DFXIs.

Said alteration is selected from an insertion, a replacement and/or a deletion, and preferably is an insertion, more preferably an insertion combined with an alteration of at least one amino acid in the region between Gly-289 and Asp-320 of SEQ ID NO: 1.

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Particularly preferred is a protein of the invention wherein the insertion is 1-50, preferably 1-20, amino acid residues. The insertion in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320, preferably between His-311 and Asp-320, of SEQ ID NO: 1 in a protein of the invention comprises or consists of between 1-50, preferably between 1-20, amino acid residues. The insertion preferably comprises, or consists of, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues, resulting in a total of 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20, 21, 22, 23, 24, 25, 26, 27, or 28, respectively, amino acids between His-311 and Asp-320.

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Particularly preferred is the insertion of at least 5 amino acid residues, such as an insertion of 9, 12 or 13 amino acid residues. The person skilled in the art will understand that the amino acid residues can be inserted at any position in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1. An amino acid residue suitable for insertion is selected from the group of twenty amino acid residues as listed in Table 1. The person skilled in the art will understand that said inserted amino acid residues may undergo a post-translational chemical alteration *in vivo* or *in vitro*. As is indicated herein above, the person skilled in the art can use synthetic DNA, PCR technology and molecular cloning to obtain recombinant DNA

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constructs having a DNA sequence encoding a protein of the present invention having an insertion of between 1-50 amino acid residues in the region of amino acid residues corresponding to the region between Gly-289 and Asp-320 of SEQ ID NO: 1.

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The insertion in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1 in a protein of the invention is preferably between Thr-315 and Lys-316, between Lys-316

and Glu-317, between Glu-317 and Thr-318, and/or between Thr-318 and Tyr-319 of SEQ ID NO: 1 or between two amino acid residues corresponding to these amino acid residues in a non-human coagulation FXa polypeptide.

- 5 Particularly preferred is a protein of the invention wherein the replacement is 1-30, preferably 1-8, more preferably 6 or 7, amino acid residues. The replacement of amino acid residues in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1 in a protein of the invention preferably comprises, or consists of, between 1-30 amino
10 acid residues in a region corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1. Said replacement preferably comprises, or consists of, 1, 2, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acid residues. It is preferred that conserved amino acid residues such as, for example, Glu-297, Val-305 and/or His-
15 311 as indicated in SEQ ID No:1. are not replaced. Particularly preferred is the replacement of either 6 or 7 amino acid residues.

- An amino acid residue present in a region corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1 of a protein of the
20 invention is preferably replaced by any one of the amino acid residues listed in Table 1, preferably by an amino acid of the same group as is indicated in the columns "side chain polarity" and "side chain charge" in Table 1. Preferably, one or more of Asn-312, Arg-313, Phe-314, Thr-315, Lys-316, Glu-317, Thr-318 and Tyr-319 of SEQ ID NO: 1, or their corresponding amino acid residues in a non-
25 human protein of the invention, are replaced by any one of the amino acid residues as indicated in Table 1. Asn-312 of SEQ ID NO: 1 is preferably replaced by a Thr or Lys residue. Arg-313 is preferably replaced by an amino acid residue with a basic polarity and positively charged side-chain (see Table 1), more preferably by a Lys residue. Amino acid residue Thr-315 is preferably replaced by
30 a polar amino acid residue with a neutral side-chain or by a nonpolar amino acid residue with a neutral side-chain, more preferably by a Val residue. Lys-316 of SEQ ID NO: 1 is preferably replaced by a Pro residue. Glu-317 of SEQ ID NO: 1

is preferably replaced by a Val residue. Thr-318 is preferably replaced by a polar amino acid residue with a neutral side-chain or by a nonpolar amino acid residue with a neutral side-chain, more preferably by a Ser or Ala residue.

5 The replacement of amino acid residues in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1 in a protein of the invention preferably comprises, or consists of, at least two amino acid residues. Any combination of at least two amino acid residues is envisaged in the invention, for example, a replacement of Asn-312 of
10 SEQ ID NO: 1 and Lys-316 of SEQ ID NO: 1 by a Pro residue and an Ala residue, respectively, or replacement of Asn-312, Arg-313, Thr-315, Lys-316, Glu-317, Thr-318 and Tyr-319 of SEQ ID NO: 1 by any one of the amino acid residues listed in Table 1. Particularly preferred is a protein of the invention having a replacement of (i) Asn-312, (ii) Arg-313, (iii) Thr-315, (iv) Lys-316, (v) Glu-317
15 and (vi) Thr-318 of SEQ ID NO: 1 by a (i) Thr or Pro residue, (ii) Lys residue, (iii) Val residue, (iv) Pro residue, (v) Val residue and (vi) Ser or Ala residue, respectively.

The person skilled in the art will understand that when amino acid residues are
20 replaced in a region of amino acid residues corresponding to the region between Gly-289 and Asp-320 of SEQ ID NO: 1 of a non-human protein of the invention, only those amino acid residues are replaced that are not already present in a preferred protein of the invention. The person skilled in the art will know that the aforementioned reference to SEQ ID NO: 1 is only made in the context of
25 exemplifying the replacement of amino acid residues in a specified region of amino acid residues. He will therefore have an indication which one or more amino acid residues he may replace in a non-human coagulation FXa for what other amino acid residue or residues.

30 A protein of the invention may further comprise a deletion of at least one amino acid residue in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO:1. Particularly

preferred is a protein of the invention having a deletion of at least 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20 or 30 amino acid residues.

A preferred protein of the invention comprises a combination of an insertion and a replacement, or a combination of an insertion, a replacement, and/or a deletion. Insertions and deletions may occur independently of each other and it is thus possible that, for example, an insertion of 5 amino acid residues and a deletion of 5 amino acid are present at different amino acid positions in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO:1, without affecting the total number of amino acid residues in a coagulation FX. The skilled person will understand that an insertion or deletion changes the amino acid residue numbering in a protein. With regard to a convenient assessment of where an alteration is located and what the alteration constitutes, the skilled person can perform a multiple alignment of the amino acid sequence of different coagulation FX proteins as shown in figure 8. The skilled person can deduce from such an alignment which amino acid residues are altered. The skilled person can use conserved amino acid residues, for example Glu-297, Val-305 and/or His-311 as markers to assess the amino acid residue number where the alteration took place.

Particularly preferred is a protein of the invention wherein the insertion is 1-50, preferably 1-20, amino acid residues and wherein the replacement is 1-7, preferably 6, amino acid residues. Said preferred protein has an insertion of between 1-50, preferably between 1-20, amino acid residues, combined with a replacement of between 1-8, preferably either 6 or 7, amino acid residues in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1

A more preferred protein of the invention has an insertion of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues and a replacement of at least 1, 2, 3, 4, 5, 6 or 7 amino acid residues in a region of amino acid residues corresponding to the region of amino acid residues between

Gly-289 and Asp-320 of SEQ ID NO: 1, meaning that the insertion of at least 1-20 amino acid residues is combined with a replacement of at least 1, 2, 3, 4, 5, 6 or 7 amino acid residues. The invention is directed to all possible combinations of the aforementioned insertion and replacement. Particularly preferred is a protein
5 having an insertion of 12-13 amino acid residues and a replacement of 6 amino acid residues in a region of amino acid residues corresponding to the region of amino acid residues between Gly-289 and Asp-320 of SEQ ID NO: 1.

A protein of the invention most preferably comprises a region of amino acid
10 residues having the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11 between amino acid residues corresponding to the amino acid residues His-311 and Asp-320 of SEQ ID NO: 1.

Furthermore, alteration of Arg-366, Glu-369, Phe-396, Asp-413, Ala-414, Cys-
15 415, Gln-416, Ser-419, Val-437, Ser-438, Trp-439, Gly-440, Glu-441, Gly-442, Cys-443, Gly-450, Ile-451 and Tyr-452 of SEQ ID NO:1 is likely to result in a protein that is desensitized to DFXIs. Without being bound by theory, Arg-366, Glu-369, Phe-396, Asp-413, Ala-414, Cys-415, Gln-416, Ser-419, Val-437, Ser-438, Trp-439, Gly-440, Glu-441, Gly-442, Cys-443, Gly-450, Ile-451 and Tyr-452 of
20 SEQ ID NO: 1 are likely to be DFXI-coordinating residues. Literature indirectly supports this view, as it is shown that at least some of these residues are involved in binding of DFXIs (Roehrig et al., 2005. J Med Chem 48: 5900–5908; Pinto et al., 2007. J Med Chem 50: 5339–5356). A protein of the invention preferably has replaced or deleted an amino acid residue corresponding to Arg-
25 366, Glu-369, Phe-396, Asp-413, Ala-414, Cys-415, Gln-416, Ser-419, Val-437, Ser-438, Trp-439, Gly-440, Glu-441, Gly-442, Cys-443, Gly-450, Ile-451 or Tyr-452 of SEQ ID NO:1. Amino acid residues Arg-366, Glu-369, Phe-396, Asp-413, Ala-414, Cys-415, Gln-416, Ser-419, Val-437, Ser-438, Trp-439, Gly-440, Glu-441, Gly-442, Cys-443, Gly-450, Ile-451 and/or Tyr-452 of SEQ ID NO:1 of SEQ ID
30 NO:1, or the corresponding amino acid residues in a related protein, are preferably replaced by any one of the amino acid residues as listed in Table 1. Also, a protein of the invention preferably has an insertion of at least one amino

acid residue, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 amino acid residues in a region of amino acid residues corresponding to the region between the amino acid residues located 15 amino acid residues N-terminal and 15 amino acid residues C-terminal from Arg-366, Glu-369, Phe-396, Asp-413, Ala-414, Cys-415, Gln-416, Ser-419, Val-437, Ser-438, Trp-439, Gly-440, Glu-441, Gly-442, Cys-443, Gly-450, Ile-451 and/or Tyr-452. Said alteration of Phe-396, Arg-366, Glu-369, Asp-413, Ala-414, Cys-415, Gln-416, Ser-419, Val-437, Ser-438, Trp-439, Gly-440, Glu-441, Gly-442, Cys-443, Gly-450, Ile-451 and/or Tyr-452 of SEQ ID NO:1. Said insertion in a region as indicated in this paragraph is preferably combined with an alteration in the region between Gly-289 and Asp-320 of SEQ ID NO: 1 as defined hereinabove.

The present invention also encompasses proteins that are substantially homologous and biologically equivalent to a protein of the invention. A protein of the invention preferably has an amino acid sequence that is more than 60%, preferably more than 70%, more preferably more than 80% and most preferably more than 90% homologous to SEQ ID NO: 1 or to the activated form thereof, wherein said protein is catalytically active (procoagulant), or catalytically active after processing/activation, and has a decreased sensitivity to DFXIs, preferably a DFXI selected from the group consisting of rivaroxaban, apixaban, edoxaban and betrixaban. The person skilled in the art knows how the preproprotein or proprotein of coagulation FX is processed to its catalytically active form. The UniProt database provides an overview, under Accession Number P00742, of the processing of human coagulation FX to activated human coagulation FXa. The skilled person will thus be able to determine which amino acid residues are present or absent in coagulation FXa.

The term “decreased sensitivity to DFXIs”, as used in the context of the present invention, refers to the concentration of a DFXI that is required to produce 50% of the maximum inhibition (K_i), that is higher for a polypeptide of the present invention than for a native coagulation FXa, wherein said native coagulation FXa is preferably derived from blood plasma or is recombinantly produced. The K_i of a

DFXI is preferably determined by pre-incubating a protein of the invention with 0.001 to 100 μ M of a DFXI and subsequently performing an experiment wherein the catalytic activity towards Spectrozyme Xa (Sekisui Diagnostics; Stamford, CT, USA) by peptidyl substrate conversion is assayed. The K_i of a protein of the invention is preferably more than 2x increased, more preferably between 50x and 100x increased, and most preferably more than 100x increased as compared to the K_i of said native coagulation FXa without an alteration of at least one amino acid residue in a region of amino acid residues corresponding to the region of amino acid residues between Gly-298 and Asp-320 of SEQ ID NO: 1.

It was unexpectedly found that a protein of the invention has an increased binding affinity for coagulation FVa, the binding partner of coagulation FXa in the prothrombinase complex, as compared to the binding affinity of native coagulation FXa for coagulation FVa. The binding affinity of a human or humanized protein of the invention for FVa is at least two times higher than the binding affinity of native human FXa for FVa.

Assays for determining the binding affinity are known in the art, for example by using a binding partner (such as FVa or FXa) with a radiolabel. The amount of radiation emitted upon binding can be used to calculate the binding affinity. Also, non-radioactive methods such as surface plasmon resonance and dual polarisation interferometry can be used to quantify the binding affinity from concentration based assays but also from the kinetics of association and dissociation and, in the latter, the conformational change induced upon binding. Recently, Microscale Thermophoresis (MST), an immobilization-free method was developed, that allows the determination of the binding affinity between two proteins (Wienken et al., 2010. Nature Communications 1: 100). Preferably, the binding affinity of the coagulation FVa-FXa complex is determined via either the kinetics of prothrombin or prothrombin derivatives (prethrombin-1, prethrombin-2) conversion (Bos et al, 2009. Blood 114: 686-692), fluorescence intensity/anisotropy measurements (Bos et al, 2012. J Biol Chem 287: 26342-51); or isothermal titration calorimetry (ITC).

The invention further provides a nucleic acid molecule comprising a DNA sequence that encodes a protein of the invention. The person skilled in the art will understand how to generate a DNA sequence that encodes an amino acid sequence of a protein of the present invention and how to manufacture and isolate a nucleic acid molecule with said DNA sequence using generally known recombinant DNA techniques. The sequence of the nucleic acid molecule is preferably codon-optimized for expression in a host cell of the invention. In this way codons are used that are favored for high level expression in a specific host cell.

The present invention also provides an expression vector comprising a nucleic acid molecule of the invention.

Nucleic acid molecules are preferably inserted in an expression vector using recombinant DNA techniques known by the person skilled in the art. Expression vectors in the context of the invention direct the expression of a protein of the invention in a host cell. These expression vectors are preferably replicable in a host cell, either as episomes or as part of the chromosomal DNA. Further, the expression vector preferably comprises (i) a strong promoter/enhancer, such as the CMV or SV40 promoter, (ii) an optimal translation initiation sequence, such as a ribosomal binding site and start codon, preferably a KOZAK consensus sequence and (iii) a transcription termination sequence, including a poly(A) signal when the protein is expressed in eukaryotic cells. Suitable expression vectors include plasmids and viral vectors such as adenoviruses, adeno-associated viruses and retroviruses. The person skilled in the art will understand that the expression vector to be used is dependent on the host cell that is used for expression of a recombinant protein. An expression vector of the invention is preferably suited for expression of a nucleic acid molecule of the invention in a prokaryotic cell including a bacterial cell, or, more preferred, in a eukaryotic host cell, such as a yeast cell and a mammalian cell. Particularly preferred is mammalian expression vector pCMV4.

As an alternative, a nucleic acid molecule of the invention may be inserted in the genome of a host cell. Said insertion preferably is at a locus or within a region that ensures expression of a nucleic acid molecule of the invention in the host cell.

5

The invention further provides a host cell comprising a nucleic acid molecule of the invention. The invention preferably provides a host cell expressing a nucleic acid molecule of the invention thereby producing a protein of the invention. Said protein is either produced within the host cell or, preferably secreted from the host cell.

10

Suitable host cells for use in the present invention include prokaryotic and eukaryotic cells, such as bacterial cells, yeast cells, insect cells, animal cells, mammalian cells, murine cells, rat cells, sheep cells, simian cells and human cells. Examples of suitable eukaryotic host cells include, but are not limited to HEK 293 cells, the hamster cell line CHO and BHK-21; the murine host cells NIH3T3, NSO and C127; the simian host cells COS and Vero; and the human host cells HeLa, PER.C6, U-937 and Hep G2. Suitable cells are available from public sources such as ATCC and Life Technologies. A number of transfection techniques are known in the art, see, e.g., Graham et al., 1973. Virology 52: 456; Green et al., 2012. "Molecular Cloning: A Laboratory Manual", CSHL Press; Davis et al., "Basic Methods in Molecular Biology", 1986, Elsevier; and Chu et al., 1981. Gene 13: 197. The person skilled in the art preferably employs techniques as described in these references to introduce one or more exogenous nucleic acid molecules into suitable host cells.

15

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A particularly preferred host cell for the production of a protein of the invention is a HEK 293 cell.

30

The invention further provides a pharmaceutical composition comprising a protein of the invention, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier or excipient. A pharmaceutical composition

of the invention preferably comprises one or more of diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials known in the art. The characteristics of the carrier will depend on the route of administration, as is known to the skilled person. To reduce the potential thrombotic risk of

5 administering the serine protease FXa, a pharmaceutical composition of the invention preferably comprises a protein of the invention that is activated after administering to the subject.

The term “subject” refers to the group of mammals, preferably humans.

10

The term “pharmaceutical composition” refers, in the context of the invention, to a combination of a protein of the invention with a carrier, inert or active, making the composition suitable for therapeutic use *in vivo* or *ex vivo*.

15 The term “pharmaceutically acceptable”, as used herein, refers to a nontoxic material that is compatible with the physical and chemical characteristics of a protein of the invention and does not interfere with the effectiveness of the biological activity of said protein.

20 A pharmaceutical composition of the invention may be adapted for enteral administration of the composition, wherein the composition is absorbed through the digestive tract, e.g., oral ingestion or rectal administration. Said composition is preferably encapsulated, for example by liposomes, to prevent proteolytic degradation.

25

A pharmaceutical composition of the invention preferably is applied locally, for example at or in a wound or to a blood vessel, preferably an artery, that supplies the wounded region with blood. Said local administration is a topical

administration, for example in the form of a cream, foam, gel, lotion or ointment,
30 or a parenteral administration, for example by injection or infusion, to generate a local or systemic therapeutic effect. Topical administration of a protein of the

invention for a local effect reduces the risk of a potential systemic thrombotic incident.

A pharmaceutical composition of the invention, preferably comprising
5 coagulation FX or a mature coagulation FX that comprises an altered coagulation factor Xa polypeptide is preferably systemically administered, preferably by parenteral administration. Systemic administration of an inactive preproprotein or inactive proprotein will result in the formation of an active prothrombinase complex that consists of coagulation FXa in association with FVa on negatively
10 charged phospholipid membranes where it converts inactive prothrombin into the active serine protease thrombin.

A pharmaceutical composition of the invention is preferably adapted for parenteral administration, wherein the composition is intravenously, intra-
15 arterial, subcutaneously, and/or intramuscularly introduced. Parenteral administration involves the injection or infusion of a pharmaceutical composition of the invention into a body tissue or body fluid, whereby preferably a syringe, needle, or catheter is used. As an alternative, needle-less high-pressure administration may be used as means for parenteral administration.
20 For injectable compositions (e.g., intravenous compositions), the carrier may be an aqueous or oily solution, dispersions, emulsions and/or suspension. Preferably, the carrier is an aqueous solution, preferably distilled sterile water, saline, buffered saline, or another pharmaceutically acceptable excipient for injection.

25 A pharmaceutical composition of the invention is preferably used in a variety of therapeutical applications. For example, the pharmaceutical composition can be used as bypassing agent in the treatment or amelioration of disorders wherein normal blood coagulation is impaired, such as in hemophilia A and B, including in hemophilia A and B inhibitor patient groups, or in factor X deficiency.

30

The invention further provides a protein according to the invention or pharmaceutical composition according to the invention for use in a method of

completely or partially reversing an anti-coagulant effect of a coagulation inhibitor in a subject.

The term “anti-coagulant effect” refers to the therapeutic effect, such as the prevention of blood clotting, that is the result of the action of coagulation inhibitors.

The invention further provides the use of a protein of the invention for the manufacture of a medicament for completely or partially reversing an anti-coagulant effect of a coagulation inhibitor in a subject.

The coagulation inhibitor preferably is a direct FXa inhibitor (DFXI), more preferably a direct FXa inhibitor selected from the group formed by rivaroxaban (5-chloro-N-[[[(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-5-oxazolidinyl]methyl]-2-thiophenecarboxamide), apixaban (1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide), edoxaban (N'-(5-chloropyridin-2-yl)-N-[(1S,2R,4S)-4-(dimethylcarbamoyl)-2-[(5-methyl-6,7-dihydro-4H-[1,3]thiazolo[5,4-c]pyridine-2-carbonyl)amino]cyclohexyl]oxamide; 4-methylbenzenesulfonic acid) and/or betrixaban (N-(5-chloropyridin-2-yl)-2-[[4-(N,N-dimethylcarbamimidoyl)benzoyl]amino]-5-methoxybenzamide).

The invention further provides a method of completely or partially reverting an anti-coagulant effect of a coagulation inhibitor in a subject, said method

comprising administering to said subject a therapeutically effective amount of a protein of the invention or a pharmaceutical composition of the invention.

Preferably, a method of the invention is applied for preventing or ameliorating bleeding complications that are associated with anticoagulant therapy.

The term “therapeutically effective amount” as used herein means that the amount of the active ingredient contained in the pharmaceutical composition to be administered is of sufficient quantity to achieve the intended purpose, such as,

in this case, to completely or partially reverse an anti-coagulant effect of a coagulation inhibitor. The amount of active ingredient, i.e. a protein of the invention, in a pharmaceutical composition according to the invention preferably is in the range of about 50 mg to about 600 mg. A pharmaceutical composition
5 according to the invention is preferably administered only once, twice or three times, preferably only once, to a subject in need of complete or partial reversal of an anti-coagulant effect of a coagulation inhibitor.

For the purpose of clarity and a concise description, features are described herein
10 as part of the same or separate embodiments, however, it will be appreciated that the scope of the invention may include embodiments having combinations of all or some of the features described.

SEQ ID NO: 1 (human coagulation factor X protein)

1 mgrplhlvl1 saslagllll geslfirreq annilarvtr ansfleemkk ghlerecmee
61 tcsyeearev fedsdktnef wnkykdgdqc etspcqnqgk ckdglgeytc tclegfegkn
5 121 celftrklcs ldngdcdqfc heeqnsvvcs cargytladn gkaciptgpy pcgkqtlerr
181 krsvaqatss sgeapdsitw kpydaadldp tenpfdlldf nqtqpergdn nltrivggqe
241 ckdgecpwqa llineenegf cggtilsefy iltaahclyq akrfkvrvgd rnteqeegge
301 avhevevvik hnrftketyd fdiavlrlkt pitfrmnavp aclperdwae stlmtqktgi
361 vsgfgrthek grqstrlkml evpyvdrnsc klsssfiitq nmfcagydtk qedacqgdsg
10 421 gphvtrfkdt yfvtgivswg egcarkgkyg iytkvtaflk widrsmktrg lpkakshape
481 vitssplk

SEQ ID NO: 2 (Light chain of human coagulation factor Xa)

15 1 ansfleemkk ghlerecmee tcsyeearev fedsdktnef wnkykdgdqc etspcqnqgk
61 ckdglgeytc tclegfegkn celftrklcs ldngdcdqfc heeqnsvvcs cargytladn
121 gkaciptgpy pcgkqtlr 139

SEQ ID No 3 (Heavy chain of human coagulation factor Xa)

20 1 ivggqeckdg ecpwqallin eenegfcggt ilsefyilta ahclyqakrf kvrvgdrnte
61 qeeggeavhe vevvikhnrf tketydfdia vlrlktpitf rmnvapaclp erdwaestlm
121 tqktgivsgf grthekgrqs trlkmlevpy vdrnscklss sfiitqnmfc agydtkqeda
181 cqgdsggphv trfkdtlyfvt givswgegca rkgkygiytk vtaflkwidr smktrglpka
25 241 kshapevits splk

SEQ ID NO: 4

1 tkfvppnyyyvhqnfdrvay
30

SEQ ID NO: 5

1 kkfvppkksqefyekfdlvsy

SEQ ID NO: 6 (human coagulation FX gene; 1-1473 bp)

atggcgacgtccgaggttgcagctgacctggctgacctggccctggctgacctgtgtagccttgtgcacagccag
catgtgttctctggctcctcagcaagcacggctcgctgctccagcgggtccggcgagccaattcctttcttgaagag
5 atgaagaaaggacacctcgaaagagagtgcatggaagagacctgctcatacgaagaggcccgaggtctttgag
gacagcgacaagacgaatgaattctggaataaatacaaaagatggcgaccagtgtgagaccagtccttgccagaac
cagggcaaatgtaaagacggcctcggggaatacacctgcacctgtttagaaggattcgaaggcaaaaactgtgaa
ttattcacacggaagctctgcagcctggacaacggggactgtgaccagttctgccacgaggaacagaactctgtg
gtgtgctcctgcgcccggggtacacctggctgacaacggcaaggcctgcattcccacagggccctacctctgt
10 gggaaacagacctggaacgcaggaagaggtcagtgggccaggccaccagcagcagcggggaggccctgacagc
atcacatggaagccatatgatgcagcgcacctggacccccaggagaaccttgcacctgcttgacttcaaccag
acgcagcctgagagggggcgacaacaacctcacgcgtatcgtgggaggccaggaatgcaaggacggggagtgtccc
tggcagggcctgctcatcaatgaggaaaaagaggggtttctgtggtggaactattctgagcgagttctacatccta
acggcagcccactgtctctaccaagccaagagattcaaggtgagggtaggtgaccggaacacggagcaggaggag
15 ggcgggtgagggcgggtgcacgaggtggaggtggtcatcaagcacaaccggttcacaaggagacctatgacttcgac
atcgccgtgctccggctcaagacccccatcaccttcgcgtgaacgtggcgacctgacctgacctccccgagcgtgac
tgggcccaggtccacgctgatgacgcagaagacggggattgtgagcggcttcgggcgaccccacgagaaggggccgg
cagtcaccaggtcaagatgctggaggtgcacctacgtggaccgcaacagctgcaagctgtccagcagcttcac
atcaccagaacatgttctgtgcgggtacgcaccaagcaggaggatgacctgccagggggacagcggggggcccg
20 cacgtcacccgcttcaaggacacctacttcgtgacaggtcgtcagctggggagagggctgtgcccgtaagggg
aagtacgggatctacaccaaggtcacgccttctcaagtggatcgacaggtccatgaaaaccaggggcttgccc
aaggccaagagccatgccccggaggtcataacgtcctctccattgaaa

SEQ ID NO: 7 (DNA sequence of modified human FX - type A; 1-1509 bp)

Atggcgacgtccgaggttgcagctgacctggctgacctggccctggctgacctgtgtagccttgtgcacagccag
catgtgttctctggctcctcagcaagcacggctcgctgctccagcgggtccggcgagccaattcctttcttgaagag
atgaagaaaggacacctcgaaagagagtgcatggaagagacctgctcatacgaagaggcccgaggtctttgag
gacagcgacaagacgaatgaattctggaataaatacaaaagatggcgaccagtgtgagaccagtccttgccagaac
30 cagggcaaatgtaaagacggcctcggggaatacacctgcacctgtttagaaggattcgaaggcaaaaactgtgaa
.ttattcacacggaagctctgcagcctggacaacggggactgtgaccagttctgccacgaggaacagaactctgt
gggtgtgctcctgcgcccggggtacacctggctgacaacggcaaggcctgcattcccacagggccctacctctg
tgggaaacagacctggaacgcaggaagaggtcagtgggccaggccaccagcagcagcggggaggccctgacag
catcacatggaagccatatgatgcagcgcacctggacccccaggagaaccttgcacctgcttgacttcaacca
35 gacgcagcctgagagggggcgacaacaacctcacgcgtatcgtgggaggccaggaatgcaaggacggggagtgtcc
ctggcagggcctgctcatcaatgaggaaaaagaggggtttctgtggtggaactattctgagcgagttctacatcct
aacggcagcccactgtctctaccaagccaagagattcaaggtgagggtaggtgaccggaacacggagcaggagga
gggagggtgagggcgggtgcacgaggtggaggtggtcatcaagcacaccaagttcgtgccccctaactactattacgt
ccaccagaattttgaccgggtggcctatgaacttcagacatcgccgtgctccggctcaagacccccatcaccttcgc
40 catgaacgtggcgacctgacctgacctccccgagcgtgactgggcccaggtccacgctgatgacgcagaagacggggat

tgtgagcggcttcggggcgacccacgagaagggccggcagtcaccaggctcaagatgctggaggtgccctacgt
ggaccgcaacagctgcaagctgtccagcagcttcacatcacccagaacatgttctgtgccggctacgacaccaa
gcaggaggatgcctgccagggggacagcggggcccgccacgtcaccgccttcaaggacacctacttcgtgacagg
catcgtcagctggggagagggctgtgcccgtaaggggaagtacgggatctacaccaagggtcaccgccttcctcaa
5 gtggatcgacagggtccatgaaaaccaggggcttgcccaaggccaagagccatgccccggagggtcataacgtcctc
tccattgaaa

SEQ ID NO: 8 (DNA sequence of modified human FX - type B; 1-1512 bp)

10 atggcgacgtccgaggcttgacagctgcctggctgcctggccctggctgcctgtgtagccttgtgcacagccag
catgtgttcctggctcctcagcaagcaaggtecgctgctccagcgggtccggcgagccaattcctttcttgaagag
atgaagaaaggacacctcgaaagagagtgcatggaagagacctgctcatacgaagaggcccgcgagggtctttgag
gacagcgacaagacgaatgaattctggaataaatacaaaagatggcgaccagtggtgagaccagtccttgccagaac
cagggcaaatgtaaagacggcctcggggaatacacctgcacctgtttagaaggattcgaaggcaaaaaactgtgaa
15 ttattcacacggaagctctgcagcctggacaacggggactgtgaccagttctgccacgaggaacagaactctgtg
gtgtgctcctgcgcccgcggttacacctggctgacaacggcaaggcctgcattcccacagggccctaccctgt
gggaaacagaccctggaacgcaggaagaggtcagtggcccaggccaccagcagcagcggggaggccctgacagc
atcacatggaagccatatgatgcagccgacctggacccaccgagaaccccttcgacctgcttgacttcaaccag
acgcagcctgagagggggcgacaacaacctcacgcgtatcgtagggaggccaggaatgcaaggacggggagtgccc
20 tggcaggccctgctcatcaatgaggaaaacgagggtttctgtggtggaactattctgagcgagttctacatccta
acggcagcccactgtctctaccaagccaagagattcaagggtgagggtaggtgaccggaacacggagcaggaggag
ggcgggtgaggcgggtgcacgaggtggaggtggtcatcaagcacaagaaattcgtgccccctaagaaaagccaggag
ttctacgaaaagtttgacctggtctcctatgacttcgacatcgccgtgctccgggtcaagacccccatcaccttc
cgcatgaacgtggcgccctgcctgcctccccgagcgtgactgggcccaggtccacgctgatgacgcagaagacgggg
25 attgtgagcggcttcggggcgacccacgagaagggccggcagtcaccaggctcaagatgctggaggtgccctac
gtggaccgcaacagctgcaagctgtccagcagcttcacatcacccagaacatgttctgtgccgggtacgacacc
aagcaggaggatgcctgccagggggacagcggggcccgccacgtcaccgccttcaaggacacctacttcgtgaca
ggcatcgtcagctggggagagggctgtgcccgtaaggggaagtacgggatctacaccaagggtcaccgccttcctc
aagtggatcgacagggtccatgaaaaccaggggcttgcccaaggccaagagccatgccccggagggtcataacgtcc
30 tctccattgaaa

SEQ ID NO: 9

1 kkfvppkksqefyekfdlaay

SEQ ID NO: 10

1 kkfvppnyyyvhqnfdaay

35 SEQ ID NO: 11

1 kkfvppqkaykfdlaay

FIGURE LEGENDS

Figure 1. Blood coagulation FXa structure. A: Schematic γ -carboxyglutamic ('GLA'), EGF-1 and -2 ('EGF'), and serine protease domain ('SP') structure of coagulation FXa. B: Crystal structure of the human FXa serine protease (pdb 2W26). Indicated are the catalytic triad His-276, Asp-322, Ser-419, rivaroxaban/apixaban contact residues Try-319 and Phe-396, position of residues 316-317 (in spheres), and residues Gly-289, Glu-297, Val-305, and His-311. C: Alignment of region 311-322 in various plasma FX species with conserved residues (highlighted), contact residue Tyr-319, and catalytic residue Asp-322 indicated. *Indicates venom coagulation FX with insertion in the region corresponding to the region between Gly-289 and Asp-320 of SEQ ID NO: 1.

Figure 2. Inhibition of chromogenic FXa activity by direct FXa inhibitors. A: Peptidyl substrate conversion (SpecFXa, 250 μ M) by recombinant human coagulation FXa (hFXa, 2 nM, circles) or venom *P. textilis* coagulation FXa (vptFXa, 10 nM, triangles) in the presence of increasing concentrations (1nM – 10 μ M) rivaroxaban ('riva', closed symbols) or apixaban ('api', open symbols). Substrate conversion is plotted as the % of incubations in the absence of inhibitor. B,C: Thrombin generation in coagulation FX-deficient plasma was initiated with 0.5 nM hFXa (panel B) or vptFXa (panel C) in the absence (grey line / grey column) or presence of 0.4 μ M rivaroxaban ('riva', black line / black column) or 2 μ M apixaban ('api', dotted line / white column). Thrombin formation was assessed using a fluorogenic substrate and peak thrombin concentrations of the various incubations are shown in the insets.

Figure 3. (A) Fluorescent western blot of recombinant FX (200 ng) obtained from HEK293 cell lines which stably express either recombinant human FX (r-hFX, lane 1, 5), modified human FX-A (mod A, lane 2, 6) or modified human FX-B (mod B, lane 3, 7), before (lanes 1,2, 3) or after (lanes 5, 6, 7) incubation with RVV-X activator. The heavy chain of endogenous plasma-derived human FXa migrates at ~29 kDa (lane 9). Relative weight (kDa) of the protein markers (lanes

4, 8) are indicated. **(B)** Recombinant FX in conditioned media from HEK293 cell lines stably expressing either recombinant human FX (black column), modified human FX-A (white column) or modified human FX-B (grey column) was quantified using a FX-specific ELISA. Each individual bar represents a single stable cell line with the highest attainable expression per FX variant.

Figure 4. Macromolecular substrate activation. Prothrombin conversion (1.4 μ M) in the presence of 50 μ M PCPS, 20 nM FV (FV810, recombinant B-domain truncated FV) and 0.1 nM of modified human coagulation FXa type A (m-hFXa A), type B (m-hFXa B), recombinant (r-hFXa), or plasma derived (pd-hFXa) FXa. The substrate conversion is plotted in nM/min/nM Enzyme and data are the mean value of two independent experiments $\pm$ S.D.

Figure 5. Inhibition of FXa chimer type-A by DFXIs. Peptidyl substrate conversion (SpecFXa, 250 μ M) of RVV-X activated modified human coagulation FXa type A (m-hFXa A, 1 nM) in comparison to recombinant human coagulation FXa (r-hFXa, 3 nM), plasma derived human coagulation FXa (pd-FXa, 2 nM), and venom *P. textilis* (vptFXa, 1 nM) FXa. Conversion rates were determined in the presence of 0.001 – 100 μ M of Rivaroxaban (panel A) or Apixaban (panel B). The data represent the mean value of two independent experiments, except for r-hFXa (n=1).

Figure 6. Inhibition of modified human FX –A and modified human FX –B by DFXIs. Peptidyl substrate conversion (SpecFXa, 250 μ M) by RVV-X activated modified human FX –A (m-hFXa A, 1 nM) and modified human FX –B (m-hFXa B, 7 nM), in comparison to RVV-X activated recombinant human coagulation FXa (r-hFXa, 6 nM). Conversion rates were determined in the presence of 0.001 – 100 μ M of rivaroxaban (panel A) and apixaban (panel B). The data are the means of two independent experiments.

Figure 7. Inhibition of modified human FX –A or modified human FX –B by DFXIs in the presence of cofactor Va and phospholipids. Peptidyl substrate

conversion (SpecFXa, 250 μ M) by RVV-X activated modified human FX –A (m-hFXa A, 2 nM) and activated modified human FX –B (m-hFXa B, 4 nM) in comparison to by RVV-X activated recombinant human coagulation FXa (r-hFXa, 3 nM), in the presence of 50 μ M PCPS and 30 nM FV (FV810, recombinant B-domain truncated). Conversion rates were determined in the presence of 0.001 – 100 μ M of Rivaroxaban (panel A) or Apixaban (panel B). The data are the means of two independent experiments.

Figure 8. Multiple alignment of coagulation FX proteins of different species. The amino acid sequence of human coagulation FX (Genbank Accession No.: AAH46125.1) (HUM) is compared to the amino acid sequences of *M. musculus* coagulation FX (Genbank Accession No.: AAC36345.1) (MUS), *X. tropicalis* coagulation FX (Genbank Accession No.: NP_001015728) (Xtr), *D. rerio* coagulation FX (Genbank Accession No.: AAM88343.1) (Dre), *T. rubripes* coagulation FX (Genbank Accession No.: NP_001027783.1) (Tru), *P. textilis* coagulation FX isoform 1 (UniprotKB accession No.: Q1L659) (Pte1), *P. textilis* coagulation FX isoform 2 (UniprotKB accession No.: Q1L658) (Pte2), *P. textilis* coagulation FX (pseutarin C catalytic subunit precursor; Genbank Accession No.: AAP86642.1) (Pte3) and *N. scutatus* coagulation FX (UniProtKB accession No.: P82807.2) (Nsc). In this figure, Gly-289, Asp-320, Tyr-319, Glu-297, Val-305 and His-311 of SEQ ID NO: 1 are indicated in bold and are underlined. This figure shows that there is variation in the region of amino acid residues corresponding to the region between Gly-289 and Asp-320 of SEQ ID NO: 1 between coagulation FX proteins of different species. Amino acid residues that are conserved in all species are indicated in the consensus sequence.

Figure 9. Amino acid composition of endogenous hFX and chimeric FX variants. Serine protease domain residues Histidine91 and Tyrosine99 (chymotrypsin numbering; corresponding to His 311 and Tyrosine 319, respectively, of FX as depicted in SEQ ID NO:1) of endogenous human (hFX) in alignment with chimeric FX type A (c-FX A, middle; sequence between His 311 and Asp 320 corresponds to SEQ ID NO:9), type B (c-FX B; ; sequence between His 311 and

Asp 320 corresponds to SEQ ID NO:10), and type C (c-FX C; sequence between His 311 and Asp 320 corresponds to SEQ ID NO:11).

Figure 10. Characterization of FXa: A: Coomassie staining of 5 µg FXa variants on 4-12% Bis-Tris gels. From left to right: plasma-derived Factor Xa (pd-FXa), r-hFXa, chimeric factor Xa type A, B and C (– A, – B, – C). B: Prothrombin conversion (1.4µM) in the presence of 50µM PCPS (75% phosphatidylcholine, 25% phosphatidylserine) and 20nM FV (FV810, recombinant B-domain truncated FV) and 0.1nM of pd-FXa, r-hFXa, c-FXa – A, c-FXa – B and c-FXa – C. Data points are the mean value of two independent experiments.

Figure 11. Inhibition of FXa variants by DOACs. Normalized prothrombin conversion by 1 nM of pd-FXa (triangles), r-hFXa (circles), chimeric FXa – A (squares), – B (diamonds) and – C (crosses) was assessed in the presence of 0.001 – 100 µM of Apixaban (left, closed symbols) or Edoxaban (right, open symbols). Inhibitory constants (determined with Graphpad Prism 6 software suite) of Apixaban for pd-FXa: 2nM, r-hFXa: 4nM, c-FXa – A: 130nM, – B: 760nM – C: 1270nM and of Edoxaban for r-hFXa: 0.5nM, c-FXa – A: 3nM, – B: 140nM – C: 270nM.

Figure 12. FXa-initiated thrombin generation (TG) profiles for FXa variants. Plasma TG in the absence (A) and presence (B) of DOAC Apixaban (2µM). Initiation of TG by pd-FXa, r-hFXa, c-FXa – A, c-FXa – B and c-FXa – C in FX-depleted plasma. Curves are the average of at least 3 independent experiments

Figure 13. Tissue factor (TF)-initiated TG profile for r-hFX and c-FX – C. A: Plasma TG at low TF (2pM) in the absence and presence of 2µM DOAC Apixaban (Apixa) by 1 unit r-hFX, r-hFX plus Apixaban, c-FXa – C or c-FXa – C plus Apixaban. One unit of r-hFX (7 µg/ml) or c-FXa – C (16 µg/ml) was defined by a prothrombin time-based clotting assay using normal human plasma as reference. Curves represent the average of at least 3 independent experiments. B: Plasma TG at high TF (20pM).

Figure 14. TF-initiated TG profile for r-hFX and c-FX – C. (Upper graph):

Plasma TG at low TF (2pM) in the absence (dotted line) and presence of 200 nM (light grey), 600 nM (dark grey) and 2000 nM (black) DOAC Edoxaban by 1 unit r-hFX (7 µg/ml). (Lower graph): Plasma TG at low TF (2pM) with similar

5 concentrations of Edoxaban by 1 unit of c-FXa – C (16 µg/ml). Curves represent the average of 2 independent experiments.

Table 1

Amino Acid	3-Letter	1-Letter	Side-chain polarity ^{2,3,4}	Side-chain charge (pH 7.4) ^{1,5}	Hydrophobic Index ^{2,3,6}	Absorbance $\lambda_{max}(nm)$ ^{1,6}	ϵ at λ_{max} ($\times 10^{-3} M^{-1} cm^{-1}$) ^{1,6}
Alanine	Ala	A	nonpolar	neutral	1.8		
Arginine	Arg	R	Basic polar	positive	-4.5		
Asparagine	Asn	N	polar	neutral	-3.5		
Aspartic acid	Asp	D	acidic polar	negative	-3.5		
Cysteine	Cys	C	nonpolar	neutral	2.5	250	0.3
Glutamic acid	Glu	E	acidic polar	negative	-3.5		
Glutamine	Gln	Q	polar	neutral	-3.5		
Glycine	Gly	G	nonpolar	neutral	-0.4		
Histidine	His	H	Basic polar	positive(10% neutralizing)	-3.2	211	0.9
Isoleucine	Ile	I	nonpolar	neutral	4.5		
Leucine	Leu	L	nonpolar	neutral	3.8		
Lysine	Lys	K	Basic polar	positive	-3.8		
Methionine	Met	M	nonpolar	neutral	1.9		
Phenylalanine	Phe	F	nonpolar	neutral	2.8	257, 259, 193	0.2, 0.5, 00.8
Proline	Pro	P	nonpolar	neutral	-1.6		
Serine	Ser	S	polar	neutral	-0.8		
Threonine	Thr	T	polar	neutral	-0.7		
Tryptophan	Trp	W	nonpolar	neutral	-0.8	280, 219	5.8, 47.8
Tyrosine	Tyr	Y	polar	neutral	-1.3	274, 222, 193	1.4, 0.8, 48.8
Valine	Val	V	nonpolar	neutral	4.2		

EXAMPLES

Example 1

5 Materials and methods

Rivaroxaban and Apixaban were obtained from Alsachim (Illkirch, France) and dissolved in DMSO (~30mg/ml). The peptidyl substrate methoxycarbonylcyclohexylglycylglycylarginine-p-nitroanilide (Spec-Xa) was
10 obtained from Sekisui Diagnostics (Stamford, CT, USA). All tissue culture reagents were from Life Technologies (Carlsbad, CA), except insulin-transferrin-sodium selenite (ITS), which was from Roche (Basel, Switzerland). Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg L-phosphatidylcholine and 25% (w/w) porcine brain L-phosphatidylserine (Avanti
15 Polar Lipids, Alabaster, AL) were prepared and characterized as described previously (Higgins et al., 1983. J Biol Chem 258: 6503–6508). FX-depleted human plasma was obtained from Diagnostica Stago (Paris, France). All functional assays were performed in HEPES buffered Saline (20mM Hepes, 0.15M NaCl, pH 7.5) supplemented with 5mM CaCl₂ and 0.1% polyethylene
20 glycol 8000 (assay buffer). Mammalian expression vector pCMV4 (Andersson et al, 1989. J Biol Chem. 264: 8222-8229, carrying recombinant human FX (r-hFX) was a generous gift from Rodney M. Camire (Camire et al. 2000. Biochemistry 39: 14322-14329). The pcDNA3 vector was obtained from Invitrogen and the PACE cDNA was a generous gift from Genetics Institute, Boston, MA. A vector carrying
25 Furin proprotein convertase has been described (US patent number 5,460,950).

Human recombinant Factor V (FV) was prepared, purified, and characterized as described previously (Bos et al, 2009. Blood 114: 686-692). Recombinant *P. textilis* venom FXa (vpt-FXa) was prepared, purified, and characterized as described
30 previously (Verhoef et al., Toxin Reviews (2013) (doi:10.3109/15569543.2013.844712). Plasma derived human Factor Xa (pd-hFXa), DAPA, human prothrombin and Anti-Human Factor X monoclonal mouse

IgG (AHX-5050) were from Haematologic Technologies (Essex Junction, VT, USA). FX antigen paired antibodies for ELISA were obtained from Cedarlane (Burlington, Canada). RVV-X activator was obtained from Diagnostica Stago (Paris, France), or Haematologic Technologies. Restriction endonuclease Apa1
5 was obtained from New England Biolabs (Ipswich, MA, USA). T4-DNA ligase was obtained from Roche (Roche Applied Science, Indianapolis, IN, USA).

The DNA sequence encoding modified human FX –A is provided as SEQ ID NO:

7. The DNA sequence encoding modified human FX –B is provided as SEQ ID

10 NO: 8. Nucleotides encoding the SEQ ID NO: 4 (to generate modified human FX –A) or SEQ ID NO: 5 (to generate modified human FX –B) sequences flanked by

ApaI restriction sites were synthesized by Genscript (Piscataway, NJ, USA),

subcloned into pCMV4 mammalian expression vector using Apa1 and T4-DNA
ligase and sequenced for consistency. Modified human FX – A and modified

15 human FX – B are also referred to as mod-hFX-A and mod-hFX-B, respectively.

Stable HEK293 cell lines expressing r-hFX or modified hFX were obtained as described previously (Larson et al., 1998. Biochemistry 37, 5029-5038). HEK293 cells were cotransfected with pCMV4 and pcDNA-PACE vectors using

Lipofectamine2000 according to the manufacturer's instructions. FX expression

20 of transfectants was assessed by a modified one-step clotting assay using FX-depleted human plasma. Transfectants with the highest expression levels were expanded into T175 culture flasks and conditioned for 24 hours on expression media (DMEM-F12 nutrient mixture without Phenol-red supplemented with:

Penicillin/Streptomycin/Fungizone, 2 mM L-glutamine, 10 µg/ml ITS, 100 µg/ml

25 Geneticin-418 sulphate and 6 µg/ml vitamine K). Conditioned media was

collected, centrifuged at 10.000 g to remove cellular debris, concentrated in a 10-kDa cut-off filter (Millipore, Darmstadt, Germany), washed with HEPES-

buffered saline and stored in 50% glycerol at -20°C. FX antigen levels of glycerol stocks were assessed by sandwich ELISA according to the manufacturer's

30 instructions using human pooled plasma as reference, assuming a plasma FX concentration of 10µg/ml.

Expression media were conditioned for 24 hours on stable cell lines expressing either r-hFX, modified human FX –A or modified human FX –B. An aliquot of conditioned media was incubated with RVV-X (10 ng/μl; Haematologic Technologies) for 120 minutes at 37°C. After activation, modified human FX –A or modified human FX –B are also referred to as m-hFXa A or m-hFXa B, respectively. Assuming similar substrate affinities for all FXa variants, we subsequently determined the concentration of FXa in media by peptidyl substrate conversion (Spec-Xa, 250 μM) using known concentrations of pd-hFXa as reference. Steady-state initial velocities of macromolecular substrate cleavage were determined discontinuously at 25°C as described (Camire, 2002. J Biol Chem 277: 37863-70). Briefly, progress curves of prothrombin activation were obtained by incubating PCPS (50μM), DAPA (10μM), and prothrombin (1.4μM) with human recombinant FV-810 (B-domain truncated, constitutively active), and the reaction was initiated with either 0.1 nM of pd-hFXa, r-hFXa, m-hFXa B, or 0.033 nM of m-hFXa A. The rate of prothrombin conversion was measured as described (Krishnaswamy et al., 1997. Biochemistry 36, 3319–3330).

Recombinant FX and modified human FX –A and modified human FX –B (200 ng) were activated by RVV-X (0.5 U/ml) for 60 minutes at 37°C and subjected to electrophoresis under reducing (30 mM dithiothreitol) conditions using pre-cast 4-12% gradient gels and the MES buffer system (Life Technologies) and transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). The blot was probed with an anti-heavy chain FX antibody and protein bands were visualized using a Dyelight-800 anti-mouse fluorescent antibody (Thermo Scientific, Rockford, IL USA). Plasma-derived hFXa (200 ng) was used as a reference.

Thrombin generation was adapted from protocols earlier described (Hemker et al., 2003. Pathophysiol Haemost Thromb, 33: 4-15). Briefly, FX-depleted plasma was mixed with Corn Trypsin Inhibitor (70 μg/ml), buffer (25 mM HEPES, 175 mM NaCl, 5 mg/ml BSA, pH 7.5) and PCPS (20 μM) and incubated for 10 minutes at 37°C in a 96-wells microplate. Thrombin formation was initiated by

addition of pd-hFXa (0.5 nM) or vpt-FXa (0.5 nM) preincubated with Rivaroxaban (0.4 μ M) or Apixaban (0.2 μ M), supplemented with FluCa and immediately transferred to the plasma mix. The final reaction volume was 120 μ l, of which 64 μ l was FX-depleted plasma. Thrombin formation was determined every 20 s for 30 minutes and corrected for the calibrator using a software suite (Thrombinoscope, version 5.0). The mean endogenous thrombin potential (the area under the thrombin generation curve) was calculated from at least 2 individual experiments. Calibrator and fluorescent substrate (FluCa) were purchased from Thrombinoscope (Maastricht, The Netherlands).

Peptidyl substrate conversion (Spec-Xa, 250 μ M final) of each FXa variant was performed in the absence or presence of direct FXa inhibitors Rivaroxaban and Apixaban (0,001 μ M – 100 μ M final) at ambient temperature. Calcium-free stocks of pd-hFXa (2 nM final) or vpt-FXa (10 nM final) were diluted in assay buffer and incubated in a 96-wells microplate in the presence of assay buffer or inhibitor for 2 minutes. Substrate conversion was initiated with Spec-Xa and absorption was monitored for 10 minutes at 405 nm in a SpectraMax M2e microplate reader equipped with the Softmax Pro software suite (Molecular Devices, Sunnyvale, CA, USA). In order to assay DFXI sensitivity of each recombinant FX variant, glycerol stocks (5-40 μ l) of r-hFX, modified human FX –A and modified human FX –B were diluted in assay buffer and incubated with RVV-X (0.5 U/ml) for 60 minutes at 37°C. Activated stocks were subsequently diluted in assay-buffer, incubated for 2 minutes in a 96-wells microplate in the presence of assay buffer or inhibitor and assayed for substrate conversion as described. The relative concentration of rhFX, m-hFXa A and m-hFXa B was assessed from the rate of substrate conversion in the absence of inhibitor using known concentrations of pd-hFXa as reference.

Results

Venom-derived P. textilis (Vpt)-FXa is resistant to inhibition by DFXIs.

Biochemical characterization of purified recombinant venom-derived *P. textilis* FXa (vptFXa) revealed that this protease, unlike any other FXa species known to

date, is resistant to inhibition by the direct anticoagulants rivaroxaban and apixaban, which have been designed to reversibly block the active site of FXa. Consistent with previous observations, the K_i for human FXa (hFXa) inhibition was approximately 1 nM (Perzborn, 2005. J Thromb Haemost, 3, 514-521),
5 whereas vptFXa inhibition was at least a 1000-fold reduced (Fig. 2A). These findings were corroborated in a plasma system mimicking in vivo fibrin generation, demonstrating that physiological concentrations of the FXa inhibitors hardly affected vptFXa-initiated thrombin formation, while a significant reduction was observed with hFXa present (Figs. 2B,C).

Human-venom *P. textilis* FXa chimeras.

A striking structural element that is not only limited to vptFXa, but also present in venom FX from the Australian snake *Notechis scutatus*, is an altered amino acid composition at a position close to the hFXa active site (Fig. 1C). Given its
15 location, we hypothesized that this unique helix may not only modulate the interaction with rivaroxaban and/or apixaban, but also with FVa, as the FVa binding site is C-terminal to this helix (Lee et al., 2011. J Thromb Haemost 9: 2123-2126). To test this hypothesis, we prepared the two protein coding DNA constructs as listed in SEQ ID NO: 7 and 8. The mod-hFX-A chimera as provided
20 in SEQ ID NO: 7 comprises the relevant part of the *N. scutatus* DNA sequence (indicated in bold and underlined) and the mod-hFX-B chimera as provided in SEQ ID NO: 8 comprises the relevant part of the *P. textilis* sequence (indicated in bold and underlined).

25 Using these DNA constructs we generated HEK293 cell lines that stably produced both chimeric proteins and subsequently assessed the expression levels of modified human FX from HEK293 cells by conditioning the cells on expression media for 24 hours. Western blot analysis revealed expression of full-length FX for both chimeric variants similar to wild-type FX (Fig. 3A). Incubation with
30 activator from Russell's Viper Venom (RVV-X) resulted in proteolytic activation of approximately 30% of zymogen FX to FXa, indicated by the appearance of the ~29 kDa heavy chain band. The heavy chain of both modified human FXa-A and

modified human FXa-B migrated at a slightly higher molecular weight, which is consistent with the insertion of a snake sequence that is 12 or 13 residues longer as compared to that of human FXa, respectively. Analysis of the FX antigen levels in conditioned media indicated that whereas the expression of mod-hFX-A was approximately ~7-fold reduced, that of mod-hFX-B was similar to wild-type human FX (Fig. 3B). The low FX antigen levels of mod-hFX-A correlated with the similarly low FX activity levels we observed employing a modified clotting assay. This indicates that while the protein expression of mod-hFX-A is suboptimal as compared to that of the other FX variants, its FX function is not perturbed.

To test zymogen activation of FX we converted rFX and modified human FX-A and modified human FX-B to FXa using FX activator from Russell's Viper Venom (RVV-X). Both modified human FXa-A and modified human FXa-B displayed protease activity upon RVV-X activation, as assessed by conversion of the small FXa-specific peptidyl substrate SpectroZyme Xa. In addition, the prothrombin conversion rates in the presence of the human cofactor FVa of both chimeras were similar to human FXa (both pd-hFXa and r-hFXa) (Fig. 4). Collectively, these observations suggest that the snake sequence insertions do not severely hamper the enzymatic properties of human FX.

Inhibition of FXa chimeras by DFXIs.

To estimate the inhibitory constant (K_i) of Rivaroxaban and Apixaban for RVV-X activated modified human FX-A, the activated recombinant protein was pre-incubated with 0.001 to 100 μ M of inhibitor and subsequently assayed for its catalytic activity towards SpectroZyme Xa. While incubation with 0.5 μ M Rivaroxaban resulted in full inhibition of r-hFXa and pd-hFXa, mod-hFXa-A remained fully active under these conditions (Fig. 5A). Moreover, the chimeric variant still displayed partial chromogenic activity following incubation with 100 μ M Rivaroxaban, similar to the *P. textilis* venom FXa. These data indicate that the K_i for inhibition of mod-hFXa-A is at least 100-fold increased as compared to that of human FXa. We observed a similar reduced sensitivity for inhibition by Apixaban (Fig. 5B).

Assessment of the inhibition of mod-hFXa-B by rivaroxaban and apixaban resulted in a K_i similar to that observed for mod-hFXa-A (Figs. 6A and 6B). Thus, a reduced sensitivity for inhibition by apixaban and rivaroxaban was shown. Finally, DFXI-inhibition of the chimeric FXa variants was not altered in the presence of the cofactor FVa and negatively charged phospholipid vesicles, suggesting that both the free protease as well as that assembled into a FVa-FXa-lipid-bound complex are equally resistant to inhibition by Rivaroxaban and Apixaban (Figs. 7A, B).

Example 2

Materials and methods

Unless indicated otherwise, materials and methods as used in this example were the same or similar to the materials and methods indicated in Example 1.

Construction and expression of recombinant FX: DNA encoding chimeric FX-A (c-FX A), chimeric FX-B (c-FX B) and chimeric FX-C (c-FX C) were synthesized at Genscript (Piscataway, NJ, USA), subcloned into pCMV4 mammalian expression vector using Apa1 and T4-DNA ligase and sequenced for consistency. Stable HEK293 cell lines expressing recombinant human or recombinant chimeric FX were obtained as described previously (Larson et al, 1998. Biochemistry 37, 5029-5038). HEK293 cells were cotransfected with pCMV4 and pcDNA-PACE vectors by Lipofectamine2000 according to the manufacturer's instructions.

Purification of chimeric FX(a): Recombinant chimeric FX products A, B and C were prepared, purified and characterized as described previously (Camire et al, 2000), with the exception that the immunoaffinity purification was replaced by a calcium gradient purification of FX on a POROS HQ20-sepharose column. The typical yield of fully γ -carboxylated recombinant FX was 0.9 mg/liter conditioned medium. Purified recombinant chimeric FX was activated with RVV-X (0.1 U/mg

FX), isolated by size-exclusion chromatography on a Sephacryl S200 HR column (Vt 460 ml) and stored at -20°C in HBS containing 50% vol/vol glycerol. Purified products were visualized by Coomassie staining.

- 5 Macromolecular substrate activation: Steady-state initial velocities of macromolecular substrate cleavage were determined discontinuously at 25°C as described (Camire, 2002). Briefly, progress curves of prothrombin activation were obtained by incubating PCPS (50µM), DAPA (10µM), and prothrombin (1.4µM) with human recombinant FV-810 (20 nM, B-domain truncated, constitutively
- 10 active FV), and the reaction was initiated with either 0.1 nM of pd-hFXa, r-hFXa, c-FXa A, c-FXa B or c-FXa C. The rate of prothrombin conversion was measured as described (Krishnaswamy et al , 1997). Prothrombin conversion was assayed in absence or presence of direct FXa inhibitors Edoxaban (CAS Registry Number 912273-65-5; manufactured by Daiichi Sankyo, marketed as Savaysa) and
- 15 Apixaban (0,001 µM – 100 µM final) in order to determine DOAC sensitivity of each recombinant FXa variant.

- Thrombin generation assays: Thrombin generation was adapted from protocols earlier described (Hemker et al, 2003). Briefly, thrombin generation curves were
- 20 obtained by supplementing FX-depleted plasma with Tissue Factor (TF, 2 or 20 pM final), Corn Trypsin Inhibitor (70 µg/ml), PCPS (20 µM) and 1 Unit (prothrombin time specific clotting activity) of r-hFX (7 µg/ml) or chimeric FX-C (16 µg/ml). Thrombin formation was initiated by adding Substrate buffer (Fluca) to the plasma. FXa thrombin generation curves were obtained by supplementing
- 25 FX-depleted plasma with Corn Trypsin Inhibitor (70 µg/ml), assay buffer and PCPS (20 µM). Thrombin formation was initiated by addition of FXa premixed with Rivaroxaban or Apixaban, assay buffer without calcium and supplemented with Fluca. The final reaction volume was 120 µl, of which 64 µl was FX-depleted plasma. Thrombin formation was determined every 20s for 30 minutes and
- 30 corrected for the calibrator, using the software of Thrombinoscope. The lag time, mean endogenous thrombin potential (the area under the thrombin generation

curve), time to peak and peak thrombin generation, was calculated from at least 3 individual experiments.

Results

5

The 9-13 residue insertion in the serine protease domains of *P. textilis* venom, *P. textilis* isoform and *N. scutatis* venom FXa has prompted us to construct chimeras of human and snake FX. We made three protein coding DNA constructs that incorporate each of these insertions in human FXa. (Fig. 9). Using these DNA
10 constructs we generated HEK293 cell lines that stably produce either recombinant normal human FX (r-hFX) or three types of chimeric FX (c-FX A, c-FX B and c-FX C). Expression levels of recombinant human and chimeric FX from HEK293 cells were determined by culturing the cells on expression media for 24 hours after which the clotting activity of conditioned medium was assessed
15 by a modified one-step PT clotting assay in FX-depleted plasma. Recombinant γ -carboxylated FX was purified from conditioned media by successive ion-exchange chromatography steps. A fraction of the FX pool was subsequently activated with the FX activator from Russell's Viper venom, isolated by size-exclusion chromatography and characterized by SDS-PAGE. The heavy chain of purified
20 plasma derived factor Xa migrates as a 50/50 mixture of FXa- α and FXa- β at ~34-31 kDa. While autoproteolytic excision of the C-terminal portion of FXa- α (residues 436–447) yields the β form of FXa, both isoforms are functionally similar with respect to prothrombinase assembly, prothrombin activation, antithrombin recognition, and peptidyl substrate conversion (Prydzial and
25 Kessler, 1996).

The purified products of r-hFXa and chimeric FXa – B and – C migrated predominantly as FXa- β , chimeric FXa – A migrates as a 50/50 mixture of α and β FXa instead. (Fig. 10A). Kinetics of macromolecular substrate activation by r-
30 hFXa and chimeric FXa (A/B/C) on negatively charged phospholipid vesicles (PCPS) in the presence of the cofactor FVa shows that all chimeric variants assemble into the prothrombinase complex. However, the catalytic rate of

chimeric FXa variants – A, – B and – C is respectively 8.2–, 6.8–, and 2.3–fold reduced compared to recombinant human FXa. Furthermore, recombinantly prepared human FXa shows a modest decrease in catalytic efficiency compared to plasma-derived FXa. (Fig. 10B).

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To determine the inhibitory constant (K_i) of DOACs (Apixaban, Edoxaban) for chimeric FXa (A/B/C), we assayed the kinetics of prothrombin activation in the presence of 0.001 to 100 μ M of DOAC. While plasma-derived FXa and recombinant human FXa are fully inhibited at near equimolar concentrations of DOAC, all chimeric FXa variants were able to sustain prothrombin conversion at significantly higher FXa-inhibitor concentrations (K_i Apixaban: 130-1270 nM, K_i Edoxaban: 3-270 nM). (Fig. 11). Given that the chimeric FXa variants comprise similarly positioned insertions with a varying length and composition of amino acids, we speculate that the close proximity of these insertions to the DOAC-coordinating residue Tyr99 and/or the active site is of direct consequence to the decreased sensitivity for the DOACs.

In order to assess the potential of chimeric FXa to restore thrombin generation in DOAC spiked plasma we performed a thrombin generation (TG) assay. FXa-initiated (5nM) thrombin generation in FX-depleted human plasma demonstrated a normal TG profile for c-FXa variant C, and near normal profiles for c-FXa variants A and B. (Fig. 12A). While Apixaban (2 μ M) dramatically prolonged the lag time and reduced peak thrombin generation in pd-FXa- and r-hFXa-initiated TG, these parameters were unperturbed with the chimeric FXa variants present. (Fig. 12B). (Table 2). These results show that chimeric FXa variants are able to restore hemostasis in DOAC inhibited plasma. In addition, the zymogen form of chimeric FX – C is also able to sustain thrombin generation in FX-depleted plasma. Initiation of coagulation by a low Tissue Factor (TF, 2pM) concentration generates a robust TG curve for chimeric FX – C that is not affected by Apixaban, unlike TG by r-hFX (Fig. 13). At low TF concentration chimeric FX – C displays a short delay in the onset of TG and time to peak, in addition, chimeric FX – C has an larger endogenous thrombin potential (ETP)

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and higher peak thrombin generation (Table 3). However, these values normalize at high TF (20 pM) concentrations (Fig. 13) (Table 3). Based on the observations made in the FXa-initiated TG assay we expect that zymogen forms of chimeric FX variants A and B also sustain TF-initiated TG in DOAC spiked plasma.

- 5 Figure 14 in combination with Table 4 provide further evidence the effect of chimeric FXa variants on restoring hemostasis in DOAC inhibited plasma. Taken together, these results show that chimeric FX(a) is able to restore hemostasis in DOAC inhibited plasma both in zymogen and protease form.

Table 2. Effect of Apixaban on FXa-initiated TG parameters. Values represent experimental TG values obtained in the presence of Apixaban corrected for TG values obtained in the absence of Apixaban.

	pd-FXa	r-hFXa	c-FXa -A	c-FXa -B	c-FXa -C
Lagtime arrest (seconds)	299	293	61	12	3
Delay in time to peak (seconds)	515	467	120	30	7
Peak Thrombin Generation (% of no Apixaban)	26	33	78	85	99
Area under the curve (% of no Apixaban)	67	76	89	92	101

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Table 3. Summary of low and high TF-initiated TG experiments.

Low TF (2 pM)	r-hFX	r-hFX + Apixaban	c-FX -C	c-FX -C + Apixaban	High TF (20 pM)	r-hFX	r-hFX + Apixaban	c-FX -C	c-FX -C + Apixaban
Lagtime (seconds)	132 ± 5	696 ± 162	185 ± 12	186 ± 6	Lagtime (seconds)	48 ± 1	138 ± 12	72 ± 2	78 ± 2
Time to peak (seconds)	324 ± 5	no peak	480 ± 24	492 ± 23	Time to peak (seconds)	114 ± 6	804 ± 36	138 ± 6	144 ± 9
Peak thrombin (nM)	61 ± 4	8 ± 4	78 ± 1	72 ± 4	Peak thrombin (nM)	338 ± 8	32 ± 4	334 ± 15	321 ± 15
ETP (nM)	567 ± 61	no ETP	830 ± 131	756 ± 38	ETP (nM)	973 ± 18	694 ± 67	1027 ± 19	1012 ± 33

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Table 4. Effect of Edoxaban on TF-initiated TG parameters for r-hFX and c-FX – C. Values represent experimental TG values obtained in the presence of increasing concentrations of Edoxaban.

r-hFX								
Edoxaban (nM)	controls	50n	100n	200n	400n	600n	1000n	2000n
Lagtime (s)%	115n	247n	297n	397n	538n	679n	874n	1180n
ETP-remaining%	100.5n	99.3n	87.1n	no-ETPn	no-ETPn	no-ETPn	no-ETPn	no-ETPn
Peak-height-%	100.5n	41.2n	30.8n	23.1n	15.9n	13.0n	10.1n	7.1n
Time-to-peak (s)n	265n	618n	756n	1290n	1890n	1932n	2472n	2562n
c-FX—C								
Edoxaban (nM)	controls	50n	100n	200n	400n	600n	1000n	2000n
Lagtime (s)%	188n	161n	161n	172n	162n	197n	212n	232n
ETP-remaining%	100.5n	87.9n	92.5n	88.3n	92.7n	96.8n	92.7n	82.0n
Peak-height-%	100.5n	109.3n	112.3n	101.0n	94.6n	88.7n	77.5n	65.3n
Time-to-peak (s)n	433n	382n	388n	418n	448n	483n	538n	578n

Claims

1. A recombinant protein comprising a coagulation Factor Xa polypeptide, said polypeptide having an alteration in a region of amino acid residues
5 corresponding to the region of amino acid residues between Gly-289 and Asp-320, preferably between His-311 and Asp-320 of SEQ ID NO: 1; wherein the alteration is an insertion of at least one amino acid residue, which altered protein has a decreased sensitivity to inhibition by a direct Factor Xa inhibitor.
- 10 2. The protein according to claim 1, wherein the insertion is 1-50 amino acid residues.
3. The protein according to claim 1 or claim 2, wherein the insertion is 1-20 amino acid residues.
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4. The protein according to any one of claim 1-3, wherein said insertion is between two amino acid residues corresponding to Lys-316 and Glu-317 of SEQ ID NO: 1.
- 20 5. The protein according to any one of claims 1-4, wherein the insertion of at least one amino acid residue is combined with a replacement of between 1-8 amino acid residues in the region of amino acid residues between Gly-289 and Asp-320, preferably between His-311 and Asp-320 of SEQ ID NO: 1.
- 25 6. The protein according to any one of claims 1-5, wherein said insertion is 9-13 amino acid residues.
7. The protein according to any one of claims 1-6, wherein the region of amino acid residues corresponding to the region of amino acid residues between His-311
30 and Asp-320 of SEQ ID NO: 1 has the amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11.

8. A nucleic acid molecule comprising a DNA sequence that encodes the protein according to any one of claims 1-7.
9. An expression vector comprising the nucleic acid molecule according to claim 8.
10. A host cell comprising the nucleic acid molecule according to claim 8 or the expression vector according to claim 9.
11. A pharmaceutical composition comprising the protein according to any of claims 1-7, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.
12. The altered recombinant protein according to any one of claims 1-7, wherein the alteration is an insertion of 1-50 amino acid residues, or the pharmaceutical composition according to claim 11, comprising an altered recombinant protein having an insertion of 1-50 amino acid residues, for use as a medicament.
13. The altered recombinant protein according to any one of claims 1-7, wherein the alteration is an insertion of 1-50 amino acid residues, or the pharmaceutical composition according to claim 11, comprising an altered recombinant protein having an insertion of 1-50 amino acid residues, for use in a method of completely or partially reversing an anti-coagulant effect of a direct Factor Xa inhibitor in a subject.
14. Use of the altered recombinant protein of any one of claims 1-7, wherein the alteration is an insertion of 1-50 amino acid residues, or of the pharmaceutical composition according to claim 11, comprising an altered recombinant protein having an insertion of 1-50 amino acid residues, for the manufacture of a medicament for completely or partially reversing an anti-coagulant effect of a direct Factor Xa inhibitor in a subject.

15. The use according to claim 14, wherein the direct Factor Xa inhibitor, is
rivaroxaban (5-chloro-N-[[[(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-5-
oxazolidinyl]methyl]-2-thiophenecarboxamide), apixaban (1-(4methoxyphenyl)-7-
5 oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-
c]pyridine-3-carboxamide), edoxaban (N'-(5-chloropyridin-2-yl)-N-[(1S,2R,4S)-4-
(dimethylcarbamoyl)-2-[(5-methyl-6,7-dihydro-4H-[1,3]thiazolo[5,4-c]pyridine-2-
carbonyl)amino]cyclohexyl]oxamide;4-methylbenzenesulfonic acid), or betrixaban
10 (N-(5-chloropyridin-2-yl)-2-[[4-(N,N-dimethylcarbamimidoyl)benzoyl]amino]-5-
methoxybenzamide).