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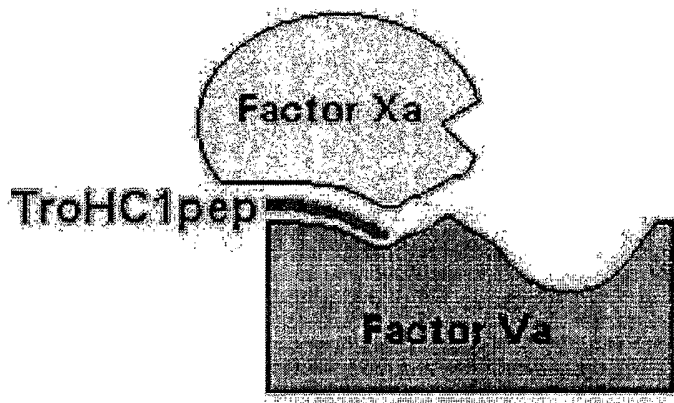
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(54) Title: ANTICOAGULANT PEPTIDES



(57) Abstract: The present invention provides an isolated peptide having an amino acid sequence of: (a) $[\Sigma]_n \text{NYYYVHQNFDVAYDYDI}[U]_m$; or (b) $[\Sigma]_n \text{KIX}^3 \text{X}^4 \text{X}^5 \text{X}^6 \text{KX}^8 \text{X}^9 [U]_m$, wherein X^3 , X^4 and X^8 , and are independently an amino acid selected from A, F, I, L, M, V, W and Y; each of X^5 , X^6 and X^9 is any amino acid; $[\Sigma]$ is any amino acid, and n is an integer from (0) to (15); $[U]$ is any amino acid, and m is an integer from (0) to (15). Further, the use of these peptides as anticoagulants is also provided.

Anticoagulant Peptides

Field of the invention

The invention relates to peptides which inhibit the assembly of the prothrombinase complex. These peptides are useful as anticoagulants. The present invention also relates to the identification of peptides which inhibit the assembly of the prothrombinase complex.

Background of the invention

- 10 In the blood coagulation cascade, thrombin is directly involved in the formation of fibrin, activation of platelets and feedback activation of other components of the cascade. Thrombin is formed when prothrombin is activated by prothrombinase complex, an enzymatic complex assembled from factor Xa (FXa), factor Va (FVa), phospholipids (PL) and calcium ions (Ca^{2+}).
- 15 While FXa alone has very low prothrombin-activating ability, the formation of prothrombinase enhances the rate of the reaction by six orders of magnitude (Joseph et al., 1999).

Current established anticoagulants (heparin and warfarin) have some major limitations, including narrow therapeutic window, highly variable dose-response relation and extensive drug-drug or drug-food interactions. This is mainly due to their mechanisms of action that affect multiple steps in the coagulation cascade. Consequently usage of the drugs has to be under close monitoring. There is a pressing need for a more efficacious and safer anticoagulant.

- 25 Newer anticoagulants are emphasizing on selectivity and specificity in the inhibition of coagulation factors. FXa is one of the most targeted coagulation factors. However, FXa and the other serine proteinases in the coagulation

cascade possess multiple roles both within the cascade, as well as in other physiological processes. FXa induces the release of growth factors from the endothelium (Gajdusek et al., 1986) and the proliferation of vascular smooth muscle cells (Gasic et al., 1992; Ko et al., 1996) and endothelial cells (Nicholson et al., 1996). It triggers an increase in cytosolic free Ca^{2+} ions in Madin-Darby canine kidney cells (Camerer et al., 1999), activates nitric oxide synthase in endothelial cells (Papapetropoulos et al., 1998) and induces the synthesis of cytokines and the expression of adhesion molecules (Papapetropoulos et al., 1998; Senden et al., 1998). Therefore even the use of specific active site inhibitors of FXa as anticoagulants would result in undesirable interference with the pleiotropic functions of FXa.

Summary of the invention

The present invention addresses the problems above, and in particular provides new peptides which are able to interact with the Factor Va (FVa) and inhibit the assembly of prothrombinase complex. Accordingly, the peptides of the invention are useful as anticoagulants.

According to one aspect, the invention provides an isolated peptide having an amino acid sequence of:

(a) $[\Sigma]_n \text{NYYYVHQNFD RVAYDYDI}[\text{U}]_m$; or

(b) $[\Sigma]_n \text{KIX}^3\text{X}^4\text{X}^5\text{X}^6\text{KX}^8\text{X}^9[\text{U}]_m$

wherein

X^3 , X^4 and X^8 , and are independently an amino acid selected from A, F, I, L, M, V, W and Y;

each of X^5 , X^6 and X^9 is any amino acid;

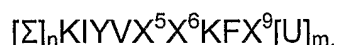
$[\Sigma]$ is any amino acid, and n is an integer from 0 to 15;

$[\text{U}]$ is any amino acid, and m is an integer from 0 to 15.

For example, m and/or n may be an integer from 1 to 14, 2 to 13, 3 to 12, 4 to 11, 5 to 10, 6 to 9 or 7 to 8.

The peptides (a) and (b) may be obtained by fragmentation and/or amino acid derivation of the protein trocarin or of other proteins. They may also be
5 prepared by chemical synthesis.

In particular, the peptide (b) has the following amino acid sequence:



10 More in particular, in the peptide of the invention:

X^5 is H or A;

X^6 is T or A; and

X^9 is V or A.

15 For example, the peptides have the following amino acid sequence:

$[\Sigma]_n \text{KIYVX}^5 \text{TKFV} [\text{U}]_m$ and X^5 is H or A;

$[\Sigma]_n \text{KIYVHX}^6 \text{KFV} [\text{U}]_m$ and X^6 is H or A; or

$[\Sigma]_n \text{KIYVHTKFX}^9 [\text{U}]_m$ and X^9 is H or A.

20 Preferably, the peptide of the invention is: $[\Sigma]_n \text{KIYVHTKFV} [\text{U}]_m$.

In particular, $[\Sigma]_n$ and $[\text{U}]_m$ are such that: $[\Sigma]_n$ is selected from ETRLLSVD, GPLLSVD, LLSVD, SVD, D, P, PP, PPP, PPPP, PPPPP, VP, VPP, VPPP, VPPPP, F, FV, FVP, FVPP and $[\text{U}]_m$ is selected from P, PP, PPP, PPPP, PPPPP, PN, PPN, PPPN, PPPPN.

25

Preferably, the amino acid sequence of the peptide of the invention is selected from:

SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:26.

- 5 According to another aspect, the invention provides an isolated nucleic acid molecule encoding any peptide of the invention, a vector comprising the nucleic acid molecule, and a host cell comprising the vector or the nucleic acid molecule. It is further provided a method of producing a peptide comprising cultivating the host cell and expressing the peptide.

10

According to another aspect, the invention provides a composition comprising at least one peptide of the invention, the nucleic acid molecule encoding the peptide of the invention, or the vector. In particular, the composition is a pharmaceutical composition. The composition may further comprise a
15 pharmaceutically acceptable excipient, diluent and/or carrier.

According to another aspect, the invention provides any peptide of the invention for use in medicine. In particular, for use as an anticoagulant. The invention also relates to the use of at least one peptide of the invention for the
20 preparation of an anticoagulant.

According to another aspect, the invention provides a method for inhibiting the assembly of prothrombinase complex in a subject comprising administering to a subject at least one peptide and/or a composition according to the invention.

25

Further, the invention provides a method for the prevention and/or treatment of formation of intravascular blood clots in a subject comprising administering to the subject at least one peptide and/or a composition according to the invention. In particular, the method is for the prevention and/or treatment of
30 ischemic events. More in particular, the ischemic event is coronary syndrome,

myocardial infarction, stroke, venous thromboembolism and/or peripheral arterial occlusion. The subject may be a mammal, for example human.

According to another aspect, the invention provides a method for identifying
5 anticoagulant peptides, comprising:

- (a) providing at least one peptide;
- (b) determining the minimum interaction site (MIS) between the at least one peptide and Factor Va (Fva);
- 10 (c) contacting *in vitro* the peptide comprising the MIS with the prothrombinase complex; and
- (d) determining the ability of the peptide to inhibiting the assembly of the prothrombinase complex.

15 The method may further comprise the step of contacting the peptide comprising the MIS to plasma, and determining the ability of the peptide to interfere with the blood coagulation cascade in plasma.

The method may further comprise the step of selecting the peptide comprising
20 the MIS, modifying at least one amino acid residue inside and/or outside the MIS, and determining the ability of the modified peptide to interfere with the assembly of the prothrombinase complex and/or with the blood coagulation cascade in plasma.

25 The method may further comprise the step of isolating or synthesising the modified peptide capable of interfering with the assembly of the prothrombinase complex and/or with the blood coagulation cascade in plasma.

In particular, the at least one peptide provided which comprises the MIS is obtained from fragmentation of a protein. The protein may be trocarin. The peptide which is used for the identification of derived or improved peptide is preferably at least one peptide according to the invention.

5

Brief description of the figures

Figure 1: Alignment of amino acid sequence of trocarin with bovine (*B taurus*) and human (*H sapiens*) FXa

The sequences are taken from following sources: trocarin – SWISS-PROT no. P81428; bovine FXa – SWISS-PROT no. P00743; human FXa – SWISS-PROT no. P00742.

Identical residues are marked with (*) while homologous residues are marked with (:) and (.). Shaded segments represent the two regions whereby trocarin is markedly different from the other two FXa. The first region is in the EGF-II between Arg101 and Gln107 and the second region is in the heavy chain between Glu246 and Ala278.

In the light chain sequences, the amino acid denoted by 'J' refers to gamma carboxy glutamic acid, which is a special modification of glutamic acid residue, E.

Figure 2: Synthetic peptides compete in the formation of FXa-FVa complex

(A) Dose response curves of FVa at varying concentrations of TroHCpep1 peptide: (♦) 0 μ M, (■) 50 μ M, (●) 100 μ M, and (▲) 200 μ M. (B) Dose response curves of FVa at varying concentrations of TroHCpep2 peptide: (■) 0 μ M, (●) 50 μ M, and (▲) 100 μ M. In both (A) and (B), the K_d for FXa-FVa remains the same irrespective of the amount of inhibitor present, while the V_{max} decreases

with increasing concentration of inhibitor. Thus TroHCpep1 and TroHCpep2 compete in the formation of FXa-FVa complex.

Figure 3: Non-competitive inhibition of FXa-FVa complex by synthetic peptides

- 5 (A) Dose response curves of bovine prothrombin at varying concentration of TroHCpep1 peptide: (■) 0 μ M, (●) 50 μ M, (▲) 100 μ M. (B) Dose response curves of bovine prothrombin at varying concentrations of TroHCpep2 peptide: (■) 0 μ M, (●) 50 μ M, and (▲) 100 μ M. In both (A) and (B), it can be observed that the $V_{\max}$ remains constant at different concentrations of inhibitor
10 while the K_m varies with the concentration of inhibitor. This is characteristic of non-competitive inhibition. The experiment was done three times in triplicate each time.

Figure 4: Mechanism of inhibition of FXa-FVa complex by trocarin D peptides

- 15 (A) Normal prothrombinase complex. In the physiologic system, FXa binds to FVa and converts prothrombin to thrombin. TroHCpep1 (B) and TroHCpep2 (C) bind specifically to FVa. Both peptides compete with FXa for the formation of the complex, indicating that they bind to the same site as FXa. Thus, the complex formation and thrombin generation is inhibited by the
20 peptides.

Figure 5: Anticoagulant effects of synthetic peptides on human plasma

- The recalcification time of citrated plasma was prolonged by 50% of the control clotting time at 50 μ M concentration of TroHCpep1 (■) and 100 μ M concentration of TroHCpep2 (□). The recalcification time was ~250% of the
25 control clotting time at a peptide concentration of 500 μ M.

Figure 6: Dose response curve of 13VP_A7_PN and 13VP_A8_PN, compared to 13VPK_VPN

IC₅₀ of the peptides shown in Table 1 and Table 3 were all obtained from such dose response curves. In this curve, (■) are data points for 13VP_A7_PN (VPKIYVATKFVPN). (◆) are data points for 13VP_A8_PN (VPKIYVHAKFVPN). (●) are data points for 13VPK_VPN (VPKIYVHTKFVPN). 13VP_A7_PN (IC₅₀ = 0.25 μM) and 13VP_A8_PN (IC₅₀ = 1.25 μM) showed much higher activity compared with 13VPK_VPN (IC₅₀ = 300 μM). 13VP_A7_PN and 13VP_A8_PN are good leads for further development of anticoagulant peptides due to their high potency.

Detailed description of the invention

Definitions and Nomenclature

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting in nature.

The amino acid abbreviations used herein are conventional one letter codes for the amino acids and are expressed as follows: A or Ala, alanine; B, asparagine or aspartic acid; C or Cys, cysteine; D or Asp, aspartic acid; E or Glu, glutamic acid; F or Phe, phenylalanine; G or Gly, glycine; H or His, histidine; I or Ile, isoleucine; K or Lys, lysine; L or Leu, leucine; M or Met, methionine; N or Asn, asparagine; P or Pro, proline; Q or Gln, glutamine; R or Arg, arginine; S or Ser, serine; T or Thr, threonine; V or Val, valine; W or Trp, tryptophan; Y or Tyr, tyrosine; Z, glutamine or glutamic acid. It should also be noted that amino acid referred to as 'J' denotes gamma carboxy glutamic acid, which is a special modification of glutamic acid residue, E.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include hydrophobic, hydrophilic, basic and acid amino acids as described below. Thus, a predicted nonessential amino acid residue in the peptide of the invention is preferably replaced with another amino acid residue from the same category. For example, a hydrophobic amino acid is replaced by another hydrophobic amino acid, etc. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of the coding sequence of the nucleic acid encoding the peptide of the invention, such as by saturation mutagenesis, and the resultant mutants can be screened for anticoagulant activity to identify mutants that retain that activity.

"Hydrophobic" amino acids are: A, V, L, I, P, W, F and M; "hydrophilic" amino acids are: G, S, T, Y, C, N and Q; however Y has a hydrophobic aromatic group and may be considered to be acting as a hydrophobic amino acid depending on the circumstances and uses; "basic" amino acids are: K, R and H; and "acidic" amino acids are: D and E.

"Peptide" as used herein refers to and encompasses any amino acid molecule, a peptide or polypeptide. The "peptide" can be obtained as a gene product, a purified and/or isolated product, an expression product, from fragmentation of protein(s) or a synthetic peptide. An "isolated peptide" encompasses naturally occurring, a gene expression product and a synthetic peptide.

The phrase "nucleic acid" or "nucleic acid molecule" as used herein refers to a naturally occurring or synthetic oligonucleotide or polynucleotide, whether DNA or RNA or DNA-RNA hybrid, single-stranded or double-stranded, sense or antisense, which is capable of hybridization to a complementary nucleic

acid by Watson-Crick base-pairing. Nucleic acids of the invention can also include nucleotide analogs (e.g., BrdU), and non-phosphodiester internucleoside linkages (e.g., peptide nucleic acid (PNA) or thiodiester linkages). In particular, nucleic acids can include, without limitation, DNA,
 5 RNA, cDNA, gDNA, ssDNA, dsDNA or any combination thereof.

As used herein, "reverse analog" or "reverse sequence" refers to a peptide having the reverse amino acid sequence as another reference peptide. For example, if one peptide has the amino acid sequence ABCDE, its reverse analog or a peptide having its reverse sequence is as follows: EDCBA.

10 The position of the amino acids in a peptide is denoted by the superscript. For example, one of the basic peptide of the invention is denoted by $K^1I^2Y^3V^4A^5T^6K^7F^8V^9$. In $V^{-2}P^{-1}K^1I^2Y^3V^4A^5T^6K^7F^8V^9P^{10}N^{11}$, the negative superscript indicates an amino acid positioned to the left of the first amino acid of the basic peptide.

15 *Compounds, Compositions, Uses and Methods of the Invention*

Snake venom is a rich source of exogenous haemostatic factors, including prothrombin activators. Prothrombin activators from snake venoms proteolytically activate prothrombin and are classed into four groups based on their structural characteristics, functional properties and cofactor requirements
 20 in prothrombin activation (Kini et al., 2001). Among these, Group D prothrombin activators are very similar to mammalian blood coagulation FXa. Group D prothrombin activators, like FXa, cleave two peptide bonds of prothrombin (Arg-274-Thr-275 and Arg-323 and Ile-324) to yield thrombin. Their activities are comparable to FXa in the presence of FVa, phospholipids
 25 and Ca^{2+} (Kini et al., 2001). One of the Group D prothrombin activators, trocarin, a venom procoagulant of the Australian rough-scaled snake *Tropidechis carinatus*, is a structural homologue of FXa. Functionally, trocarin is also similar to FXa. Like FXa, trocarin cleaves both peptide bonds of

prothrombin, and the activation of prothrombin is at a comparable rate with FXa in the presence of FVa, phospholipids and Ca^{2+} .

In WO 03/082914, Masci, Paul Pantaleone et al. describes snake venom protease polypeptides that are prothrombin activators and that are useful,
5 e.g., as reagents to increase coagulation, to promote haemostasis and prevent blood loss such as during surgery or for treatment of wounds.

The amino acid sequence of trocarin was determined recently (Kini et al., 2001) and was found to be highly similar to that of mammalian FXa.

The present inventors have further investigated trocarin, and based on kinetic
10 studies, have found that trocarin, when compared to bovine FXa, has higher affinity to bovine FVa. Short peptides which contain the sequence of residues involved in the binding to FVa but lack the functional prothrombin activating catalytic residues are able to inhibit the assembly of prothrombinase complex. The peptides can be either synthetic or obtained from naturally occurring
15 protein(s) or polypeptide(s). As a result, the activation of prothrombin can be minimized and the propagation of coagulation cascade can be inhibited. Based on this principle, the peptides can be used as anticoagulants which prevent spontaneous formation of intravascular blood clots for the prevention and treatment of ischemic events, including coronary syndromes, myocardial
20 infarction, stroke, venous thromboembolism and peripheral arterial occlusion.

Accordingly, the present inventors have found that two fragments of trocarin bind to FVa and inhibit the prothrombinase complex. Accordingly, these two peptides may be particularly useful as anticoagulants. These two peptides are identified as TroHCpep1 (ETRRLLSVDKIYVHTKFVPPN) (SEQ ID NO:1) and
25 TroHCpep2 (FVPPNYYYVHQNFDRAVDYDI) (SEQ ID NO:2). These two peptides are obtained from portions of trocarin (as shown in Figure 1) and bind the FVa in different positions (as shown in Figure 4). The ability of

TroHCpep1 and TroHCpep2 inhibiting the prothrombinase complex assembly is shown in Table 1.

TroHCpep1 and TroHCpep2 have been synthesized based on the sequence of trocarin and used for the determination of the minimum interaction segment
5 (MIS) for FVa. It was found that the MIS on trocarin for FVa is located within this sequence. Kinetic studies and binding studies confirmed the peptide interfered with prothrombinase complex by binding to cofactor FVa. In assays done using human plasma, the anticoagulant effect was retained. TroHCpep1 was used to further delineate the MIS which was identified to be KIYVHTKFV
10 based on *in vitro* activity of synthetic peptides. This segment corresponds to residues 255-263 on Trocarin.

Substitutions, deletion and/or modifications of the amino acids of TroHCpep1 and TroHCpep2 have been carried out according to the standard technologies. For example, Y, V and F, which are mostly hydrophobic have
15 been substituted with other hydrophobic amino acids (overall Y is hydrophilic but has a hydrophobic aromatic group). Substituting amino acids with other amino acids of similar properties is acceptable, as the overall properties of the peptide are not altered. For example, substituting one hydrophobic or hydrophilic amino acid with another hydrophobic or hydrophilic amino acid
20 respectively is acceptable as the overall property of the peptide is not altered.

Further, it has been found that the attachment of one or more residue of proline, further attached to one or more amino acidic residue, for example N and/or V, at either side of the selected peptide improves the prothrombinase complex inhibitory activity. In particular, the attachment of one proline at either
25 side of the MIS (VPKIYVHTKFVPN) to form 'Proline bracket' was found to improve the activity of MIS by 3-folds (Evans and Kini, US patents 6,100,044 and 6,258,550, herein incorporated by reference).

Alanine scans performed on MIS ($K^1IYVHTKFV^9$) indicate that side chains of K^1 , I^2 and K^7 are essential for the peptide's inhibitory activity.

Side chains of Y^3 , V^4 and F^8 are important, although not essential for interaction.

- 5 Side chains of H^5 , T^6 and V^9 are not important for activity. Among these three, H^5 and T^6 hindered the optimum binding between peptides and FVa. When H^5 and T^6 are replaced with alanine separately, the activity increased 1200-folds and 240-folds respectively.

- 10 For example, two peptides 13VP_A7_PN (VPKIYVATKFVPN) and 13VP_A8_PN (VPKIYVHAKFVPN) are good leads for further development of anticoagulant peptides due to their high potency ($IC_{50} = 0.25 \mu M$ and $1.25 \mu M$ respectively).

Accordingly, the invention provides an isolated peptide having an amino acid sequence of:

- 15 (a) $[\Sigma]_n N Y Y Y V H Q N F D R V A Y D Y D I [U]_m$; or

- (b) $[\Sigma]_n K I X^3 X^4 X^5 X^6 K X^8 X^9 [U]_m$

wherein

X^3 , X^4 and X^8 , are independently an amino acid selected from A, F, I, L, M, V, W and Y;

- 20 each of X^5 , X^6 and X^9 is any amino acid;

$[\Sigma]$ is any amino acid, and n is an integer from 0 to 15;

$[U]$ is any amino acid, and m is an integer from 0 to 15.

More in particular, n may be an integer from 0 to 15, from 1 to 14, from 2 to 13, from 3 to 12, from 4 to 11, from 5 to 10, from 6 to 9, or from 7 to 8. Further, m 0 to 15, from 1 to 14, from 2 to 13, from 3 to 12, from 4 to 11, from 5 to 10, from 6 to 9, or from 7 to 8.

- 5 The peptides (a) and (b) may be obtained by fragmentation and/or amino acid derivation of the protein trocarin or from other proteins. The peptides (a) and (b) may also be prepared by chemical synthesis according to standard methods known in the art.

In particular, the peptide (b) has the following amino acid sequence:



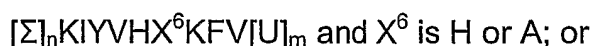
More in particular, in the peptide of the invention:

X^5 is H or A;

X^6 is T or A; and

X^9 is V or A.

- 15 For example, the peptides have the following amino acid sequence:



Preferably, the peptide of the invention is: $[\Sigma]_n \text{KIYVHTKFV} [\text{U}]_m$.

- 20 In particular, $[\Sigma]_n$ and $[\text{U}]_m$ are such that: $[\Sigma]_n$ is selected from ETRLLSVD, GPLLSVD, LLSVD, SVD, D, P, PP, PPP, PPPP, PPPPP, VP, VPP, VPPP, VPPPP, F, FV, FVP, FVPP and $[\text{U}]_m$ is selected from P, PP, PPP, PPPP, PPPPP, PN, PPN, PPPN, PPPPN.

Preferably, the amino acid of the peptide of the invention is selected from:

SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6,
SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17,
SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:26.

- 5 Peptide Production – peptide of the invention may be produced by any method known in the art. One method of producing the disclosed peptides is to link two or more amino acid residues together by protein chemistry techniques. For example, peptides are chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (tert-butyloxycarbonyl) chemistry (Applied Biosystems, Inc.,
10 Foster City, Calif.). A peptide can be synthesized and not cleaved from its synthesis resin, whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group, which is functionally blocked on the other fragment. By
15 peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, (Grant G A (1992) Synthetic Peptides: A User Guide. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) Principles of Peptide Synthesis. Springer-Verlag Inc., NY). Alternatively, the peptide is independently
20 synthesized in vivo. Once isolated, these independent peptides may be linked to form a peptide or fragment thereof via similar peptide condensation reactions.

According to another aspect, the invention provides an isolated nucleic acid molecule encoding any peptide of the invention, a vector comprising the
25 nucleic acid molecule, and a host cell comprising the vector or the nucleic acid molecule. It is further provided a method of producing a peptide comprising cultivating the host cell and expressing the peptide.

Nucleic Acid and Vectors - The invention is also directed to an isolated nucleic acid encoding any one or more of the peptides disclosed herein. In one embodiment, the nucleic acid comprises DNA, RNA and/or cDNA. It would be routine for one with ordinary skill in the art to make a nucleic acid that encodes the peptides disclosed herein since codons for each of the amino acids that make up the peptides are known. As non-limiting examples, the nucleic acids of the invention can be produced by recombinant, in vitro methods, or by chemical synthetic means using machines and standard chemistry which would be known to one with skill in the art, or by in vivo cellular synthesis. Methods of synthesizing nucleic acids would be well known to one with skill in the art. For example, US 6,472,184 entitled "Method for producing nucleic acid polymers" and US 6,444,111 entitled "Electrochemical solid phase synthesis of polymers describes such synthesis" describe such synthetic methods. These references are hereby incorporated by reference in their entireties.

Additionally, the invention provides a vector comprising the nucleic acid encoding any one or more of the peptides described herein. In certain embodiments, the invention provides a vector comprising a nucleic acid encoding at least one of the peptides of the present invention. The vector can be a viral vector, a plasmid vector, a cosmid vector, an adenoviral vector, a phage vector, a retroviral vector, an adeno-associated viral (AAV) vector, or any other vector capable of including a nucleic acid encoding a peptide of the invention. The vector can be an expression vector that is intended and capable of integrating into a cell genome. Other useful virus vectors include retroviruses such as Moloney murine leukemia virus (MoMuLV); papovaviruses such as JC, SV40, polyoma, adenoviruses; Epstein-Barr Virus (EBV); papilloma viruses, e.g. bovine papilloma virus type I (BPV); vaccinia and poliovirus and other human and animal viruses. Useful vectors and their

construction are disclosed in Sambrook and Russel, (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, US.

Host cell -The invention also provides for a host cell containing the nucleic acid, polypeptide, peptide, and/or the vector of the invention. Such a host cell
5 is a eukaryotic cell or a prokaryotic cell. In the case of eukaryotic cells, retrovirus or adenovirus based vectors can be used to put the nucleic acid of the invention into the host cell. Methods known to one with skill in the art to insert the nucleic acids or polypeptides in host cells are encompassed within this invention. The following are non-limiting examples of such methods:
10 naked DNA transfection, lipofectin-mediated transfer, transformation, micro-injection of nucleic acid into a cell, or calcium-phosphate precipitation transfection methods. Host cells can be obtained from commercial sources such as the American Type Culture Collection (ATCC). Host cells can be grown in liquid media culture or on tissue culture plates. The growth
15 conditions will be dependent upon the specific host cells used and such conditions would be known to one with skill in the art. Transfection and growth of host cells is described in Sambrook and Russel, id. The invention provides for a recombinant cell expressing a nucleic acid encoding the polypeptide of the claimed invention. The invention also provides for a recombinant cell
20 producing the polypeptide of the invention.

According to another aspect, the invention provides a composition comprising at least one peptide of the invention, the nucleic acid molecule encoding the peptide of the invention, or the vector. In particular, the composition is a pharmaceutical composition. The composition may further comprise a
25 pharmaceutically acceptable excipient, diluent and/or carrier.

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art. In general, such preparatory methods include the step of bringing the active

ingredient into association with a carrier, excipient and/or diluent, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

5 Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions that are suitable for administration to humans, the person skilled in the art will understand that such compositions are generally suitable for administration to animals of all
10 to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modifications. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates.

15

According to another aspect, the invention provides any peptide of the invention for use in medicine. In particular, for use as an anticoagulant. The invention also relates to the use of at least one peptide of the invention for the preparation of an anticoagulant. The anticoagulant peptides and/or
20 polypeptides of the present invention may block only the hemostatic function but not other non-hemostatic roles of the coagulation enzymes, overcoming the limitations of other anticoagulants currently available or under development.

According to another aspect, the invention provides a method for inhibiting the
25 assembly of prothrombinase complex in a subject comprising administering to a subject at least one peptide or a composition according to the invention.

Further, the invention provides a method for the prevention and/or treatment of formation of intravascular blood clots in a subject comprising administering

to the subject at least one peptide or a composition thereof according to the invention. In particular, the method is for the prevention and/or treatment of ischemic events. More in particular, the ischemic event is coronary syndrome, myocardial infarction, stroke, venous thromboembolism and/or peripheral
5 arterial occlusion. The subject may be a mammal, for example human.

According to another aspect, design and development of the peptides and/or polypeptides of the invention may be used for the identification of further and/or improved anticoagulants peptides and/or polypeptides, which specifically interfere with the assembly of prothrombinase complex by preventing the
10 binding between FXa and FVa.

Accordingly, the invention provides a method for identifying anticoagulant peptides, comprising:

- providing at least one peptide;
- determining the minimum interaction site (MIS) between the at least one
15 peptide and FVa;
- contacting *in vitro* the peptide comprising the MIS with the prothrombinase complex; and
- determining the ability of the peptide to inhibit the assembly of the prothrombinase complex.

20 The method may further comprise the step of contacting the peptide comprising the MIS to plasma, and determining the ability of the peptide to interfere with the blood coagulation cascade in plasma.

The method may further comprise the step of selecting the peptide comprising the MIS, modifying at least one amino acid residue inside and/or outside the
25 MIS, and determining the ability of the modified peptide to interfere with the

assembly of the prothrombinase complex and/or with the blood coagulation cascade in plasma.

The method may further comprise the step of isolating or synthesising the modified peptide capable of interfering with the assembly of the prothrombinase complex and/or with the blood coagulation cascade in plasma.

In particular, the at least one peptide provided, which comprises the MIS is obtained from fragmentation of a protein. The protein may be trocarin. The peptide which is used for the identification of derived or improved peptides is preferably at least one peptide according to the invention.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

Examples

1.1.1. Peptide synthesis

Peptides used were chemically synthesized using solid phase peptide synthesis (SPPS) based on 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. SPPS is based on sequential addition of α -amino and side chain protected amino acid residues to an insoluble polymeric support. Synthesis of peptides was carried out on PerSeptive BioSystems Pioneer Peptide Synthesis System. Fmoc amino acid derivatives, chemical reagents and resins used were of peptide synthesis grade purchased from Applied Biosystems.

Temporary α -amino-protecting Fmoc group was removed using 20% v/v piperidine in N,N-dimethylformamide (DMF), coupling reagent used was 0.5 M of N-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) in DMF. Side-chain protecting groups were cleaved simultaneously with detachment of the peptide from the resin. The cleavage was carried out in a cleavage cocktail of trifluoroacetic acid (TFA): ethanedithiol (EDT): thioanisole: water (45:2:2:1) for 3 hours. All sequences of peptides synthesized are listed in Table 1 and Table 3.

10

1.1.2. Purification of peptides

Synthesized peptides were subjected to purification using reverse-phase high performance liquid chromatography (RP-HPLC) system on a Phenomenex Jupiter C18 (300Å, 5μ) 250 x 10 mm column. The RP-HPLC systems were equilibrated with two solvents. Solvent A was 0.1 % v/v TFA in water and Solvent B was 80 % v/v acetonitrile (HPLC grade) with 0.1 % v/v TFA in water.

Masses of peptides were verified using electrospray ionization mass spectrometry (ESI-MS). The spectra were then analyzed using software BioMultiview to obtain the mass of the peptides.

20

1.1.3. Assay of inhibition of prothrombinase complex assembly

To test for the inhibitory effects of synthetic peptides on the assembly of prothrombinase complex, an inhibition assay modified from previously reported method (Joseph et. al., 1999) was carried out. Pure bovine FXa, bovine FVa and bovine prothrombin were purchased from Haematologic Technologies Inc., bovine serum albumin (BSA) was purchased from Sigma Chemical Co., H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline

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dihydrochloride (S-2238) was purchased from Chromogenic and all other chemicals and reagents used were of the highest quality available.

1.1.3.1. Principle

- 5 The principle of the assay is based on the observations that assembly of the prothrombinase complex is an important step in the activation of prothrombin to thrombin. The thrombin formed will then be added to S-2238, a chromogenic substrate for thrombin. The rate of coloured product formation (i.e. the increase in absorbance per second) at 405 nm is proportional to
- 10 thrombin activity. This increase in absorbance can be monitored using an ELISA plate reader.

1.1.3.2. Assay

- Reactions were carried out in 96-well microtiter plates. 8 wells of reaction
- 15 mixtures, each made up to a final volume of 50 μ l, were prepared. Lyophilized peptides were reconstituted in buffer containing 50 mM Tris-HCl, 50 mM CaCl_2 , 0.5 mg/ml BSA, pH 8.0. Suitable amount of peptides were added to the 8 reaction wells such that eight different concentrations of peptides were obtained in the final reaction mixtures: 0 μ M, 0.1 μ M, 1.0 μ M, 10 μ M, 100 μ M,
- 20 500 μ M, 800 μ M and 1000 μ M. FVa was then added to the reaction wells such that each well contained 13 nM of FVa in the final mixture of 50 μ l. The reaction mixtures were incubated at room temperature for 20 minutes. FXa was then added to each reaction well such that a concentration of 11 nM of FXa in the final mixture of 50 μ l was obtained in each well. The reaction
- 25 mixtures were incubated at room temperature for 20 minutes. Prothrombin was then added to each well such that a concentration of 15 μ M of prothrombin in the final mixture of 50 μ l was achieved. At sampling time of 5, 10 and 20 min after addition of prothrombin, 2.5 μ l aliquots of each reaction mixture was taken out and added to 197.5 μ l of buffer containing 50 mM of
- 30 Tris-HCl, 100 μ M of S-2238, 100 μ M of EDTA (to inhibit further conversion of

prothrombin to thrombin). The OD was monitored for 5 min at 405 nm using ELISA plate reader SpectraMax 340 and the thrombin activity was obtained from the gradient of the absorbance plotted against time. Averages of the readings obtained in three independent experiments were taken.

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1.1.4. Binding sites on Trocarin

Despite being the structural homologue of FXa, amino acid sequence of trocarin has only 70 % similarity with that of FXa. There are mainly two regions where the sequences share minimum similarity. For trocarin, the first
10 region is in the EGF-II between Arg101 and Gln107 and the second region is in the heavy chain between Glu246 and Ala278 (Figure 1). These two sites were predicted to be the possible binding sites on Trocarin for FVa and account for the higher binding affinity compared to that of FXa. Three peptides were synthesized based on the sequences of these two regions and assayed
15 for activity. TroLCpep (Lys100 to Glu107: KRVQSETQ) was synthesized based on the first region (light chain EGF-II domain). TroHCpep1 (Glu246 to Asn266: ETRLLSVDKIYVHTKFVPPN), and TroHCpep2 (Phe262 to Ile283: FVPPNYYYVHQNFDREVAYDYDI), were synthesized based on the second region (heavy chain). Both TroHCpep1 and TroHCpep2 effectively inhibit FXa-
20 mediated formation of thrombin while TroLCpep had no significant inhibitory effects. TroHCpep1 recorded IC₅₀ of 70 µM and 93% inhibition at 500 µM while TroHCpep2 recorded IC₅₀ of 150 µM and 94% inhibition at 500 µM (Table 1). TroLCpep did not show any inhibition even at 500 µM.

25 1.1.5. Kinetic studies of peptides

To verify if TroHCpep1 and TroHCpep2 interfered with the FXa-FVa interaction by competing for FXa binding site on FVa, competitive inhibition studies were carried out using the same system as the assay of inhibition of prothrombinase complex assembly, varying concentrations of FVa, peptides
30 and prothrombin accordingly.

The dissociation constant (K_d) and maximum velocity (V_{max}) for the interaction between FXa and FVa in the presence of a fixed concentration of a peptide inhibitor was estimated from double reciprocal plots of reaction velocity versus increasing concentrations of FVa. The inhibition constant, K_i , for the peptide was also determined from the double reciprocal graph using the formula: $K_i = xK_d [I]/(1 - xK_d)$, where x = x-intercept, $[I]$ = inhibitor concentration, K_d = affinity constant for FXa- FVa interaction.

Both peptides competitively inhibited the interaction between FXa and FVa; since the K_d values for interaction between FXa and FVa were different at different concentrations of peptides while the V_{max} values were equal, a characteristic of competitive inhibition (Figure 2). The average inhibition constants (K_i) for the competitive inhibition reactions were found to be 73.6 μ M for TroHCpep1 and 112.5 μ M for TroHCpep2 (Table 2). In similar inhibition studies, varying the concentration of prothrombin, it was observed that the K_m 's were the same for different concentrations of peptides while the V_{max} values were different, indicating non-competitive inhibition (Figure 3). This indicated that peptides did not bind to the substrate binding site of the FXa-FVa complex and did not compete for the interaction between prothrombin and the active complex. The average inhibition constants (K_i) for the non-competitive inhibition were calculated as 102.8 μ M for TroHCpep1 and 200 μ M for TroHCpep2 (Table 2). These results strongly suggest that these peptides interfered with the assembly of FXa-FVa complex, most likely by binding to FVa, but did not bind to the substrate prothrombin or the active site.

1.1.6. Binding studies using surface plasmon resonance (SPR)

Real time biomolecular interaction analysis (BIA) studies were performed to obtain the affinity constants to determine the binding affinity of TroHCpep1 and TroHCpep2 for FVa, FXa and prothrombin. These interactions were monitored by surface plasmon resonance (SPR) using a BIACORE instrument (Pharmacia Pte Ltd). Briefly, the system was primed with 10 mM HEPES buffer, 150 mM NaCl, pH 7.4 at 25°C. A CM5-sensor chip was pre-activated by amine coupling by injecting 55 µl of a mixture of equal volumes of 0.1 M N-hydrosuccinimide (NHS) and 0.4 M N-ethyl-N'-dimethylaminopropylcarbodiimide. For immobilization on the chip, the ligands were dissolved in 10 mM HEPES, 150 mM NaCl, at a pH ~1 to 1.5 units below the isoelectric point of the ligand. Accordingly, TroHCpep1 (pI = 9.7) was dissolved in 55 µl buffer of pH 8.5 and TroHCpep2 (pI = 4.5) in 55 µl buffer of pH 3.5, both to 1 mg/ml. After immobilization of ligands, excess reactive carboxyl groups on the chip were deactivated by injecting 55 µl of 1 M ethanol amine hydrochloride, pH 8.5. Then, 100 µl of a regeneration buffer, 1 M NaCl, was injected to remove ligand molecules that were not properly immobilized on the chip surface. All the above steps were carried out at a flow rate of 5 µl/min of the running buffer.

20

Kinetic studies were carried out on the immobilized peptides using 40 µl of six different concentrations (100 µg/ml, 75 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml and 1 µg/ml) of three sets of analytes (FVa, FXa and prothrombin), which were passed over each peptide at a running buffer flow rate of 35 µl/min. After each concentration of each analyte was injected, the chip was regenerated using the regeneration buffer, 1 M NaCl. Affinity constants were calculated from interaction curves using the BIAevaluation software.

Both TroHCpep1 and TroHCpep2 bound specifically to FVa. The K_d for the TroHCpep1-FVa interaction was 23.8 µM, while that for the TroHCpep2-FVa

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interaction was 90.3 μ M. As for interaction with FXa, TroHCpep1 showed very weak but negligible binding, and TroHCpep2 did not show any detectable binding. Both TroHCpep1 and TroHCpep2 did not bind to prothrombin. The K_d values for the TroHCpep1-FXa and TroHCpep2-FVa interaction was undetectable by the SPR. These results indicate that TroHCpep1 and TroHCpep2 bind to FVa (Figure 4). Since they compete with FXa for the formation of the complex they bind to the same site as FXa (Figure 4).

1.1.7. Anticoagulant effect of peptides in human plasma

Since both the peptides were effective in specifically inhibiting the FXa-FVa interaction, their anticoagulant effect on human plasma was determined. Anticoagulant activity of the peptides was determined by their effects on the recalcification time of human plasma using a fibrometer (Becton-Dickinson Microbiology Systems, Sparks, MD, USA). Freshly obtained human whole blood was centrifuged at 1500 rpm for 10 minutes, to separate blood cells from plasma. Clotting of a mixture of 100 μ l human plasma, 250 μ l of various concentrations peptide in 50 mM Tris-HCl buffer, pH 7.5 containing 150 mM sodium chloride, at 37°C, was initiated by the addition of 50 μ l of 50 mM CaCl_2 and the recalcification time was measured. Dose effect curves for the peptides were plotted.

Both peptides prolonged the recalcification time of citrated human plasma, though TroHCpep1 was marginally more potent than TroHCpep2. The recalcification time was prolonged by 50% of the control clotting time at 50 μ M concentration of TroHCpep1 and 100 μ M concentration of TroHCpep2 (Figure 5). The recalcification time was ~250% of the control clotting time at a peptide concentration of 500 μ M. Hence the peptides are efficacious not only in a system consisting of purified factors, but also interfere in the blood coagulation cascade in plasma. They therefore have the potential to be developed into novel anticoagulants that specifically inhibit the assembly of

the enzyme-cofactor prothrombinase complex. TroHCpep1 was chosen for further investigation to delineate MIS.

5 **1.1.8. Stepwise deletion of residues from amino and carboxylic acid terminal**

To delineate the MIS, peptides were synthesized with stepwise deletion of residues from the amino terminal end of TroHCpep1 (ETRRLLSVDKIYVHTKFVPPN) and assayed for activity. Results showed that
10 residues ETRRLLSVD could all be removed without significantly affecting the activity of the peptide (IC_{50} of the active peptides = 500 μ M). The same process deletion was done on the carboxyl terminal. PPN was removed with the resultant peptide showing about a 50 % drop (IC_{50} = 1000 μ M) in activity. Removal of any residues beyond PPN from the carboxyl terminal resulted in a
15 significant drop in activity. This showed that PPN at the carboxyl terminal may play a structural role of enhancing the activity while not being directly involved in the binding (Table 1). Previous studies have shown that the presence of prolines in the flanking regions of synthetic bioactive peptides significantly enhances their potency by limiting backbone flexibility, hence stabilizing
20 favorable conformations (Kini and Evans, 1995a; Kini and Evans, 1995b). This explains the moderate drop in activity upon the removal of PPN. Combining these results, the MIS found contained 9 residues, KIYVHTKFV.

1.1.9. Enhancing the activity of MIS by 'proline bracket'

25 To enhance the activity of MIS both the amino and carboxyl terminal were attached with one proline residue each to form the 'proline bracket'. The 'proline bracket' was suggested to impose conformational restrictions and stabilizing favorable conformations of the peptides. The results showed that the activity of MIS was enhanced by 3-folds to the IC_{50} of 300 μ M by inclusion
30 of the 'proline bracket' (Table 1).

1.1.10. Alanine scan

Alanine scan was used to identify the role of side chain of each residue of MIS. Sequential replacements of one residue at a time with alanine for all the residues spanning the MIS were done. Alanine was chosen to replace the residues as it has only a methyl group as side chain thus eliminating any group beyond the β -carbon. Unlike glycine and proline, it does not alter the main chain conformation and does not impose extreme electrostatic or steric effects. Since the replacement of alanine only disrupts the side chain structure, any difference in activity observed should be due to the loss of the original side chain. The peptide sequence V⁻²P⁻¹K¹I²Y³V⁴H⁵T⁶K⁷F⁸V⁹P¹⁰N¹¹ (IC₅₀ = 300 μ M) was used as prototype in this alanine scan (underlined portion of the sequence represents the MIS for binding and prolines were added at both sides to enhance the activity) (Table 3).

K¹, I² and K⁷ of the peptide were found to possess side chains that are essential for binding. Loss of either one of the side chains resulted in a significant drop of activity as there was minimal inhibitory effect (~10 %) even at a concentration of 1000 μ M. Since a single replacement of residue side chain caused such a significant loss in activity, these side chains are believed to be directly involved in the binding to their complementary residues in the receptor site. K¹ and K⁷ both possess basic side chains and are likely to be protonated in the assay. This means that the side chains are positively charged and could bind to their complementary binding residues through ionic interaction. I² possesses a bulky aliphatic side chain, which is neutral and very hydrophobic. Side chain of I² could bind to its complementary binding residue through hydrophobic interaction (Table 3).

Y³, V⁴ and F⁸ were found to possess side chains that are important but not essential for interaction. This observation is based on the results that although

some activities were lost when these residues were replaced, they were to a lesser extent compared to when K¹, I² and K⁷ were replaced. These side chains might not be involved directly in binding to a specific residue at the receptor site, but their presence helps in maintaining the optimum structure for the interaction. As Y³, V⁴, and F⁸ are mostly hydrophobic (overall, Y is hydrophilic but has a hydrophobic aromatic group), the effects of these side chains are most likely brought about by hydrophobic interactions and steric effects either with each other to lock the specific binding residues in position or to interact with hydrophobic residues in the receptor site to bring the specific binding residues closer to their target. All in all, the intermediate loss of activity suggested that the role of these 3 residues were not essential (Table 3).

V⁹, H⁵ and T⁶ did not record any loss of activities when replaced with alanine. Although V⁹ proved to be essential in the C-terminal deletion scan, it had a similar activity when replaced with alanine. The side chain of V⁹ is not important in interaction but if a residue is eliminated from this position the activity is lost. The steric effect brought about by the presence of a residue is possibly needed in the backbone for interaction (Table 3).

Most interestingly, when H⁵ and T⁶ were replaced with alanine separately, the resultant peptides recorded 1200-folds (IC₅₀ = 0.25 µM) and 240-folds (IC₅₀ = 1.25 µM) increase in activity respectively. Based on these results the side chains of the two residues hindered the optimum binding of the peptides to receptor site. The bulky histidine side chain might introduce steric hindrance in the interaction. Threonine causes a bend in the secondary structure of proteins, and therefore replacement with alanine might have eliminated this bend and opened up the peptide structure for optimum binding (Table 3, Figure 6).

These two peptides: 13VP_A7_PN ($V^2P^1K^1I^2Y^3V^4A^5T^6K^7F^8V^9P^{10}N^{11}$) and 13VP_A8_PN ($V^1P^1K^1I^2Y^3V^4H^5A^6K^7F^8V^9P^{10}N^{11}$) are good leads for further development of anticoagulant peptides due to their high potency.

Peptide	Sequence	IC ₅₀ /μM	Percentage of inhibition at 1000 μM
TroHCpep1	ETRRLLSVDKIYVHTKFVPPN (SEQ ID NO:1)	70	93% at 500 μM
TroHCpep2	FVPPNYYYVHQNFDRVAYDYDI (SEQ ID NO:2)	150	94% at 500 μM
TroLCpep	KRVQSETQ (SEQ ID NO:3)	> 500	0 % at 500 μM
Deletions from the amino terminal			
19GP_PN	GPLLSVDKIYVHTKFVPPN (SEQ ID NO:4)	~ 600	59 %
17LL_PN	LLSVDKIYVHTKFVPPN (SEQ ID NO:5)	~ 600	56 %
15SV_PN	SVDKIYVHTKFVPPN (SEQ ID NO:6)	~ 600	58 %
13DK_PN	DKIYVHTKFVPPN (SEQ ID NO:7)	~ 600	59 %
12KI_PN	KIYVHTKFVPPN (SEQ ID NO:8)	500	70 %
11IY_PN	IYVHTKFVPPN (SEQ ID NO:9)	> 1000	11 %
9VH_PN	VHTKFVPPN (SEQ ID NO:10)	> 1000	12 %
7TK_PN	TKFVPPN (SEQ ID NO:11)	> 1000	16 %
Deletions from carboxyl terminal			
11D_KPPN	DKIYVHTKPPN (SEQ ID NO:12)	> 1000	6 %
9D_HPPN	DKIYVHPPN (SEQ ID NO:13)	> 1000	8 %
11K_FPPN	KIYVHTKFPPN (SEQ ID NO:14)	> 1000	12 %
Presence of proline at the termini			
10D_V	DKIYVHTKFV (SEQ ID NO:15)	1000	50 %
11K_VPN	KIYVHTKFVPN (SEQ ID NO:16)	900	58 %
13VPK_VPN	VPKIYVHTKFVPN (SEQ ID NO:17)	300	75%

5

Table 1. Sequences of peptides synthesized to determine MIS, their corresponding IC₅₀/μM and percentage of inhibition at 1000 μM in the assay of inhibition prothrombinase complex assembly.

		TroHCpep1	TroHCpep2
Assay of inhibition of prothrombinase complex assembly		70 μ M	150 μ M
Kinetic studies	Competitive inhibition	73.6 μ M	112.5 μ M
	Non-competitive inhibition	102.8 μ M	200 μ M
Binding studies		23 μ M	90 μ M
Anticoagulant effect in human plasma		50 μ M	100 μ M

Table 2. A comparison of inhibitory properties of TroHCpep1 and TroHCpep2.

5

Peptide	Sequence	IC ₅₀ / μ M	Percentage of inhibition at 1000 μ M
13VPK_VPN	VPKIYVHTKFVPN (SEQ ID NO:17)	300	75 %
13VP_A3_PN	VPAIYVHTKFVPN (SEQ ID NO:18)	> 1000	7 %
13VP_A4_PN	VPKAYVHTKFVPN (SEQ ID NO:19)	> 1000	15 %
13VP_A5_PN	VPKIAVHTKFVPN (SEQ ID NO:20)	> 1000	31 %
13VP_A6_PN	VPKIYAHTKFVPN (SEQ ID NO:21)	> 1000	29 %
13VP_A7_PN	VPKIYVATKFVPN (SEQ ID NO:22)	0.25	92 %
13VP_A8_PN	VPKIYVHAKFVPN (SEQ ID NO:23)	1.25	95 %
13VP_A9_PN	VPKIYVHTAFVPN (SEQ ID NO:24)	> 1000	12 %
13VP_A10_PN	VPKIYVHTKAVPN (SEQ ID NO:25)	> 1000	40 %
13VP_A11_PN	VPKIYVHTKFAPN (SEQ ID NO:26)	300	75 %

Table 3. Sequence of peptides synthesized for alanine scan, their corresponding IC₅₀/ μ M and percentage of inhibition at 1000 μ M in the assay of inhibition prothrombinase complex assembly.

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Claims

1. An isolated peptide having an amino acid sequence of:
 - (a) $[\Sigma]_n \text{NYYYVHQNFDREVAYDYDI}[\text{U}]_m$; or
 - (b) $[\Sigma]_n \text{KIX}^3\text{X}^4\text{X}^5\text{X}^6\text{KX}^8\text{X}^9[\text{U}]_m$

5 wherein

X^3 , X^4 and X^8 , are independently an amino acid selected from A, F, I, L, M, V, W and Y;

each of X^5 , X^6 and X^9 is any amino acid;

$[\Sigma]$ is any amino acid, and n is an integer from 0 to 15;

10 $[\text{U}]$ is any amino acid, and m is an integer from 0 to 15.
2. The peptide of claim 1, wherein the amino acid sequence is:

$$[\Sigma]_n \text{KIYVX}^5\text{X}^6\text{KFX}^9[\text{U}]_m.$$
- 15 3. The peptide of claims 1-2, wherein

X^5 is H or A;

X^6 is T or A; and

X^9 is V or A.
- 20 4. The peptide of claims 1-3, wherein the amino acid is:

$[\Sigma]_n \text{KIYVX}^5\text{TKFV}[\text{U}]_m$ and X^5 is H or A;

$[\Sigma]_n \text{KIYVHX}^6\text{KFV}[\text{U}]_m$ and X^6 is H or A;

$[\Sigma]_n \text{KIYVHTKFX}^9[\text{U}]_m$ and X^9 is H or A.
- 25 5. The peptide of claims 1-4, wherein the amino acid sequence is:

$$[\Sigma]_n \text{KIYVHTKFV}[\text{U}]_m.$$
6. The peptide of claims 1-5, wherein

$[\Sigma]_n$ is selected from ETRRLLSVD, GPLLSVD, LLSVD, SVD, D, P, PP, PPP, PPPP, PPPPP, VP, VPP, VPPP, VPPPP, F, FV, FVP, FVPP and $[U]_m$ is selected from P, PP, PPP, PPPP, PPPPP, PN, PPN, PPPN, PPPPN.

5

7. The peptide of claims 1-6, wherein the amino acid is selected from:

SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:26.

10

8. The peptide of claims 1-7, which is a synthetic peptide.

9. An isolated nucleic acid molecule encoding the peptide of claims 1-8.

15

10. A vector comprising the nucleic acid molecule of claim 9.

11. A host cell comprising the vector of claim 10 or a nucleic acid of claim 9.

20 12. A method of producing a peptide comprising cultivating the cell of claim 11 and expressing the peptide.

13. A composition comprising at least one peptide of claims 1-8, the nucleic acid molecule of claim 9 or the vector of claim 10.

25

14. The composition of claim 13, wherein the composition is a pharmaceutical composition.

30 15. The composition of claim 14, further comprising a pharmaceutically acceptable excipient, diluent and/or carrier.

16. The peptide of claims 1-8 for use in medicine.
17. The peptide of claim 16, for use as an anticoagulant.
- 5 18. Use of at least one peptide of claims 1-8 for the preparation of an anticoagulant.
- 10 19. A method for inhibiting the assembly of prothrombinase complex in a subject comprising administering to a subject at least one peptide of claims 1-8 or a composition of claims 14-15.
- 15 20. A method for the prevention and/or treatment of formation of intravascular blood clots in a subject comprising administering to the subject at least one peptide of claims 1-8 or a composition of claims 14-15.
21. The method of claim 20, wherein the method is for the prevention and/or treatment of ischemic events.
- 20 22. The method of claim 21, wherein the ischemic event is coronary syndrome, myocardial infarction, stroke, venous thromboembolism and/or peripheral arterial occlusion.
23. The method of claims 20-22, wherein the subject is a mammal.
- 25 24. The method of claim 23, wherein the mammal is human.
25. A method for identifying anticoagulant peptides, comprising:
- 30 - providing at least one peptide;

- determining the minimum interaction site (MIS) between the at least one peptide and FVa;
- contacting *in vitro* the peptide comprising the MIS with the prothrombinase complex; and
- 5 - determining the ability of the peptide in inhibiting the assembly of the prothrombinase complex.

26. The method of claim 25, further comprising the step of contacting the peptide comprising the MIS to plasma, and determining the ability of the peptide to interfere with the blood coagulation cascade in plasma.

27. The method of claims 25-26, further comprising selecting the peptide comprising the MIS, modifying at least one amino acid residue inside and/or outside the MIS, and determining the ability of the modified peptide to interfere with the assembly of the prothrombinase complex and/or with the blood coagulation cascade in plasma.

28. The method of claim 27, further comprising the step of isolating or synthesising the modified peptide capable of interfering with the assembly of the prothrombinase complex and/or with the blood coagulation cascade in plasma.

29. The method of claims 25-28, wherein the provided at least one peptide comprising the MIS is obtained from fragmentation of a protein.

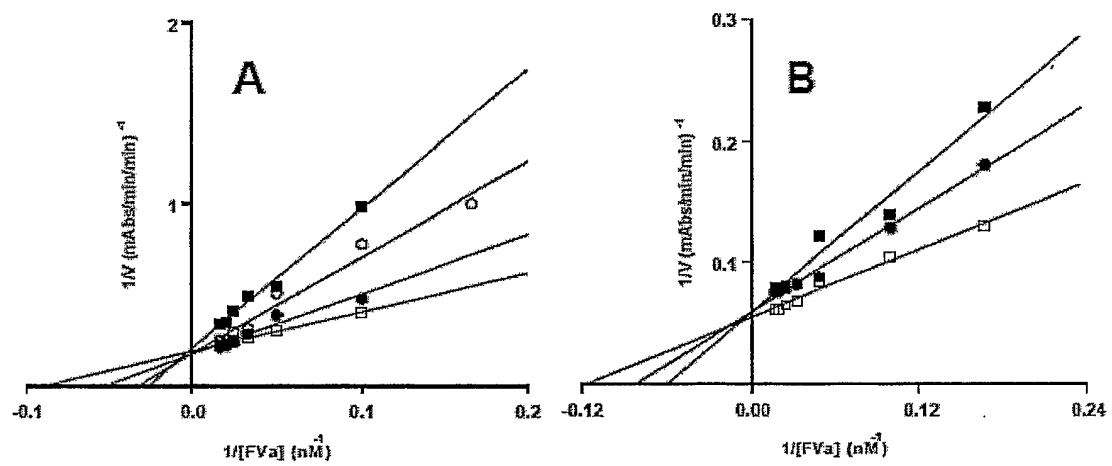
30. The method of claim 29, wherein the protein is trocarin.

31. The method of claims 29-30, wherein the peptide is at least one peptide of claims 1-8.

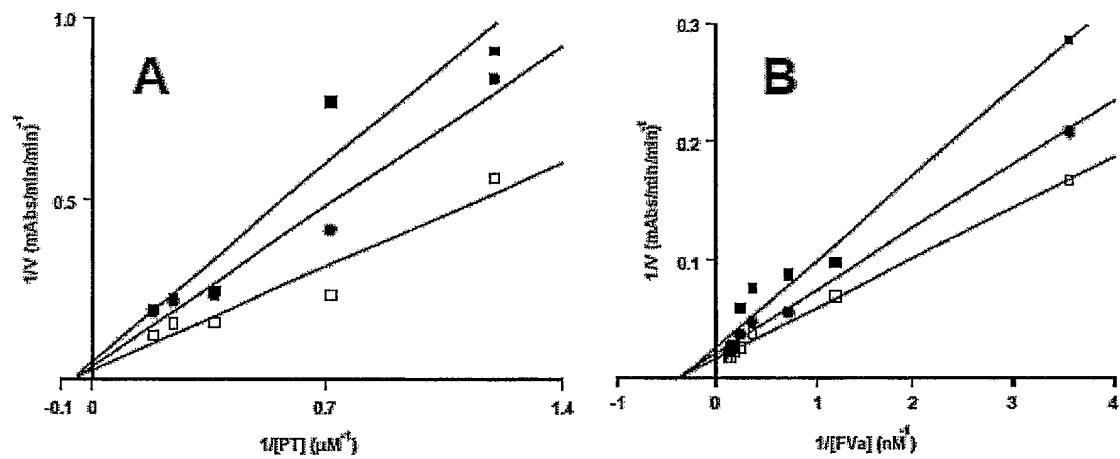
HEAVY CHAIN SEQUENCES

FIGURE 1

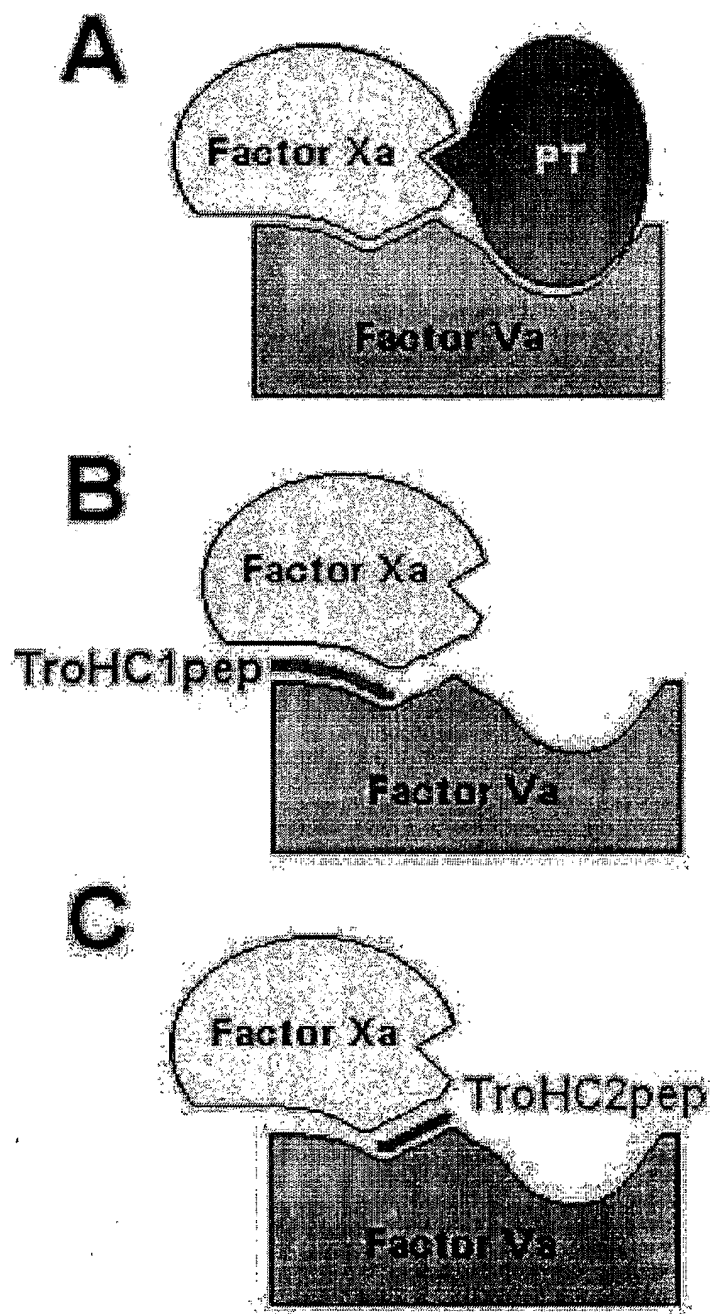
2/6

**FIGURE 2(A, B)**

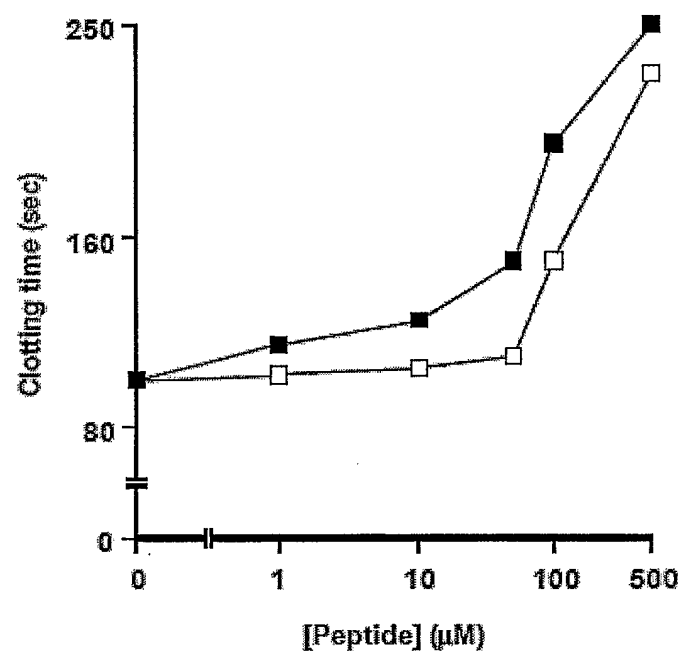
3/6

**FIGURE 3 (A, B)**

4/6

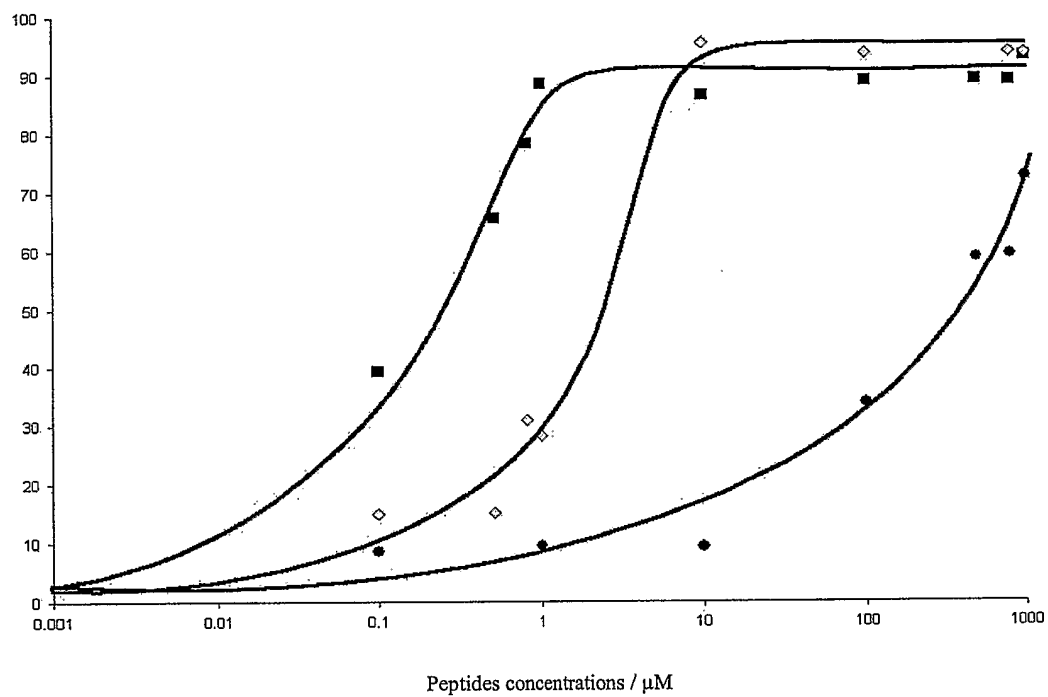
**FIGURE 4 (A, B, C)**

5/6

**FIGURE 5**

6/6

Percentage of inhibition

**FIGURE 6**

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Phe Val Pro Pro Asn
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Asn

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<211> 13

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5

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Cys Leu Xaa Xaa Ala Cys Ser Leu Xaa Xaa Ala Arg Xaa Val Phe Xaa
20 25 30

Asp Ala Xaa Gln Thr Asp Xaa Phe Trp Ser Lys Tyr Lys Asp Gly Asp
35 40 45

Gln Cys Glu Gly His Pro Cys Leu Asn Gln Gly His Cys Lys Asp Gly
50 55 60

Ile Gly Asp Tyr Thr Cys Thr Cys Ala Glu Gly Phe Glu Gly Lys Asn
65 70 75 80

Cys Glu Phe Ser Thr Arg Glu Ile Cys Ser Leu Asp Asn Gly Gly Cys
85 90 95

Asp Gln Phe Cys Arg Glu Glu Arg Ser Glu Val Arg Cys Ser Cys Ala
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His Gly Tyr Val Leu Gly Asp Asp Ser Lys Ser Cys Val Ser Thr Glu
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			20					25					30		

Asp	Ser	Asp	Lys	Thr	Asn	Xaa	Phe	Trp	Asn	Lys	Tyr	Lys	Asp	Gly	Asp
		35					40					45			

Gln	Cys	Glu	Thr	Ser	Pro	Cys	Gln	Asn	Gln	Gly	Lys	Cys	Lys	Asp	Gly
	50					55					60				

Leu	Gly	Glu	Tyr	Thr	Cys	Thr	Cys	Leu	Glu	Gly	Phe	Glu	Gly	Lys	Asn
65					70					75					80

Cys	Glu	Leu	Phe	Thr	Arg	Lys	Leu	Cys	Ser	Leu	Asp	Asn	Gly	Asp	Cys
				85				90						95	

Asp Gln Phe Cys His Glu Glu Gln Asn Ser Val Val Cys Ser Cys Ala
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Asp Asn Xaa Lys Thr Glu Thr Phe Trp Asn Val Tyr Val Asp Gly Asp
35 40 45

Gln Cys Ser Ser Asn Pro Cys His Tyr Arg Gly Thr Cys Lys Asp Gly
50 55 60

Ile Gly Ser Tyr Thr Cys Thr Cys Leu Pro Asn Tyr Glu Gly Lys Asn
65 70 75 80

Cys Glu Lys Val Leu Tyr Gln Ser Cys Arg Val Asp Asn Gly Asn Cys
85 90 95

Trp His Phe Cys Lys Arg Val Gln Ser Glu Thr Gln Cys Ser Cys Ala
100 105 110

Glu Ser Tyr Arg Leu Gly Val Asp Gly His Ser Cys Val Ala Glu Gly
115 120 125

Asp Phe Ser Cys Gly Arg Asn Ile Lys Ala Arg Asn Lys
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 20 25 30

Asn Glu Phe Tyr Val Leu Thr Ala Ala His Cys Leu His Gln Ala Lys
 35 40 45

Arg Phe Thr Val Arg Val Gly Asp Arg Asn Thr Glu Gln Glu Glu Gly
 50 55 60

Asn Glu Met Ala His Glu Val Glu Met Thr Val Lys His Ser Arg Phe
 65 70 75 80

Val Lys Glu Thr Tyr Asp Phe Asp Ile Ala Val Leu Arg Leu Lys Thr
 85 90 95

Pro Ile Arg Phe Arg Arg Asn Val Ala Pro Ala Cys Leu Pro Glu Lys
 100 105 110

Asp Trp Ala Glu Ala Thr Leu Met Thr Gln Lys Thr Gly Ile Val Ser
 115 120 125

Gly Phe Gly Arg Thr His Glu Lys Gly Arg Leu Ser Ser Thr Leu Lys
 130 135 140

Met Leu Glu Val Pro Tyr Val Asp Arg Ser Thr Cys Lys Leu Ser Ser
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Ser Phe Thr Ile Thr Pro Asn Met Phe Cys Ala Gly Tyr Asp Thr Gln
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Pro Glu Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro His Val Thr Arg
 180 185 190

Phe Lys Asp Thr Tyr Phe Val Thr Gly Ile Val Ser Trp Gly Glu Gly
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Arg Phe Lys Val Arg Val Gly Asp Arg Asn Thr Glu Gln Glu Glu Gly
50 55 60

Gly Glu Ala Val His Glu Val Glu Val Val Ile Lys His Asn Arg Phe
65 70 75 80

Thr Lys Glu Thr Tyr Asp Phe Asp Ile Ala Val Leu Arg Leu Lys Thr
85 90 95

Pro Ile Thr Phe Arg Met Asn Val Ala Pro Ala Cys Leu Pro Glu Arg
100 105 110

Asp Trp Ala Glu Ser Thr Leu Met Thr Gln Lys Thr Gly Ile Val Ser
115 120 125

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130 135 140

Met Leu Glu Val Pro Tyr Val Asp Arg Asn Ser Cys Lys Leu Ser Ser
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Cys Ala Arg Lys Gly Lys Tyr Gly Ile Tyr Thr Lys Val Thr Ala Phe
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 20 25 30

Ser Pro Ile His Val Leu Thr Ala Ala His Cys Ile Asn Gln Thr Lys
 35 40 45

Ser Val Lys Glu Thr Arg Arg Leu Leu Ser Val Asp Lys Ile Tyr Val
 50 55 60

His Thr Lys Phe Val Pro Pro Asn Tyr Tyr Tyr Val His Gln Asn Phe
 65 70 75 80

Asp Arg Val Ala Tyr Asp Tyr Asp Ile Ala Ile Ile Arg Met Lys Thr
 85 90 95

Pro Ile Gln Phe Ser Glu Asn Val Val Pro Ala Cys Leu Pro Thr Ala
 100 105 110

Asp Phe Ala Asn Glu Val Leu Met Lys Gln Asp Ser Gly Ile Val Ser
 115 120 125

Gly Phe Gly Arg Ile Gln Phe Lys Gln Pro Thr Ser Asn Thr Leu Lys
130 135 140

Val Ile Thr Val Pro Tyr Val Asp Arg His Thr Cys Met Leu Ser Ser
145 150 155 160

Asp Phe Arg Ile Thr Gln Asn Met Phe Cys Ala Gly Tyr Asp Thr Leu
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Pro Gln Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro His Ile Thr Ala
180 185 190

Tyr Arg Asp Thr His Phe Ile Thr Gly Ile Ile Ser Trp Gly Glu Gly
195 200 205

Cys Ala Arg Lys Gly Lys Tyr Gly Val Tyr Thr Lys Val Ser Lys Phe
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Ile Pro Trp Ile Lys Lys Ile Met Ser Leu Lys
225 230 235

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2004/000365

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: C07K 7/06, 7/08, 14/46, A61K 38/08, 38/10, 38/17, A61P 7/02, 9/10

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

ELECTRONIC DATA BASE SEARCHED: see below

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

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

STN search of claim 1 in Chemicals Abstracts and Patents

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Methe, B.A. et al, "Genome of <i>Geobacter sulfurreducens</i> : Metal Reduction in Subsurface Environments", Science, Vol. 302, pp. 1967-1969, 12 December 2003 SEQ. : MYIEGHIS KI ILIYKWI CKY DMEFSLFADP disclosed.	1(b), 8-16
X	Deng, W. et al, "Comparative Genomics of <i>Salmonella enterica</i> Serovar Typhi Strains Ty2 and CT18", J. of Bacteriology, Vol. 185, No. 7, pp. 2330-2337, April 2003 SEQ. : MNLV KI IYEL KLP KRFLQKN KYI disclosed.	1(b), 8-16
X	Parkhill, J. et al, "Complete genome sequence of a multiple drug resistant <i>Salmonella enterica</i> serovar Typhi CT18", Nature, Vol. 413, pp.848-852, 25 October 2001 SEQ. : MNLV KI IYEL KLP KRFLQKN KYI disclosed.	1(b), 8-16



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:		
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Date of the actual completion of the international search
27 January 2005

Date of mailing of the international search report 2 FEB 2005

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2004/000365

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Farquhar, M. et al, "Chimeric mastoparans: biological probes and designer secretagogues", Peptides: The wave of the future, Proceedings of the second international and the seventeenth american peptide peptide symposium, San Diego, pp. 750-751, June 9-14, 2001 MP-MP in Table 1 : INLKALAALA K KI LINLKAL AALAKKIL	1(b), 8-16
X	Mette, M. F. et al, "Endogenous viral sequences and their potential contribution to heritable virus resistance in plants", The EMBO Journal, Vol. 21, No. 3, pp.461-469, 2002 SEQ. : MP KI YILAKI I VEGYYNRY Y TPMVDT disclosed.	1(b), 8-16
X	Andrews, C. D. et al, "Stabilizing Nonpolar/polar side-chain interactions in the alpha-helix", PROTEINS: structure, function and genetics, Vol. 45, No. 4, pp.449-455, 2001 IKc in Table II: A KI AAAAKIA AAAKAKAGY	1(b), 3, 8-16
X	Casjens, S. et al, "A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete Borrelia burgdorferi", Molecular Microbiology, Vol. 35, No. 3, pp.490-516, 2000 SEQ. : MFITIKLIL YIRF KI FLKK K FV INIL VKI NINQNKKI disclosed	1(b), 8-16
X	Oh, H. et al, "Activities of synthetic hybrid peptides against anaerobic bacteria: aspects of methodology and stability", Antimicrobial agents and chemotherapy, Vol. 44, No. 1, pp. 68-72, January 2000 Camel 48, 48D : KWKL GK KI LAVL KVL in table 1.	1(b), 8-16
X	Astori. M et al, "Antibodies to the C-terminal dipeptide of mouse mammary tumour virus [MMTV(SW)] superantigen effectively inhibit T-cell activation in vivo", J. of General Virology, Vol. 79, pp. 57-60, 1998 SEQ. : KI LYNMKYT H GGRVGFDPF disclosed	1(b), 8-16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2004/000365

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Mee, P. M et al, "Design of active analogues of a 15-residue peptide using D-optimal design, QSAR and a combinatorial search algorithm", J. of Peptide Research, Vol. 49, pp. 89-102, 1997 Tables 2-3 disclose: WKLGK KI LAV LKVL; KWKLFLH KI LA VLKVL; KWHLFL KI LA VLKVL; LWHLFL KI LA VLKVL; GWKLFL KI LA VLKVL.	1(b), 8-16
X	Oppi, C. et al, "Attenuation of GTPase activity of recombinant G₀alpha by peptides representing sequence permutations of mastoparan", PNAS, Vol. 89, pp. 8268-8272, September 1992 Table 3 has peptide 2: L KI ALNLKAL IAAK	1(b), 8-16
X	WO 1998/040401 A2 (MICROLOGIX BIOTECH INC.) 17 September 1998 See claim 15 fourth sequence: ILRWPWWPWR R KI MILKKAGS	1(b), 8-16
X	EP 0 538 942 A2 (ISTITUTO GUIDO DONEGANI S.p.A) 28 April 1993 Page 2 column 2 line 13: KI LINLKALAALAK	1(b), 8-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2004/000365

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	1998 40401			AT	218579	AU	43279/97
		AU	63177/98	AU	66047/98	CA	2263799
		CA	2282807	DE	69713112	EP	0925308
		EP	0966481	EP	1174439	ES	2178000
		HK	1021824	JP	2001500477	JP	2002544759
		US	6062889	US	6180604	US	6503881
		US	6538106	US	2002035061	US	2003232750
		US	2004009910	WO	1998 07745	WO	1998 33138
EP	0538942			IT	1251961	IT	MI912770
		JP	5213993	US	5519116		
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
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