NOVEL ANTI-CLOTTING MOLECULES AND USES THEREFOR

FIELD OF THE INVENTION

The present invention relates generally to peptides and to derivatives, homologues, analogues and mimetics thereof capable of inducing or facilitating inhibition of blood clot formation and more particularly platelet aggregation. The present invention also contemplates genetic sequences encoding said peptides and derivatives, homologues, analogues and mimetics thereof. The molecules of the present invention are useful *inter alia* in a range of therapeutic and prophylactic applications.

BACKGROUND OF THE INVENTION

Platelets are essential elements involved in haemostatic events. Their role in hemostasis involves two distinct activities: (1) adhesion - the interaction of platelets with subendothelial connective tissue and (2) aggregation - platelet to platelet adhesion. Abnormal functioning of platelets i.e. abnormal adhesion and/or aggregation is thought to contribute to a variety of pathophysiological conditions such as thrombosis, atherosclerosis, myocardial infarction, stroke and pulmonary embolism.

Several inhibitors of platelet activity have been identified. These include the enzymes ADPase, fibrinogenase and phospholipase A_2 (referred to herein as "PLA_2") and the nonenzymatic proteins such as disintegrins and mambin. The mechanisms by which these inhibitors function varies. In the case of nonenzymatic proteins the mechanism appears to be simpler. Mambin and disintegrins that contain Arg-Gly-Asp (RGD) sequence exert their action by competitively blocking fibrinogen binding to platelet glycoprotein IIb/IIIA. Ca^{2+}-dependent type lectin-related proteins exhibit their effects on platelet agglutination and aggregation by specifically binding to platelet glycoprotein Ib. Among the enzymes, some proteinases have been studied for their inhibition of platelet aggregation effects. The inhibitory activity of fibrinogenase was initially thought to be due to fibrinogen degradation, since fibrinogen is involved in the final stages of platelet aggregation. Subsequent studies have shown that fibrinogenases can inhibit platelet aggregation independent of fibrinogen

degradation. Recent studies have indicated that some of the metalloproteinases inhibit platelet aggregation by cleaving glycoprotein Ib which is a receptor for von-Willebrand factor. The ADPase inhibit platelet aggregation by hydrolysis of ADP to form AMP, an inhibitor of platelet aggregation. Thus these enzymes physically destroy either ligand and/or receptor due to their inherent enzymatic activity. PLA_2 enzymes, on the other hand, interfere in platelet aggregation by specific mechanisms. They have been divided or grouped into three distinct classes; class A PLA_2s initiate platelet aggregation, class B PLA_2s inhibit platelet aggregation and class C PLA_2s both initiate as well as inhibit platelet aggregation. However, the structure-function relationship and molecular mechanism of platelet inhibition caused by PLA_2s is not understood.

In work leading up to the present invention, the inventors have identified the amino acid sites of the native PLA_2 protein which inhibit platelet aggregation. This has lead to the development of peptides which inhibit platelet aggregation.

SUMMARY OF THE INVENTION

One aspect of the present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acids in a phospholipase and which peptide is capable of inducing or facilitating the inhibition of blood clotting.

Another aspect of the present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acid in A-PLA_2 and which peptide is capable of inducing or facilitating the inhibition of blood clotting.

Yet another aspect of the present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acids in A-APL_2 from snake venom and which peptide is capable of inducing or facilitating the inhibition of blood clotting.

Still yet another aspect of the present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acids in A-PLA_2 from snake venom and which peptide is capable of inducing or facilitating the inhibition of platelet aggregation.

A further aspect of the present invention provides a peptide comprising a sequence of amino acids from about 2 to about 50 residues having at least about 50% similarity to a sequence of amino acids from A-PLA_2 isolatable from *O. hannah* snake venom wherein said peptide is capable of inducing or facilitating the inhibition of platelet aggregation or a derivative, homologue, analogue or mimetic of said peptide.

Still a further aspect of the present invention provides a peptide having the following characteristics:

(i) Induces or facilitates inhibition of platelet aggregation;
(ii) Comprises from about 2 to about 50 amino acid residues;
(iii) Its clotting ability is not due to its enzymatic activity;

or a derivative, homologue, analogue or mimetic of said peptide.

The present invention also provides a peptide comprising the amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1. Most preferably, the peptide comprises the amino acid sequences:

S S I M D S P Y (SEQ ID NO: 2);
L P A A S S I M D S P Y (SEQ ID NO: 3); and
A P S S I M D S P Y (SEQ ID NO: 4);

or derivatives, homologues, analogues or mimetics thereof.

Another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a peptide as hereinbefore defined or a derivative or homologue thereof.

Yet another aspect of the present invention provides a nucleic acid molecule which encodes or is complementary to a sequence which encodes amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1, said peptide capable of inducing or facilitating platelet aggregation.

Still yet another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence selected from:

S S I M D S P Y (SEQ ID NO: 2);
L P A A S S I M D S P Y (SEQ ID NO: 3); and
A P S S I M D S P Y (SEQ ID NO: 4).

The present invention further extends to the use of the subject peptides in the manufacture of a medicament for inducing or facilitating inhibition of platelet aggregation.

In yet another further aspect, the present invention provides peptides which comprise the amino acid sequence

$[X_1]_n [X_2]_n [A]_n [X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid, and n is 0 or 1.

Particularly preferred peptides are selected from:

S S I M D S P Y (SEQ ID NO: 2);
L P A A S S I M D S P Y (SEQ ID NO: 3); and
A P S S I M D S Y P (SEQ ID NO: 4).

Yet another aspect of the present invention provides an agent useful for inducing or facilitating platelet aggregation comprising a peptide as hereinbefore defined.

Another aspect of the present invention contemplates a method of inhibiting platelet aggregation in a subject said method comprising administering to said subject an anti-clotting effective amount of a peptide hereinbefore defined for a time and under conditions sufficient to inhibit platelet aggregation.

Still another aspect of the present invention provides a composition for use in inhibiting or facilitating inhibition of platelet aggregation comprising a peptide as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents.

Still yet another aspect of the present invention is directed to antibodies to the subject peptides and their derivatives, homologues, analogues and mimetics.

The present invention also extends to a method for detecting a subject peptide in a biological sample from a subject or culture supernatant flow or other source said method comprising contacting said biological sample with an antibody specific for said peptide or its derivative, homologue, analogue or mimetic for a time and under conditions sufficient for an antibody-peptide complex to form, and then detecting said complex.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined immediately before the bibliography.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Single and three letter abbreviations used throughout the specification are defined in Table 1.

TABLE 1

Single and three letter amino acid abbreviations

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the inhibition of collagen-induced platelet aggregation by OHV A-PLA_2. Whole blood (A) and PRP (B) was preincubated with various concentrations of OHV A-PLA_2 at 37°C for 2 min and then collagen (2 μg/ml) was added to initiate platelet activation.

Figure 2 is a graphical representation of the effects of OHV A-PLA_2 on human platelet aggregation induced by various agonists. Whole blood (A) and PRP (B) was preincubated with various concentrations of OHV A-PLA_2 at 37°C for 2 min and then the inducer ADP (10 μM●), collagen (2 μg/ml O) and arachidonic acid (0.5 Mm□) was added. Percent inhibitions are presented as mean ± SEM (n=6).

Figure 3 is a graphical representation of the effects of OHV A-PLA_2 on platelet aggregation and ATP release. ATP release was measured by the luciferin-luciferase reaction using Chromo-Lume which was added to PRP before application of collagen (2 μg/ml) (A), arachidonic acid (0.5 μM) (B). Values are presented as mean ± SEM (n=6).

Figure 4 is a photographic representation of the effects of OHV A-PLA_2 on platelet aggregation and ATP release. ATP release was measured by the luciferin-luciferase reaction using Chromo-Lume which was added to PRP before application of ADP (10 μM). Values are presented as mean ± SEM (n=6).

Figure 5 is a photographic representation of scanning electron micrographs of human platelet. (A) A control platelet with typical discoid form, the surface appeared regular and pseudopodium were not seen. Mag. x 42,000 (B) A platelet from the sample incubated with OHV A-PLA_2 (350 nM) for 5 min. The cell appeared irregular sphere, surface ruffling, and with many projections. Mag. x 31,500.

Figure 6 is a photographic representation of the effect of OHV A-PLA_2 on ultrastructure of platelets. (A) The control platelet sectioning in the equatorial plane. Circumferential band of microtubules (MT) supporting the lentiform appearance. Granules (G), dense bodies (DB), mitochondria (M), glycogen (Gly). Channels of the open canalicular system (CS) and dense tubular system (DTS), small membrane complex (MC) formed by their interaction. Mag. x 38,500. (B) The platelet from the sample incubated with OHV A-PLA_2 (360 nM) for 5 min. The cell appeared irregular with many projections (P). The canalicular system was widened and disrupted. There was an increase of the number of vacuoles in platelet. The dense tubular system was fragmented and redistributed throughout the cytoplasm (arrows). Mag. x 29,750.

Figure 7 is a photographic representation of the effect of OHV A-PLA_2 on platelets exposed to ADP. (A) Aggregation: platelets from PRP stirred on an aggregometer with ADP (10 μM) and fixed after reaching the plateau of maximum aggregation. Surface contours of the activated cells are irregular and molded to each other. Organelles are concentrated toward cell centres and contents of most organelles have been discharged. Mag. x 38,500. (B) Platelets were treated with OHV A-PLA_2 (360 nM) at 37°C for 2 min prior to the addition of ADP (10 μM) and fixed after stirring for 5 min. The platelets appeared spherical with organelle centralization, but long pseudopodia and degranulation were hardly found. The platelets failed to aggregate. Mag. x 29,750.

Figure 8 is a graphical representation of the effects of alkylation of OHV A-PLA_2 on its antiplatelet activity in PRP. PRP was incubated with various concentrations of OHV A-PLA_2 or the p-BPB-modified OHV A-PLA_2 at 37°C for 2 min then the inducer ADP (10 μM) was added to induce aggregation. Percent inhibitions are presented as mean ± SEM (n=4).

Figure 9 is a graphical representation of effects of the synthetic peptide-I on human platelet aggregation induced by collagen (2 μg/ml O). Whole blood was preincubated with various concentrations of the synthetic peptide-I at 37°C for 2 min and then the inducer collagen (2 μg/ml O) was added. Percent inhibitions are presented as mean ± SEM (n=3).

Figure 10 is a graphical representation of effects of the synthetic peptide II on human platelet aggregation induced by various agonists. Whole blood was preincubated with various concentrations of the synthetic peptide-II at 37°C for 2 min and then the inducer collagen (2 μg/ml O) and ADP (10 μM●) was added. Percent inhibitions are presented as mean ± SEM (n=4).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acids in a phospholipase and which peptide is capable of inducing or facilitating the inhibition of blood clotting. Said peptide is conveniently in isolated or purified form.

Reference hereinafter to "phospholipase" should be read as including reference to all types of phospholipases including, by way of example, A phospholipase A_2. Preferably said phospholipase is A phospholipase A_2 (referred to herein as "A-PLA_2"). Reference to A-PLA_2 should be understood as a reference to all forms of phospholipase.

Accordingly, the present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acid in A-PLA_2 and which peptide is capable of inducing or facilitating the inhibition of blood clotting.

The present invention is exemplified with respect to peptides based on A-PLA_2 isolated from King Cobra (*Ophiohagus hannah*) venom and is particularly exemplified from *O. hannah* venom. However, the present invention extends to any phospholipase and more particularly phospholipases from the venom of *O. hannah* as well as from the venom of any other snake.

Preferred snakes include snakes from the family *Colubridae, Elapidae, Viperidae* and *Crotalidae* such as species of the genera *Naja, Dendroaspis, Bungarus, Pseudechis, Ophiophagus* and *Hemachatus*. Particularly preferred snakes are from the family *Elapidae* such as but not limited to King cobra *(Ophiohagus hannah);* True cobras *(Naja* spp); Asian or Indian cobra *(N. naja);* Egyptian cobra *(N. haje);* Spitting cobra *(N. nigricolli);* Black-lipped cobra *(N. malenoleuca);* Cape cobra *(N. nivea);* Gold's tree cobra *(Pseudohaje goldii);* Desert black snakes *(Walterinnesia* spp); Shield-nose snakes *(Aspidelaps* spp); Water cobras or water snakes *(Boulengerina* spp); Black mamba *(Dendroaspis polylepis);* Mamba *(D. angusticeps);* Kraits snake *(Bungarus* spp); Oriental coral snakes *(Calliophis* spp); Long-glanded coral snakes *(Maticora* spp); American coral snakes *(Micurus* spp); Southern coral

snake *(M. frontalis);* Eastern coral snake or Harlequin snake *(M. fulvius);* Western coral snake *(Micruroides* spp); Arizona coral snake *(M. euryxanthus);* Death adder *(Acanthophis antarcticus);* Australian tiger snakes *(Notechis* spp); and Australian copperhead *(Denisomia* spp).

The present invention also extends to similar or equivalent peptides isolated from any other reptilian or non-reptilian source.

According to this most preferred embodiment, the present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acids in A-APL_2 from snake venom and which peptide is capable of inducing or facilitating the inhibition of blood clotting.

The term "blood clotting" is used in its broadest sense to include reference to any one or more steps of the blood clotting cascade such as, but not limited to, platelet aggregation. It should also be understood to refer to the aggregation of any one or more components of the blood such as, but not limited to, platelets. Reference to "inhibition" of said clotting is a reference to complete or partial prevention of blood clot formation. For example, the proteins of the present invention may completely prevent platelet aggregation or they may merely reduce the extent of platelet aggregation. Preferably said blood clotting is platelet aggregation. Reference hereinafter to platelet aggregation should be understood as a reference to all forms of blood clotting.

According to this most preferred embodiment the present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 50% similar to a sequence of amino acids in A-PLA_2 from snake venom and which peptide is capable of inducing or facilitating the inhibition of platelet aggregation.

The subject of the platelet aggregation is generally an animal or bird such as but not limited to a human primate livestock animal (e.g. sheep, cow, horse, donkey, pig) companion

animal (e.g. dog, cat) laboratory test animal (e.g. mouse, rat, guinea pig, rabbit, hampster) captive wild animal (e.g. deer, fox) caged bird (e.g. parrot) and poultry bird (e.g. chicken, duck, pheasant, goose, turkey). Preferably the subject is a human or primate. Most preferably, the subject is a human.

The term "peptide" encompasses a proteinaceous molecule comprising from about 2 amino acid residues to about 50 amino acid residues. Preferably, the peptide comprises from about 3 amino acid residues to about 40 amino acid residues. Even more preferably, the peptide comprises from about 4 amino acid residues to about 30 amino acid residues. Particularly preferred embodiments include peptides comprising from about 4 amino acid residues to about 20 amino acid residues such as from 4 to 12 and 4 to 10 amino acid residues. Although the amino acid sequence of the anti-clotting peptides is derived from or based on A-PLA_2 from snake venom, it does not extend to the full length A-PLA_2 but rather only to a part thereof.

The peptide may be glycosylated or unglycosylated and/or may contain a range of other molecules fused, linked, bound or otherwise associated to the peptide such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. Reference hereinafter to a "peptide" includes a peptide comprising a sequence of amino acids as well as a peptide associated with other molecules such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. For the purposes of the present description, a molecule comprising a sequence of at least about 50 amino acids is regarded as a peptide.

The peptides of the present invention may be produced by chemical synthetic techniques or may be produced by recombinant DNA technology as discussed further below. The peptides may also be fragments of larger phospholipase molecules and in particular, phospholipase molecules from snake venom. The fragments may be naturally occurring fragments or generated by the action of proteases, peptidases, amidases, lysins or other enzymes as well as by sonic disruption, heat, chemical disruption and/or shearing.

The amino acid sequence of the peptides of the present invention are considered to be derived from or based on a snake venom phospholipase and, more particularly, A-PLA_2.

This is not to imply, however, that the preferred peptides are necessarily derivatives of naturally occurring snake venom phospholipase since particularly useful peptides are, in accordance with the present invention, chemically synthesised based on the amino acid sequence of phospholipase molecules from sources including, but not limited to, snake venom.

Reference to a peptide "inducing or facilitating" the inhibition of platelet aggregation should be understood as a reference to the inhibition of platelet aggregation by both direct and indirect mechanisms. For example, said peptide may interact directly with platelets to prevent their aggregation or, alternatively, may act indirectly to prevent aggregation by, for example, inhibiting one or more of the biological signals which result in the induction of platelet aggregation.

According to a particularly preferred embodiment, there is provided a peptide comprising a sequence of amino acids from about 2 to about 50 residues having at least about 50% similarity to a sequence of amino acids from A-PLA_2 isolatable from *O. hannah* snake venom wherein said peptide is capable of inducing or facilitating the inhibition of platelet aggregation or a derivative, homologue, analogue or mimetic of said peptide.

The percentage similarity may be greater than 60% such as at least about 70% or at least about 80% or at least about 90% or higher.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are neverthless related to each other at the strucutural, functional, biochemical and/or conformational levels.

Preferred snakes include snakes from the family *Colubridae, Elapidae, Viperidae* and *Crotalidae* such as species of the genera *Naja, Dendroaspis, Bungarus, Pseudechis, Ophiophagus* and *Hemachatus*. Particularly preferred snakes are from the family *Elapidae* such as but not limited to King cobra *(Ophiohagus hannah);* True cobras *(Naja* spp); Asian or Indian cobra *(N. naja);* Egyptian cobra *(N. haje);* Spitting cobra *(N. nigricolli);* Black-lipped cobra *(N. malenoleuca);* Cape cobra *(N. nivea);* Gold's tree cobra *(Pseudohaje goldii);* Desert black snakes *(Walterinnesia* spp); Shield-nose snakes *(Aspidelaps* spp); Water cobras or water snakes *(Boulengerina* spp); Black mamba *(Dendroaspis polylepis);* Mamba *(D. angusticeps);* Kraits snake *(Bungarus* spp); Oriental coral snakes *(Calliophis* spp); Long-glanded coral snakes *(Maticora* spp); American coral snakes *(Micurus* spp); Southern coral snake *(M. frontalis);* Eastern coral snake or Harlequin snake *(M. fulvius);* Western coral snake *(Micruroides* spp); Arizona coral snake *(M. euryxanthus);* Death adder *(Acanthophis antarcticus);* Australian tiger snakes *(Notechis* spp); and Australian copperhead *(Denisomia* spp).

Without limiting the present invention to any one theory or mode of action the anti-clotting effects of A-PLA_2 are independent of its enzymatic activity since chemical modification of said enzyme with *p*-bromophenacyl bromide results in almost total loss of enzymatic activity but only marginal reduction of anti-clotting activity. A-PLA_2 induces morphological changes in both the shape and subcellular structure of platelets. Disorders of the open canalicular and dense tubular systems are observed indicating a change in the cell sarcoplasmic reticulum. The disorder of the cytoskeleton and sarcoplasmic reticulum lead to the loss of released reaction which is thought to play an important role in inhibiting platelet secretion and aggregation. Thus, A-PLA_2 is likely to inhibit platelet aggregation by interfering in platelet secretion or in any specific step involved in the secretion process.

The inventors have determined that A-PLA_2 contains the same loop of amino acid residues (62-66) which are found in pancreatic PLA_2S. This loop is positioned between two proline residues (Pro 59 and Pro 68) and contains several hydrophilic amino acids (Ser and Asp). This region exhibits a high degree of conformational flexibility and is positioned on the surface of the molecule. Peptide sequences based on the 62-66 loop residue sequence have been shown to inhibit platelet aggregation.

Accordingly, another preferred embodiment of the present invention provides a peptide having the following characteristics:

(i) Induces or facilitates inhibition of platelet aggregation;
(ii) Comprises from about 2 to about 50 amino acid residues;
(iii) Its clotting ability is not due to its enzymatic activity;

or a derivative, homologue, analogue or mimetic of said peptide.

A particularly preferred peptide of the present invention comprises the amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1. Most preferably, the peptide comprises the amino acid sequences:

S S I M D S P Y (SEQ ID NO: 2);
L P A A S S I M D S P Y (SEQ ID NO: 3); and
A P S S I M D S P Y (SEQ ID NO: 4);

or derivatives, homologues, analogues or mimetics thereof.

The peptides of the present invention are also useful in the treatment and prophylaxis of heart conditions and strokes. According to this embodiment, there is provided a method for the treatment or prophylaxis of a disease condition involving clotting in a vascular system in an animal such as but not limited to a heart condition (eg. heart attack) or a stroke, said method comprising administering to said animal, a platelet aggregation inhibition effective amount of a peptide having the following characteristics:

(i) Induces or facilitates inhibition of platelet aggregation;
(ii) Comprises from about 2 to about 50 amino acid residues;

(iii) Its clotting ability is not due to its enzymatic activity;

or a derivative, homologue, analogue or mimetic of said peptide.

More particularly, the present invention provides a method for the treatment or prophylaxis of a disease condition involving clotting in a vascular system in an animal such as but not limited to a heart condition (eg. heart attack) or a stroke, said method comprising administering to said animal a a platelet aggregation inhibition effective amount of a peptide comprising the amino acid sequence:

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1. Most preferably, the peptide comprises the amino acid sequences:

S S I M D S P Y (SEQ ID NO: 2);
L P A A S S I M D S P Y (SEQ ID NO: 3); and
A P S S I M D S P Y (SEQ ID NO: 4);

or derivatives, homologues, analogues or mimetics thereof.

The animal may be a human, primate, livestock animal (eg. horse, sheep, cow, pig, donkey), laboratory test animal (eg. rabbit, mouse, guinea pig), companion animal (eg. dog, cat or a captive wild animal (eg. goat).

Reference herein to "derivatives" includes parts, fragments and portions of the subject anti-clotting peptides. A derivative also includes a single or multiple amino acid substitution, deletion and/or addition. Homologues include functionally, structurally or sterochemically similar peptides from venom of the same species of snake or from within the same genus or family of snake. For example, a homologue would include a peptide having, for example, the amino acid sequence S S I M D S P Y (SEQ ID NO: 2) from another phospholipase from another member of the *Elapidae* family. Homologues also include similar peptides from

phospholipases derived from non-venom sources, such as pancreas, from any species. The amino acids S S I M D S P Y (SEQ ID NO: 2) may be replaced by amino acids having a similar charge, stereochemistry, conformation or peptide conformation influencing ability. All such homologues are contemplated by the present invention.

Analogues and mimetics include molecules which contain non-naturally occuring amino acids and which do not contain amino acids but nevertheless behave functionally the same as the peptide. Natural product screening is one useful strategy for identifying analogues and mimetics. Analogues of the subject peptides contemplated herein include modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptide molecule or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate,

4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 2.

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α–aminobutyric acid	Abu	L-N-methylalanine	Nmala
α–amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α–methyl-aminoisobutyrate	Maib

D-valine	Dval	α–methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α–napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α–methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu

N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α–methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The present invention further contemplates chemical analogues of the subject peptides capable of acting as antagonists or agonists of the anti-clotting peptides. Chemical analogues may not necessarily be derived from the peptides themselves but may share certain conformational similarities. Alternatively, chemical analogues may be specifically designed to mimic certain physiochemical properties of the peptides. Chemical analogues may be chemically synthesised or may be detected following, for example, natural product screening.

All these types of modifications may be important to stabilise the subject peptide if administered to a subject.

The present invention further contemplates recombinant anti-clotting peptides. Accordingly, another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a peptide as hereinbefore defined or a derivative or homologue thereof.

A particularly useful nucleic acid molecule is one which encodes or is complementary to a sequence which encodes amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1, said peptide capable of inducing or faciliating platelet aggregation.

Even more preferably, the nucleic acid molecule comprises a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence selected from:

S S I M D S P Y (SEQ ID NO: 2);
L P A A S S I M D S P Y (SEQ ID NO: 3); and
A P S S I M D S P Y (SEQ ID NO: 4).

The nucleic acid molecule of the present invention is generally in isolated form. It may also comprise additional nucleotide sequence information fused, linked or otherwise associated with it either at the 3′ or 5′ terminal portions or at both the 3′ and 5′ terminal portions. The nucleic acid molecule may also be part of a vector, such as an expression vector. The latter embodiment facilitates production of recombinant forms of the subject peptides which forms are encompassed by the present invention.

The present invention further encompasses host cells for the subject nucleic acid molecules and which are used to produce recombinant peptides. The host cells may be prokaryotic cells or eukaryotic cells. Examples of prokaryotic cells include *E. coli, Bacillus* sp, *Pseudomonas* sp amongst many others. Examples of eukaryotic cells include mammalian cell lines (eg. CHO cells), yeast cells, fungal cells, insect cells, plant cells and reptilian cell lines. The ability to produce recombinant peptides of the present invention permits the large scale production of vast qualities of peptides for commercial uses. As stated above, the peptide may need to be produced as part of a large peptide, polypeptide or protein which may be used as is or may first need to be processed in order to remove the extraneous proteinaceous sequences. Such processing includes digestion with proteases, peptidases and amidases or a range of chemical, electrochemical, sonic or mechanical disruption techniques. Notwithstanding that the present invention encompasses recombinant peptides, chemically synthetic techniques are particularly preferred in the synthesis of the peptides.

The peptide fragments such as those set forth in SEQ ID NO: 2 to 4 are conveniently

synthesized based on molecules isolated from snake venom. Isolation of the venom derived molecules may be accomplished by any suitable means such as by chromatographic separation, for example using CM-cellulose ion exchange chromatography followed by Sephadex (eg. G-50 column) filtration. Many other techniques are available including HPLC, PAGE amongst others. Once purified, the venom derived molecule can be partially sequenced and/or fragments produced and used directly as a source of peptides or as a template for peptide synthesis.

Peptides may be synthesized by solid phase synthesis using F-moc chemistry as described by Carpino *et al* (1991). The peptide and fragments thereof may also be synthesized by alternative chemistries including, but not limited to, t-Boc chemistry as described in Stewart *et al* (1985) or by either classical methods of liquid phase peptide synthesis.

The present invention further extends to the use of the subject peptides in the manufacture of a medicament for inducing or facilitating inhibition of platelet aggregation.

Preferred peptides are those which comprise the amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid, and n is 0 or 1.

Particularly preferred peptides are selected from:

S S I M D S P Y (SEQ ID NO: 2);
L P A A S S I M D S P Y (SEQ ID NO: 3); and
A P S S I M D S Y P (SEQ ID NO: 4).

Yet another aspect of the present invention provides an agent useful for inducing or facilitating platelet aggregation comprising a peptide as hereinbefore defined.

As stated above, the peptides of the present invention are useful in preventing platelet aggregation in a subject such as a mammal (e.g. a human).

Accordingly, another aspect of the present invention contemplates a method of inhibiting platelet aggregation in a subject said method comprising administering to said subject an anti-clotting effective amount of a peptide hereinbefore defined for a time and under conditions sufficient to inhibit platelet aggregation.

In a related aspect of the present invention, there is provided a method for the treatment or prophylaxis of a disease condition involving clotting in a vascular system in an animal such as but not limited to a heart condition (eg. heart attack) or a stroke, said method comprising administering to said subject an anti-clotting effective amount of a peptide hereinbefore defined for a time and under conditions sufficient to inhibit platelet aggregation.

In accordance with this method, more than one type of peptide may be administered or the peptide may be co-administered with a known anti-clotting compound or molecule. By "co-administered" is meant simultaneous administration in the same formulation or in two different formulations *via* the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of peptides or the peptide and the known anti-clotting compound or molecule. The peptides or the peptide and known anti-clotting compound or molecule may be administered in any order.

Routes of administration include but are not limited to intravenously, intraperitioneal, subcutaneously, intracranial, intradermal, intramuscular, intraocular, intrathecal, intracerebrally, intranasally, infusion, orally, rectally, *via* iv drip, patch and implant. Intravenous routes are particularly preferred.

Another aspect of the present invention provides a composition for use in inhibiting or facilitating inhibition of platelet aggregation comprising a peptide as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. The composition may also comprise two different types of peptides or a peptide and a known anti-clotting

compound or molecule.

Compositions suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. They must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by, for example, filter sterilization or sterilization by other appropriate means. Dispersions are also contemplated and these may be prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, a preferred method of preparation includes vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution.

When the active ingredients are suitably protected, they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions,

syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1ng and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The present invention also extends to forms suitable for topical application such as creams, lotions and gels. In such forms, the anti-clotting peptides may need to be modified to permit penetration of the surface barrier.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated.

Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved and (b) the limitations inherent in the art of compounding such an active material for induce or facilitating analgesia in living subjects.

Effective amounts of anti-clotting peptides contemplated by the present invention will vary depending on the severity of the pain and the health and age of the recipient. In general terms, effective amounts may vary from 0.01 ng/kg body weight to about 100 mg/kg body weight. Alternative amounts include for about 0.1 ng/kg body weight about 100 mg/kg body weight or from 1.0 ng/kg body weight to about 80 mg/kg body weight.

Still another aspect of the present invention is directed to antibodies to the subject peptides and their derivatives, homologues, analogues and mimetics. Such antibodies may be monoclonal or polyclonal.
In the case of small peptides, these may first need to be associated with a carrier molecule.

The antibodies of the present invention are particularly useful as therapeutic or diagnostic agents. For example, specific antibodies can be used to screen for peptides using immunoassays or used as antagonists to inhibit peptide activity under certain circumstances such as where temporary clotting inhibition is only required. Techniques for such immunoassays are well known in the art and include, for example, sandwich assays and ELIZA. Knowledge of peptide levels may be important for monitoring certain therapeutic protocols.

Antibodies to the peptides (or their derivatives, homologues, analogues or mimetics) of the

present invention may be monoclonal or polyclonal. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies.

As stated above, specific antibodies can be used to screen for the subject peptides. The latter would be important, for example, as a means for screening for levels of peptides in a cell extract or other biological fluid or purifying peptides made by recombinant means from culture supernatant fluid.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies or synthetic antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of peptide.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the enzyme or protein and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of peptide, or antigenic parts thereof, collecting serum from the animal and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art.

Another aspect of the present invention contemplates a method for detecting a subject peptide in a biological sample from a subject or culture supernatant flow or other source said method comprising contacting said biological sample with an antibody specific for said peptide or its derivative, homologue, analogue or mimetic for a time and under conditions sufficient for an antibody-peptide complex to form, and then detecting said complex.

The presence of a peptide may be accomplished in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4, 424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain an analgesic peptide including cell extract, culture supernatant tissue biopsy, serum, saliva, mucosal secretions, lymph, tissue fluid and respiratory fluid. The sample is, therefore, generally a biological sample comprising

biological fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.

In the typical forward sandwich assay, a first antibody having specificity for the peptide or antigenic parts thereof is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes or overnight if more convenient) and under suitable conditions (e.g. from room temperature to about 37°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, luciferase glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-peptide complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescene and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Effects of OHV A-PLA_2 on platelet aggregation

The effects of OHV A-PLA_2 on platelet aggregation were examined by using human whole blood and PRP. As shown in Fig 1, OHV A-PLA_2 showed a marked inhibitory effect on platelet aggregation induced by collagen in both whole blood and PRP in a dose-dependent manner. It was studied that the dose-response of OHV A-PLA_2 on platelet aggregation induced by various agonists in human whole blood and PRP, and estimated the IC_{50} values (inhibitor concentration causing 50% inhibition) for these inhibitions. OHV A-PLA_2 inhibits ADP (10 μM)-, collagen (2 μg/ml)- and arachidonic acid (0.5 mM)-induced platelet aggregation of human whole blood and PRP in a dose-dependent manner with the different IC_{50} values (Fig 2, Table 1). Relative inhibitory potencies of OHV A-PLA_2 are ADP > collagen > arachidonic acid in whole blood, and arachidonic acid > collagen > ADP in PRP.

EXAMPLE 2

Effects of OHV A-PLA_2 on ATP release reaction

It was investigated that the effect of OHV A-PLA_2 on platelet release reaction and its relationship with effect on aggregation. OHV A-PLA_2 caused the inhibition of platelet ATP release reaction in response to collagen and arachidonic acid in a dose-dependent manner with the IC_{50} of 50.4 nM and 0.91 nM, respectively. This dose-dependent inhibition of ATP secretion paralleled OHV A-PLA_2 inhibition (with IC_{50} of 40 nM and 0.74 nM respectively) of platelet aggregation induced by collagen (Fig 3A) and arachidonic acid (Fig. 3B). OHV A- PLA_2 also inhibited ADP-induced platelet ATP release reaction. In this case, however, the inhibition of ATP secretion was not parallel to the inhibition of platelet aggregation (Fig 4). Studies on the effect of OHV A-PLA_2, on platelet secretion show that the ATP release reaction induced by various agonists were inhibited by OHV A-PLA_2. These results indicate that OHV A-PLA_2 inhibits platelet secretion.

EXAMPLE 3

Ultrastructural changes of platelets

In order to analyse the effect of OHV A-PLA_2 on platelets, the changes of ultrastructure of platelet were studied. Transmission electron microscopic study showed that OHV A-PLA_2 (70 or 360 nM) induced remarkable morphological changes both in the shape and the subcellular structure distribution. After 5 minutes of OHV $APLA_2$ treatment, platelets lost their discoid shape, become irregular or relatively spherical. Portions of the surface developed rufffing and pseudopodium-like projections (Fig 5B) which are not as long and big as pseudopods caused by agonists. The surface change was also confirmed by scanning electron microscopic study (Fig 6B). The canalicular system was widened and disrupted (Fig 5B). There was an increase of the number of vacuoles in platelets. The channels of the dense tubular system lost their original arrangement, and were fragmented and redistributed throughout the cytoplasm (Fig 6B). The platelets stirred on an aggregometer with ADP (10 μM), surface contours of the activated platelet are irregular with numerous pseudopodia and molded to each other. The contents of most granule have been discharged (Fig 7A). After platelets were incubated with OHV A-PLA_2 (360 or 720 nM) for 2 min, ADP could not cause platelets to extended markedly long pseudopods, degranulate or aggregate (Fig 7B). Platelets appeared spherical with organelle centralization that keep in the first step of platelet responses, but without long pseudopods. Ultrastructural studies showed that OHV A-PLA_2 induced remarkable morphological changes both in the shape and the subcellular structure (Fig 5 and 6). Platelets lost their discoid shape, become irregular or relatively spherical with numerous pseudopodia-like projections. It has been observed that disorders in the open canalicular system and dense tubular system. These systems serve as the sarcoplasmic reticulum of the platelet, regulating calcium flux, and are also the site for enzymes involved in metabolism of arachidonic acid into endoperoxides (EPs) and thromboxane A_2 (TxA_2). The formation of large irreversible platelet aggregates is mediated by EPs and TxA_2, ADP and Ca^{2+} secreted from dense granules, and adhesive proteins secreted from -granules through the channels of the open canalicular system. The disorders in the open canalicular system and dense tubular system indicate a dramatic change in the "cell sarcoplasmic reticulum". In combination with the result of inhibition of ATP release,

this disorder may result in platelets that are unable to produce release reaction and aggregation. Thus, the disorder of the cytoskeleton as well as canalicular system and the loss of release reaction appears to play a significant role in the mechanism of antiplatelet effect of OHV A-PLA_2.

EXAMPLE 4

Role of enzymatic activity in the antiplatelet effect

In order to determine whether inhibitory effect on platelet aggregation is dependent on catalytic activity of enzyme the influence of various preincubation times on the antiplatelet effects of OHV A-PLA_2 was examined. If enzymatic activity contributes significantly to the antiplatelet effects, as the preincubation times are increased more phospholipids are hydrolyzed and more products accumulated, and hence should result in increased inhibition of platelet aggregation. For example, the inhibition of platelet aggregation bv PLA_2 enzyme from *Austrelaps superba* venom is time-dependent. As shown in Fig 8A, in collagen-induced platelet aggregation of whole blood, the inhibitions at 0.35 M of OHV A-PLA_2 after 2, 5 and 10 min of preincubations were 56.2 $\pm$ 11.1, 57.4 $\pm$ 10.9 and 57.8 $\pm$ 10.2, respectively ($P > 0.05$, n = 3). Thus, the inhibitory activity of OHV A-PLA2did not significantly change when the preincubation times of platelets and OHV A-PLA_2 were prolonged. This indicates that catalytic activity has no significant role in the antiplatelet effects of OHV A-PLA_2. To further support this proposal, it was also examined that the effect of alkylation of catalytic site (His 48) on the inhibition of platelet aggregation. It has been demonstrated that the treatment of PLA_2 with *p*-BPB results in the alkylation of His residue and loss of enzymatic activity. When OHV A-PLA_2 was modified with *p*-BPB, its PLA_2 activity was completely lost. The native PLA_2 of 4 g/ml showed marked activity (84.3 mol/min/mg), but modified PLA_2 even at 100 g/ml showed no measurable enzymatic activity. The platelet inhibitory activity of modified PLA_2 was only partially reduced (Fig 8B). Modified PLA_2- inhibited ADP (10 μM)-induced platelet aggregation of human platelet-rich plasma with a IC_{50} value of 1.25 M. This strongly supports the contention that the anti-clotting effects of OHV A-PLA_2 are independent of its enzymatic activity. This is in contrast to some other PLA_2 inhibitors which lost both of their enzymatic activity and antiplatelet activity upon alkylation by *p*-BPB. The results also suggest the presence of

separate sites responsible for the catalytic and antiplatelet activities. The partial loss of antiplatelet activity could be explained by the probable conformational changes of the enzyme caused by the alkylation.

EXAMPLE 5

Effects of the synthetic peptides on platelet aggregation

The inhibitory effects of the synthetic peptides on platelet aggregation were tested by using human whole blood. As shown in Fig 9, the peptide-I (SEQ ID NO: 3) showed an inhibitory effect on platelet aggregation induced by collagen (2 μg/ml) in whole blood with IC_{50} of 2.33 mM. The peptide-II (SEQ ID NO: 4) inhibited ADP (10 μM)- and collagen (2 μg/ml)-induced platelet aggregation of human whole blood in a dose-dependent manner with the IC_{50} values of 0.55 mM and 0.97 mM respectively (Fig. 10).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: NATIONAL UNIVERSITY OF SINGAPORE
(ii) TITLE OF INVENTION: NOVEL ANTI-CLOTTING MOLECULES AND USES THEREFOR

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

X_1 X_2 A X_3 Ser Ser Ile Met Asp Ser Pro Tyr
5 10

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Ser Ile Met Asp Ser Pro Tyr
5

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Pro Ala Ala Ser Ser Ile Met Asp Ser Pro Tyr
5 10

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Pro Ser Ser Ile Met Asp Ser Tyr Pro
5 10

CLAIMS

1. An isolated peptide comprising a sequence of amino acids having at least 50% similarity to a sequence of amino acids in a phospholipase and which peptide is capable of inducing or facilitating the inhibition of blood clotting or a derivative, homologue, analogue or mimetic of said peptide.

2. An isolated peptide according to claim 1 wherein said blood clotting is platelet aggregation.

3. An isolated peptide according to claim 1 or claim 2 wherein the snake venom is from a snake from the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.

4. An isolated peptide according to claim 3 wherein the snake venom is from a snake from the family *Elapidae*.

5. An isolated peptide according to claim 4 wherein the snake venom is from *O. Hannah*.

6. An isolated peptide according to claim 5 wherein the phospholipase is A-PLA_2.

7. An isolated peptide according to claim 6 comprising the amino acid sequence
$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)
wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1.

8. An isolated peptide according to claim 7 comprising the amino acid sequence S S I M D S P Y (SEQ ID NO: 2).

9. An isolated peptide according to claim 7 comprising the amino acid sequence L P A A S S I M D S P Y (SEQ ID NO: 3).

10. An isolated peptide according to claim 9 comprising the amino acid sequence A P S S I M D S P Y (SEQ ID NO: 4).

11. A nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a peptide or a derivative, homologue, analogue, or mimetic thereof or a nucleotide sequence capable of hybridising thereto under low stringency conditions at 42°C wherein said peptide has at least 50% similarity to a sequence of amino acids in a phospholipase and which peptide is capable of inducing or facilitating the inhibition of blood clotting.

12. A nucleic acid molecule according to claim 11 wherein said blood clotting is platelet aggregation.

13. A nucleic acid molecule according to claim 11 or claim 12 wherein the phospolipase is derived from snake venom.

14. A nucleic acid molecule according to claim 13 wherein the snake venom is from a snake from the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.

15. A nucleic acid molecule according to claim 14 wherein the snake venom is from a snake from the family *Elapidae*.

16. A nucleic acid molecule according to claim 15 wherein the snake venom is from *O. Hannah*.

17. A nucleic acid molecule according to claim 16 wherein the phospholipase is A-PLA_2.

18. A nucleic acid molecule according to claim 17 wherein the peptide comprises the amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1.

19. A nucleic acid molecule according to claim 18 wherein said peptide comprises the amino acid sequence

S S I M D S P Y (SEQ ID NO: 2).

20. A nucleic acid molecule according to claim 18 wherein said peptide comprises the amino acid sequence

L P A A S S I M D S P Y (SEQ ID NO: 3).

21. A nucleic acid molecule according to claim 18 wherein said peptide comprises the amino acid sequence

A P S S I M D S P Y (SEQ ID NO: 4).

22. A method of inhibiting blood clotting in a subject said method comprising administering to said subject an anti-clotting effective amount of a peptide comprising a sequence of amino acids having at least 50% similarity to a sequence of amino acids in phospholipase.

23. A method according to claim 22 wherein said blood clotting is platelet aggregation.

24. A method according to claim 22 or claim 23 wherein the phospholipase is derived from snake venom.

25. A method according to claim 24 wherein the snake venom is from a snake from the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.

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* C L M S *

26. A method according to claim 25 wherein the snake venom is from a snake from the family *Elapidae*.

27. A method according to claim 26 wherein the snake venom is from *O. Hannah*.

28. A method according to claim 27 wherein the phospholipase is A-PLA_2.

29. A method according to claim 28 wherein the peptide comprises the amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1.

30. A method according to claim 29 wherein the peptide comprises the amino acid sequence

S S I M D S P Y (SEQ ID NO: 2).

31. A method according to claim 29 wherein the peptide comprises the amino acid sequence

L P A A S S I M D S P Y (SEQ ID NO: 3).

32. A method according to claim 29 wherein the peptide comprises the amino acid sequence

A P S S I M D S P Y (SEQ ID NO: 4).

33. A composition useful for inhibiting blood clotting said composition comprising a peptide having at least 50% similarity to a sequence of amino acids in a phospholipase.

34. A composition according to claim 33 wherein said blood clotting is platelet aggregation.

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35. A composition according to claim 33 or claim 34 wherein said phospholipase is derived from snake venom.

36. A composition according to claim 35 wherein said snake venom is from a snake from the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.

37. A composition according to claim 36 wherein said snake venom is from a snake from the family *Elapidae*.

38. A composition according to claim 37 wherein said snake venom is from *O. Hannah*.

39. A composition according to claim 38 wherein the phospholipase is A-PLA_2.

40. A composition according to claim 39 wherein the peptide comprises the amino acid sequence

$[X_1]_n$ $[X_2]_n$ $[A]_n$ $[X_3]_n$ S S I M D S P Y (SEQ ID NO: 1)

wherein X_1, X_2 and X_3 may be the same or different and each is an amino acid residue and n is 0 or 1.

41. A composition according to claim 40 wherein the peptide comprises the amino acid sequence

S S I M D S P Y (SEQ ID NO: 2).

42. A composition according to claim 40 wherein the peptide comprises the amino acid sequence

L P A A S S I M D S P Y (SEQ ID NO: 3).

43. A composition according to claim 40 wherein the peptide comprises the amino acid sequence

A P S S I M D S P Y (SEQ ID NO: 4).

44. An agent for use in inhibiting blood clotting comprising a peptide from snake venom or a derivative, homologue, analogue, mimetic thereof.

45. An agent according to claim 44 wherein said blood clotting is platelet aggregation.

46. An antibody specific for the peptide according to any one of claims 7 or 8 or 9 or 10.

47. An antibody according to claim 46 wherein the antibody is a monoclonal antibody.

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