

Bi- or Multifunctional Molecules Based
on a Dendroaspin Scaffold

The present invention relates to dendroaspin-based chimeric
5 molecules which have anticoagulant, antiplatelet and other
activities. The invention also relates to nucleic acid
molecules encoding these chimeric dendroaspin molecules,
cloning and expression vectors comprising such nucleic
acids and host cells transformed with expression vectors so
10 as to provide recombinant chimeric multifunctional
dendroaspin. The invention further relates to
pharmaceutical compositions comprising chimeric dendroaspin
for use in the prevention or treatment of disease
associated with thrombus formation or platelet aggregation.
15 The invention also further relates to the use of a
dendroaspin scaffold in the design and generation of
chimeric dendroaspin derivatives having inhibitory activity
against integrin binding activity of platelets plus some
further functionality such as an anticoagulant or
20 antithrombotic action.

The role of blood coagulation is to provide an insoluble
fibrin matrix for consolidation and stabilisation of a
haemostatic plug. Formation of a cross-linked fibrin clot
25 results from a series of biochemical interactions involving
a range of plasma proteins.

Acute vascular diseases, such as myocardial infarction,
stroke, pulmonary embolism, deep vein thrombosis and
30 peripheral arterial occlusion are caused by either partial
or total occlusion of a blood vessel by a blood clot.

The formation of a blood clot within a blood vessel is
termed thrombosis and is dependent upon platelet
35 aggregation. In the context of blood vessel injury (such
as that which might arise in surgical procedures) the
interaction of blood platelets with the endothelial surface
of injured blood vessels and with other platelets is a

major factor in the course of development of clots or thrombi.

Various agents for preventing formation of blood clots are now available, such as aspirin, dipyridamole and
5 filopidine. These products generally inhibit platelet activation and aggregation, or delay the process of blood coagulation but they have the potential side effect of causing prolonged bleeding. Moreover, the effect of such products can only be reversed by new platelets being formed
10 or provided.

Platelet aggregation is dependent upon the binding of fibrinogen and other serum proteins to the glycoprotein receptor IIb/IIIa complex on the platelet plasma membrane.
15 GP IIb/IIIa is a member of a large family of cell adhesion receptors known as integrins, many of which are known to recognise an Arg-Gly-Asp (RGD) tripeptide recognition sequence.

20 Heparin and low molecular weight heparins have been used widely to treat conditions, such as venous thromboembolism, in which thrombin activity is responsible for the development or expansion of a thrombus. Although effective, heparin produces many undesirable side effects,
25 including haemorrhaging and thrombocytopenia. A more specific and less toxic anticoagulant is therefore required.

Direct thrombin inhibitors are available and examples of
30 these are hirudin, hirugen and hirulog (the latter two being synthetic hirudin derivatives), PPACK (a synthetic tripeptide) and argatroban (an arginine derivative). The actions of these inhibitors are reviewed in Lefkovits J and Topol E J (1994), Circulation 90:1522-1536. Although in
35 theory, the bleeding risk with direct thrombin inhibitors is lower than with other antithrombotics because of their mono-target specificity, absence of direct platelet effects, and short half-life, bleeding still remains as the most concerning adverse effect.

There are a range of other thrombin inhibitors which have been developed (listed in table 1 of Lefkovits J and Topol E J *supra*) but these have turned out to be just too toxic
5 for clinical use.

Localised narrowing of an artery caused by atherosclerosis is a condition which can usually be remedied surgically by the technique of balloon angioplasty. The procedure is
10 invasive and causes some tissue damage to the arterial wall which can result in thrombus formation. Extracellular proteins such as fibronectin in the arterial wall become exposed to blood in the artery. Platelets bind to the RGD motif of fibronectin via integrin receptors which in turn
15 leads to platelet aggregation and the start of the cascade of clotting reactions. An agent which specifically inhibits platelet aggregation at the sites of damage and which also inhibits clotting at these sites is required. The agent should be non-toxic and free of undesirable side
20 effects such as a risk of generalised bleeding.

Integrins are a family of cell surface receptors that mediate adhesion of cells to each other or to the extracellular matrix (Kieffer N & Philips D R (1990) *Annu*
25 *Rev Cell Biol* 6: 329-357; Hynes R O (1992) *Cell* 69: 11-25; McEver R P (1992) *Curr Opin Cell Biol* 4: 840-849; Smyth S S et al (1993) *Blood* 81: 2827-2843; Giancotti F G and Mainiero F (1994) *Biochim Biophys Acta* 1198: 47-64). They are composed of noncovalently associated α and β
30 transmembrane subunits. There exist 16 different α and 8 different β subunits that heterodimerize to produce about 20 different kinds of receptors (Clark E A & Brugge J S (1995) *Science* 268: 233-239). Among the integrins, the platelet membrane integrin $\alpha_{IIb}\beta_3$ is one of the best
35 characterized. Upon cell activation, the $\alpha_{IIb}\beta_3$ integrin binds several glycoproteins, predominantly through the Arg-Gly-Asp (RGD) tripeptide sequence (Pierschbacher M D and Ruoslahti E (1984) *supra*; Plow E F et al (1987) *Blood* 70:

110-115; Pytela R et al (1986) *Science* 231: 1559-1562) present in fibrinogen (Nachman R L and Nachman L L K (1992) *J Clin Invest* 69: 263-269), fibronectin (Gardner J M and Hynes R O (1985) *Cell* 42: 439-448), von Willebrand factor
5 (Ruggeri Z et al (1983) *J Clin Invest* 72: 1-12), vitronectin (Pytela R M et al (1985) *Proc Natl Acad Sci USA* 82: 5766-5770), and thrombospondin (Karczewski J et al (1989) *J Biol Chem* 264: 21322-21326). The nature of the interactions between these glycoprotein ligands and their
10 integrin receptors is known to be complex, and conformational changes occur in both the receptor (Sims P J et al (1991) *J Biol Chem* 266: 7345-7352) and the ligand (Ugarova T et al (1995) *Thromb Haemostasis* 74: 253-257).

15 Recently, many proteins from a variety of snake venoms have been identified as potent inhibitors of platelet aggregation and integrin-dependent cell adhesion. The majority of these proteins which belong to the so-called "disintegrin" family share a high level of sequence
20 homology, are small (4-8 kDa), cysteine-rich and contain the sequence RGD (Gould R J et al (1990) *Proc Soc Exp Biol Med* 195: 168-171) or KGD (Scarborough R M et al (1991) *J Biol Chem* 266: 9359-9362). In addition to the disintegrin family, a number of non-disintegrin RGD proteins of similar
25 inhibitory potency, high degree of disulfide bonding, and small size have been isolated from both the venoms of the *Elapidae* family of snakes (McDowell R S et al (1992) *Biochemistry* 31: 4766-4772; Williams J A et al (1992) *Biochem Soc Trans* 21: 73S) and from leech homogenates
30 (Knapp A et al (1992) *J Biol Chem* 267: 24230-24234). All of these proteins are approximately 1000 times more potent inhibitors of the interactions of glycoprotein ligands with the integrin receptors than simple linear RGD peptides; a feature that is attributed to an optimally favourable
35 conformation of the RGD motif held within the protein scaffold. The NMR structures of several inhibitors including kistrin (Adler M et al (1991) *Science* 253: 445-448; Adler M and Wagner G (1992) *Biochemistry* 31: 1031-1039; Adler M et al (1993) *Biochemistry* 32: 282-289),

flavoridin (Senn H and Klaus W (1993) J Mol Biol 234: 907-925), echistatin (Saudek V et al (1991) Biochemistry 30: 7369-7372; Saudek V et al (1991) Eur J Biochem 202: 329-328; Cooke R M et al (1991) Eur J Biochem 202: 323-328; 5 Cooke R M et al (1992) Protein Eng 5: 473-477), albolabrin (Jaseja M et al (1993) Eur J Biochem 218: 853-860), decorsin (Krezel A M et al (1994) Science 264: 1944-1947), and dendroaspin (Jaseja M et al (1994) Eur J Biochem 226: 861-868; Sutcliffe M J et al (1994) Nature Struct Biol 1: 10 802-807) have been reported, and the only common structural feature elucidated so far is the positioning of the RGD motif at the end of a solvent exposed loop, a characteristic of prime importance to their inhibitory action.

15

Recent studies have implied a role for the amino acids around the tripeptide RGD in regulating the ligand binding specificity shown by snake venom proteins. Scarborough R M et al (1993) J Biol Chem 268: 1058-1065 examined a range of 20 disintegrins and observed that those containing RGDW were very effective at inhibiting the interactions of fibrinogen to purified $\alpha_{11b}\beta_3$ but not of vitronectin and fibronectin to purified $\alpha_v\beta_3$ and $\alpha_5\beta_1$, respectively, whereas the converse was true for disintegrins containing the sequence RGDNP. 25 Other regions of amino acid sequence divergence may also be contributory (Scarborough et al (1993) *supra*).

Dendroaspin, a short chain neurotoxin analogue containing the RGD sequence, and the disintegrin kistrin, which show 30 little overall sequence homology but have similar amino acids flanking the RGD sequence (PRGDMP), are both potent inhibitors of platelet adhesion to fibrinogen but poor antagonists of the binding of platelets to immobilised fibronectin (Lu X et al (1994) Biochem J 304: 929-936). In 35 contrast, elegantin, which has 65% sequence homology to kistrin but markedly different amino acids around RGD (ARGDNP), preferentially inhibited platelet adhesion to

fibronectin as opposed to fibrinogen and binds to an allosterically distinct site on the $\alpha_{IIb}\beta_3$ complex.

Smith J W et al (1995) Journal of Biological Chemistry 270:
5 30486-30490 undertook protein "loop grafting" experiments to construct a variant of tissue-type plasminogen activator (t-PA) which bound platelet integrin $\alpha_{IIb}\beta_3$. Amino acids in a surface loop of the epidermal growth factor (EGF) domain of t-PA were replaced with residues from a
10 complementarity-determining region (CDR) forming one CDR of a monoclonal antibody reactive against the adhesive integrin receptor $\alpha_{IIb}\beta_3$. The resulting variant of t-PA (loop-grafted-t-PA) bound $\alpha_{IIb}\beta_3$ with nanomolar affinity and had full activity to both synthetic and natural
15 substrates. The effects and applicability of loop grafting are altogether unpredictable and uncertain.

The present inventors have now discovered that the dendroaspin scaffold lends itself to modification. When
20 dendroaspin (including the RGD motif) is modified to incorporate further functional amino acid sequences eg active portions or motifs of agonists, antagonists or inhibitors of factors in the clotting cascade, the resulting molecules are particularly useful as
25 anticoagulants and do not suffer from the drawbacks associated with existing anticoagulants.

In first aspect the present invention provides a hybrid polypeptide comprising a first amino acid sequence
30 including the RGD motif and conferring dendroaspin activity and a further amino acid sequence conferring activity other than that of dendroaspin activity.

The invention also provides a hybrid polypeptide having
35 integrin binding activity comprising a dendroaspin scaffold and a further non-dendroaspin amino acid sequence, preferably of different activity.

Advantageously, the molecules of the invention have an integrin binding activity which when administered *in vivo* results in the binding of the molecules to platelets thereby inhibiting the aggregation of the platelets, at
5 sites of injury. Moreover, the non-wild-type dendroaspin domain provides secondary, optionally further functionality eg antithrombotic action, inhibiting cell migration and proliferation and regulating signal transduction. Molecules of the invention are therefore bi- or
10 multifunctional in their activities against blood coagulation, particularly thrombus formation and arterial/venous wall thickening at the sites of injury. Polypeptides of the invention may have activities against leukocyte recruitment, immune system activation, tissue
15 fibrosis and tumorigenesis.

The polypeptide of the invention may comprise at least two said further amino acid sequences, preferably the two said further sequences are the same.

20 The further amino acid sequence may comprise two or more amino acid sequence portions separated by at least one amino acid residue of dendroaspin. The two or more sequence portions may be transposed with respect to one
25 another and to the linear order of amino acids in the native further amino acid sequence. In other words, the native order of the two or more amino acid sequence portions is altered although the actual sequences of each portion may not necessarily be altered.

30 The said further sequence may be selected from platelet derived growth factor (PDGF), glycoprotein (GP) IIB α , hirudin, thrombin, thrombomodulin (particularly the fifth EGF-like domain thereof), vascular epidermal growth factor
35 (VEGF), transforming growth factor- β 1 (TGF β 1), basic fibroblast growth factor (bFGF), angiotensin II (Ang II), factor VIII and von Willebrand factor (vWF).





In this way the molecules of the invention may be rendered multifunctional so that they are active against more than just platelet aggregation, for example another component in the clotting cascade (eg thrombin activity), or the
5 intracellular signalling cascade (eg growth factor). The modified dendroaspins of the invention may be engineered so that the further amino acid sequence has integrin binding activity, thereby providing a dendroaspin based molecule with augmented integrin binding activity.

10

The polypeptide of the invention preferably comprises an amino acid sequence as shown in Figure 3. Prior to inclusion of said further amino acid sequence the dendroaspin scaffold of the invention includes homologous
15 molecules which may share about 50% amino acid sequence homology, preferably about 65%, more preferably about 75% and even more preferably about 85% homology with dendroaspin.

20 Excluding the nucleic acid sequence encoding said further amino acid sequence, nucleic acid sequences encoding the polypeptide of the invention may share about 50% nucleotide sequence homology, preferably about 65%, more preferably about 75% and even more preferably about 85% homology with
25 a dendroaspin nucleotide sequence.

The polypeptides of the invention may comprise a greater or lesser number of amino acid residues compared to the 59 amino acids of dendroaspin. For example, the molecules of
30 the invention may comprise a number of amino acid residues in the range 45 to 159, preferably about 49 to 89, more preferably about 53 to 69, even more preferably about 57 to 61.

35 The further amino acid sequence is preferably incorporated into (a) loop I and/or loop II; (b) loop I and/or loop III; (c) loop II and/or loop III; or (d) loop I, loop II and loop III of the dendroaspin scaffold. Loop I comprises amino acid residues 4-16, loop II residues 23-36 and loop

III residues 40-50. However, the further amino acids being incorporated may extend into or substitute regions external to the loops, ie residues 1-3, 17-22 and 37-39 such that residues of the non-loop regions are augmented or substituted for those of the further amino acid sequence or sequences being inserted.

The further amino acid residues are preferably incorporated into either loop I or loop II. In this way the RGD-containing loop III is unaltered and so the integrin binding function of dendroaspin is retained.

A further RGD motif may be introduced into the dendroaspin scaffold, preferably into loop I or loop II, thereby increasing dendroaspin activity.

A preferred location for the inserted further sequence is at a site in dendroaspin scaffold between amino acid residues: 4-16, 18-21, 23-36, or 52-59.

Each inserted further amino acid sequence or portion of a further amino acid sequence is preferably an amino acid sequence in the range 3-40 amino acid residues, more preferably 3-16, even more preferably 3-14 amino acid residues long. The start of the inserted further amino acid sequence may be at any one of amino acid residues 1-57 of the dendroaspin scaffold. The end of the inserted further amino acid sequence may be at any one of amino acid residues 3-59 of the dendroaspin scaffold.

When two further amino acid sequences are inserted into the dendroaspin scaffold then the linear distance between these is preferably in the range 1-35 amino acids, more preferably 1-14 amino acids. When more than two further amino acid sequences are inserted then there is preferably at least one native dendroaspin amino acid residue separating each further amino acid sequence.

The RGD-containing loop may be modified by insertion, deletion or substitution of one of more amino acid residues, preferably a maximum of 8 or a minimum of 1 amino acids can be modified within loop III of dendroaspin.

5

The RGD loop preferably has an amino acid sequence as shown in table I. An advantage of modifying the RGD loop region is that the integrin binding activity may be enhanced and become more specific for certain glycoprotein ligands.

10 Also, if one or more of the "foreign" further amino acid sequences grafted into the dendroaspin scaffold has steric effects on the RGD motif then loop III around the RGD site can be modified to overcome any steric hindrance thereby restoring, perhaps enhancing RGD functionality.

15

Loop I and/or loop II may be modified by insertion, deletion or substitution of one or more amino acid residues. Any suitable number of amino acids can be inserted into the dendroaspin scaffold to give the desired
20 bi- or multi-functional activity although a number of residues in the range 14 to 36 are preferred for insertion at one or more sites in the dendroaspin scaffold.

Modification of the loops may become necessary if a
25 "foreign" further amino acid sequence grafted into the dendroaspin scaffold has a steric hindrance effect either on another grafted domain or on the RGD-containing loop. Computer assisted molecular modelling using Insight II software (Molecular Simulations Inc) can be used to predict
30 the structure of the "loop grafted" dendroaspins of this invention. In instances where steric effects between the loops may serve to cause loss of functionality, these effects can be "designed out" by modifying appropriate parts of the dendroaspin molecule in an appropriate way.
35 Sometimes this may involve inserting a number of suitable amino acid residues to extend one or more of the loop structures.

This Sheet Has Been Replaced



Preferred modification includes the insertion of polyglycine into the loop or loops of the dendroaspin scaffold in order to extend them. Other modifications comprising repeat units of an amino acid residue or number
5 of residues can be used. Computer modelling studies can be used to design the loop modifications needed in order to extend the loops of dendroaspin.

In the design of a bifunctional or multifunctional molecule
10 in accordance with the invention, "fine tuning" of activity, stability or other desired biological or biochemical characteristic may be achieved by altering individual selected amino acid residues by way of substitution or deletion. Modification by an insertion of
15 an amino acid residue or residues at a selected location is also within the scope of this "fine tuning" aspect of the invention. The site-directed mutagenesis techniques available for altering amino acid sequence at a particular site in the molecule will be well known to a person skilled
20 in the art.

In second aspect the invention provides a nucleic acid molecule encoding a polypeptide as hereinbefore defined.

25 The nucleic acid may be linked operatively to a promoter and optionally to a nucleic acid sequence encoding a heterologous protein or peptide thereby to encode a fusion product. The promoter is preferably β -D-isopropyl-thiogalactopyranoside (IPTG) inducible and the heterologous
30 protein or peptide may be glutathione S-transferase (GST).

This aspect of the invention also includes a plasmid comprising a nucleic acid as hereinbefore defined. The plasmid is preferably pGEX-3X.
35

In third aspect the invention provides a host cell transformed with a plasmid as hereinbefore defined, preferably said host cell is *E coli*.

The invention therefore also provides a cell culture comprising transformed host cells as hereinbefore defined.

5 In fourth aspect the invention provides a method of producing a polypeptide as hereinbefore defined comprising culturing a host cell as hereinbefore defined so as to express said polypeptide, extracting the polypeptide from the culture and purifying the same.

10 In fifth aspect, the invention provides a method of producing a multifunctional anticoagulant comprising the steps of:

15 a) constructing an expression vector comprising a nucleic acid sequence encoding a dendroaspin scaffold operatively linked to a promoter and optionally linked to nucleic acid encoding an heterologous protein for co-expression therewith.

20 b) modifying at least a portion of the nucleic acid sequence of the vector encoding the dendroaspin scaffold, excluding the RGD motif, by one or more of insertion, deletion or substitution of nucleic acid residues so that on expression the dendroaspin
25 scaffold comprises a further amino acid sequence of activity other than that of dendroaspin activity.

30 c) transforming a host cell with the vector and causing the host cell to express the modified dendroaspin nucleic acid sequence.

The method preferably further comprises the steps of:

35 d) extracting the modified dendroaspin from a host cell culture,

e) purifying the modified dendroaspin from the cell culture extract, optionally including the step of

cleaving the dendroaspin from a co-expressed heterologous protein.

5 The heterologous protein is preferably GST and the purification preferably involves affinity chromatography using glutathione Sepharose 4B contained in the GST purification modules followed by factor Xa-mediated cleavage of the modified dendroaspins from GST.

10 The invention therefore provides a polypeptide as hereinbefore defined obtainable by the method of producing a multifunctional anticoagulant as defined above.

15 In sixth aspect the invention provides a pharmaceutical composition comprising a therapeutically effective amount of a polypeptide as hereinbefore defined, optionally further comprising a pharmaceutically acceptable excipient or carrier. A multiplicity of polypeptides of the invention of different functionalities may be combined
20 together in a pharmaceutically acceptable form so as to provide a desired treatment. A multiplicity of polypeptides of the invention of different functionalities may be combined together in a pharmaceutically acceptable form so as to provide a desired treatment.

25 The polypeptide of the invention is preferably formulated for intravenous injection or intravenous infusion although other methods of administration are possible, eg subcutaneous or intramuscular should it be desired to
30 provide a slow release into the circulatory system of an individual. Also possible is the formulation of the polypeptide for use with implanted controlled release devices such as those used to administer growth hormone for example.

35 One formulation may comprise extravasated blood combined with a polypeptide of the invention at a concentration in the range 1nM - 60µM. This blood may be stored in ready to use form and provides an immediate and convenient supply of

blood for transfusion in cases when clotting must be avoided such as during or immediately following surgical procedures.

- 5 In seventh aspect the invention provides a polypeptide as hereinbefore defined for use as a pharmaceutical.

In eighth aspect the invention provides for the use of a polypeptide as hereinbefore defined for the manufacture of
10 a medicament for the treatment of disease associated with thrombosis; more particularly thrombosis, myocardial infarction, retinal neovascularization, endothelial injury, dysregulated apoptosis, abnormal cell migration, leukocyte recruitment, immune system activation, tissue fibrosis and
15 tumorigenesis.

The invention also provides methods for the treatment of disease associated with thrombosis; more particularly thrombosis, myocardial infarction, retinal
20 neovascularization, endothelial injury, dysregulated apoptosis, abnormal cell migration, leukocyte recruitment, immune system activation, tissue fibrosis and tumorigenesis. The methods comprise administering a therapeutically effective amount of a polypeptide as
25 hereinbefore defined.

Preferred embodiments of the invention will now be described and with reference to the following examples and drawings in which:

30

Figure 1 shows the basic three-dimensional structure of a molecule of dendroaspin. N denotes the -NH terminus and -C denotes the -COOH terminus. Amino acid residues are numbered.

35

Figure 2A shows the nucleotide and corresponding amino acid sequences of dendroaspin. Individual synthetic oligonucleotides are indicated by numbers 1-10.

Figure 2B is a partial restriction map of plasmid pGEX-DEG.

Figure 3A comprises alignments of modified dendroaspins where the inserted amino acid sequences are listed beneath
5 the amino acid sequence of dendroaspin.

Figure 3B is like Figure 3A and further alignments of the amino acid sequences (one letter code) of various modified dendroaspins.

10

Figure 3C is like Figure 3A and shows modified molecules of the invention.

Figure 3D is a table showing various activities of the
15 modified molecules of the invention.

Figure 4 shows the expression plasmid pGEX-3X used to clone and express modified dendroaspin fusion protein.

20 Figure 5A shows a 12.5% SDS-PAGE gel comparing the total cellular lysates of either induced (lane 1) or uninduced (lane 2) bacteria transformed with the recombinant plasmid pGEX-DEG harbouring the modified dendroaspin gene.

25 Figure 5B shows a 10% SDS-PAGE gel comparing total cell lysate (lane 1) from induced bacteria transformed with pGEX-DEG and an affinity purified extract of such a lysate (lane 2).

30 Figure 5C shows a 20% SDS-PAGE gel of affinity purified and factor Xa digested GST-modified dendroaspin fusion protein. Lane 1 is dendroaspin, lane 2 is GST-Den and lane 3 is factor Xa digested GST-Den

35 Figure 6A is a reverse phase HPLC elution profile of recombinant dendroaspin purified on a Vydac C₁₈ column developed with an acetonitrile gradient (the retention time of recombinant dendroaspin at 44 minutes was indicated by an arrow).

Figure 6B is a reverse phase HPLC elution profile of the peak fraction eluting at 44 minutes in Figure 6A carried out under similar conditions.

5

Figure 7 shows immunoprecipitation of dendroaspin containing PDGF loop I using antibodies R38 and R65 raised to loop I and R51 raised to both loop I and loop III. A 32kDa protein which corresponds to the GST-mutant dendroaspin was observed. Lane 1 is DEN-PDGF probed with R38, lane 2 probed with R65 and lane 3 probed with R51.

Figure 8 is a bar chart showing the results of an experiment in which PDGF-dendroaspin is used to inhibit PDGF induced proliferation of human fibroblast cells. "Peptide 1" corresponds to the linear amino acid sequence of PDGF loop I.

Dendroaspin is a short chain neurotoxin homologue from the venom of *Elapidae* snakes, which lacks neurotoxicity. Unlike neurotoxins, it contains an Arg-Gly-Asp-(RGD)-motif and functions as an inhibitor of platelet aggregation and platelet adhesion with comparable potency to the disintegrins from the venoms of *Viperidae*. The structure of dendroaspin in solution has been determined using NMR spectroscopy (Sutcliffe M J et al (1994) Nature Struct Biol 1: 802-807). The structure contains a core similar to that of short chain neurotoxins, but with a novel arrangement of loops and a solvent-exposed RGD-motif. Dendroaspin is thus an integrin antagonist with a well defined fold different from that of the disintegrins, based on the neurotoxin scaffold.

The structure of dendroaspin consists of a core region from which three loops, denoted I, II and III (residues 4-16, 23-36 and 40-50) extend outwards (Figure 1). The core contains the four disulphide bonds which are spatially close to each other and hold the loops together. The amino acid sequence of dendroaspin is shown in Figure 2.

In the following examples materials used include:

Restriction enzymes, T4 polynucleotide kinase, T4 DNA
5 ligase, IPTG (isopropyl- β -D-thio-galactopyranoside) and
DH5 α competent cells purchased from Life Technologies Ltd
(UK) or Promega Ltd (Southampton, UK). Vent (exo-) DNA
polymerase was supplied by New England Biolabs Ltd
(Hitchin, UK). Oligonucleotides were made either in King's
10 College School of Medicine & Dentistry (London, UK) or by
Cruachem Ltd (Glasgow, UK) and further purified by
denaturing PAGE on a 15% acrylamide / 8 M urea gel.
Deoxynucleotide triphosphates (dNTP's), dideoxynucleotide
triphosphates (ddNTP's) and plasmid pGEX-3X, a vector that
15 expresses a cloned gene as a fusion protein linked to
glutathione S-transferase (GST) and Glutathione-Sepharose
CL-4B were purchased from Pharmacia Biotech Ltd (Herts,
UK). "Geneclean" kit and Plasmid maxi kit were purchased
from Bio 101 (La Jolla CA, USA) and Qiagen Ltd (Surrey, UK)
20 respectively. The sequencing Sequenase 2.0 was obtained
from Cambridge Bioscience (Cambridge, UK). [³⁵S]dATP[α S]
and ¹²⁵I (15.3 mCi / mg iodine) were supplied by NEN Dupont
(Herts, UK) and Amersham International Plc (Amersham,
Bucks, England) respectively.

25

Example 1 - Construction of expression vectors encoding
variants of dendroaspin

The wild type dendroaspin gene was inserted into a plasmid
30 pGEX-3X (Figure 2) successfully and expressed according to
the method of Lu et al, (1996) J Biol Chem 271: 289-295.
Starting with the wild type gene for dendroaspin, variants
of dendroaspin genes were engineered using recombinant DNA
technology. For the longer insertion variants, oligo-
35 nucleotides which encode the non-dendroaspin or
heterologous amino acids were simply inserted directly into
suitably restriction digested wild type dendroaspin gene
and then ligated. For minor changes such as modification

of a few amino acid residues including the insertion, substitution or deletion, the Transformer™ Site-Directed Mutagenesis kit from Clontech Laboratories was used in accordance with the manufacturers instructions.

5

Figure 2A shows the nucleotide sequence of the synthetic dendroaspin (Den) gene. The gene was designed on the basis of the known amino acid sequence (Williams J A et al ((1992)) Biochem Soc Trans 21: 73S) and the codons for each amino acid were adopted from those which were highly expressed in *E coli* (Fiers W ((1982)) Gene 18: 199-209). Ten synthetic oligonucleotides are shown in brackets and numbered individually 1 to 10 either above the coding strand or below the non-coding strand. The stop codon is indicated by an asterisk. Three-letter amino acid code is used and the total of 59 amino acids of Den are only numbered 1 for N-terminal residue arginine and 59 for C-terminal leucine.

20 Figure 2B is a partial restriction map of pGEX-DEG. Only the dendroaspin (Den) gene and its relevant upstream region are shown.

Figure 3A shows the amino acid sequences of various modified dendroaspin molecules which will be described in more detail below. In each case the modified inserted residues are set out beneath the amino acid sequence of dendroaspin.

30 Example 2 - Modified dendroaspin containing a platelet-derived growth factor (PDGF) domain

Platelet-derived growth factor (PDGF) is a 30-kDa polypeptide and a major serum mitogen and chemotactic factor for mesenchymal cells. Originally purified from human platelets, PDGF has subsequently been found to be produced by other cell types such as smooth muscle cells, placental cytotrophoblast cells, fibroblasts and connective tissue. PDGF has been shown to promote cell migration and

proliferation and these are key events in natural processes such as embryogenesis and wound healing. PDGF has also been implicated in a number of pathological states, such as arteriosclerosis, fibrosis and rheumatoid arthritis (Heldin
5 C H and Westermark B (1990) Cell Regul 1: 555-566 and Engstrom U et al (1991) J Biol Chem 267: 16581-16587). PDGF is a dimer of two similar chains called A- and B-chains.

10 Amino acid sequences derived from PDGF B chain is inserted into loop II of dendroaspin as shown in Figures 3A and 3C, maintaining the adhesive property of the RGD sequence of loop III within dendroaspin. The modified dendroaspins are then examined for competitive inhibition of PDGF activity
15 by analysing smooth muscle and fibroblast cell proliferation and integrin antagonist activity. Figure 3D summarises the results obtained with the example given in Figure 3C. The modified dendroaspin molecules inhibit ADP-induced platelet aggregation and PDGF-induced proliferation
20 of fibroblast cells.

Assembly and cloning of the gene of dendroaspin containing PDGF loop I: The gene of dendroaspin containing PDGF loop I was assembled from the fragments (77 mer, 76 mer, 42 mer
25 and 44 mer) of wild-type gene, after digestion with Bam HI, EcoR I, Hinf I and Hpa II, and a pair of complementary phosphorylated mutagenesis oligos (81 mer and 80 mer). A mixture of the total 6 fragments was heated to 85°C for 5 min and annealed by slowly cooling down to room temperature
30 (RT). Ligation was performed at 16°C for 15 hours in a total volume of 50µl containing about 1nM of each fragment, 50mM Tris-HCl (pH7.6), 10mM MgCl₂, 1mM DTT, 1mM ATP and 5% PEG 8000 and 5 units of T4 ligase. After ligation, 1µl of ligation mixture was used as template together with two 5'-
35 overhanging oligonucleotides as primers and 2 units of Vent polymerase for PCR. The single product obtained was of expected size checked by 2% agarose gel. The hybridisation gene was then cloned into the vector pGEX-3X to produce the

recombinant plasmid pGEX-3X comprising PDGF-modified dendroaspin gene.

Transformation and plasmid DNA isolation: The pGEX-3X
5 vector comprising the PDGF-modified dendroaspin gene (about 5ng) was used to transform 50µl of the *E coli* strain DH5α. Controls were set up of a vial containing competent cells only, a vial containing unligated plasmid DNA and a vial containing wild-type plasmid DNA. The incubation media
10 used for the bacteria is SOC medium (containing 20g/l bacto-tryptone, 5g/l bacto-yeast extract, 0.5g/l NaCl, 2.5mM KCl, 20µM MgCl₂ and 20mM glucose). The transformation step was followed by a recovery period in which the bacterial culture was incubated without ampicillin for an hour.
15 After transformation, a sufficient quantity of each of the bacterial cultures was plated out. SOC agar medium containing ampicillin was used to culture the bacteria. The ampicillin selection marker of pGEX-3X confers ampicillin resistance on any transformed bacteria allowing
20 them to grow whilst any untransformed bacteria are eliminated. The control plate remained clear following incubation overnight.

Individual colonies were picked from the plates, each one
25 representing a modified dendroaspin gene. These were cultured on SOC media containing ampicillin for a few hours. A small amount of each was removed and transferred to new SOC media containing ampicillin. Sterile glycerol was added to the original bacterial culture to permit
30 storage at -70°C as a stock supply. The remaining cultures were incubated overnight. The isolation of the plasmid DNA from the transformed bacterial culture is carried out by a plasmid mini prep (for quick test) or maxi-prep (for DNA sequencing) following the manufacturer's instructions
35 (QIAGEN). A plasmid mini-prep was carried out on each to isolate the plasmid DNA once more; the majority of which comprised the modified dendroaspin gene. DNA sequencing was carried out on the region of the plasmid containing the dendroaspin gene fragment to check that the modification

was present and correct. Complete DNA sequencing of the inserted fragments was made using the dideoxy chain-termination method of Sanger et al (1977) Proc Natl Acad Sci 74: 5463-5467.

5

Protein expression: *E coli* DH5 α was transformed with modified plasmid pGEX-3X using standard methods particularly as described in Sambrook et al (1989) Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY. The transformed cultures were used for protein expression. Medium were inoculated with an overnight seed culture (1%, v/v) and grown in LB/ampicillin medium (100 μ g/ml) and shaken at 37°C until an A₆₀₀ of 0.7 was obtained. IPTG was then added to a final concentration of 0.1mM for induction. The cells were grown for an additional 4 hours at a lower temperature of 30°C and harvested by centrifugation.

Purification of fusion protein: Recombinant dendroaspins were purified as follows by suspending the cell pellets in phosphate buffered saline (PBS) buffer (pH 7.4) containing 1% Triton X-100 and the protease inhibitors phenylmethylsulfonyl fluoride (1mM), pepstatin (1 μ M), aprotinin (2 μ g/ml), trypsin inhibitor from soyabean (1 μ g/ml), 1 mM EDTA, and sonicated on ice. The sonicated mixture was centrifuged at 7,800 x g at 4°C for 10 min to pellet the cell debris and insoluble material. Recombinant GST-dendroaspin and GST-modified-dendroaspins from supernatants were purified by affinity chromatography on glutathione-Sepharose CL-4B columns by adsorption in PBS containing 150 mM NaCl and elution with 50 mM Tris-HCl containing 10 mM reduced glutathione (pH 8.0). Elution of the adsorbed material with glutathione (pH 8.0) resulted in the appearance of a major band migrating at 32 kDa (GST-dendroaspin fusion protein) in polyacrylamide gels. (Figures 5A and 5B).

The appropriate fractions comprising the 32-kDa fusion proteins were then digested in the presence of 150 mM NaCl, 1 mM CaCl_2 , and Factor Xa (1:100, w/w, Factor Xa:fusion protein) at 4°C for 24h. Treatment of the purified GST-
5 proteins with Factor Xa released recombinant proteins migrating as 7-kDa bands (Figure 5C), approximating the size of dendroaspin, and free GST appearing as an intensification of a 28-kDa band on SDS-polyacrylamide gel electrophoresis. The digested mixture was loaded onto a
10 Vydac C_{18} reverse-phase HPLC analytical column (TP104) and eluted with a linear gradient of 0-26% acetonitrile (1.78% per min) containing 0.1% trifluoroacetic acid, followed by 26-36% acetonitrile in 0.1% trifluoroacetic acid (0.25% per min). When necessary, further analytical columns were run
15 under the same conditions. Figures 6A and 6B show the elution profile of reverse phase HPLC runs.

Western blotting: Fusion proteins (10µg) were electrophoresed on an SDS-PAGE (12%) gel and transferred by
20 semi-dry blotting to nitrocellulose membranes using a current density of 0.8mA/cm². The nitrocellulose membranes were blocked for 2h at RT with 5% dried milk in TBS/Tween buffer (20mM Tris, 500mM NaCl, 0.1% Tween) following which they were probed with a corresponding primary antibody
25 overnight and then with either anti-rabbit or anti-mouse second antibody linked to horseradish peroxidase for 2h. The blots were then developed with aminoethylcarbazole (Figure 7).

30 Production of R51 antibody: Peptide 1 (linear amino acid sequence of PDGF loop) was conjugated to bovine thyroglobulin (Tg) via rabbit serum albumin (RSA), via the thiol group, using the hetero-bifunctional crosslinkers, sulphosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-
35 carboxylate (Sulpho-SMCC) and γ -maleimidobutyrate succinimide (GMBS), respectively. The manufacturer's own procedure was used. The conjugates were made 1mg/ml in PBS and stored at -20°C.

New Zealand white rabbits were immunised with Tg-peptide 1 (100µg of each per rabbit injected subcutaneously in 1ml of 1:1 PBS:Freund's Complete Adjuvant). The rabbits were
5 boosted after 6 and 12 weeks using the same dose but in Freund's Incomplete Adjuvant. Final bleeds were taken 10 days after the last booster injection. The antisera were aliquoted and stored at -20°C.

10 Production of R38 and R65 antibodies: IgG (R38 and R65) was purified from the above-mentioned antisera using Protein-G affinity chromatography (Hi-TRAP Protein-G, Pharmacia, Upsala, Sweden) as described by the
15 manufacturers. Purity was checked by SDS-PAGE. The IgG solutions in PBS were filter sterilised, aliquoted and stored at -20°C.

Example 3 - Identification of RGD Function (dendroaspin activity)

20

Platelet aggregation: Platelet aggregation was measured by the increase in light transmission as described in Lu et al (1994) Biochem J 304: 929-936 and used to identify RGD function. Briefly, platelet rich plasma (PRP) was prepared
25 from citrated human blood from healthy individuals by centrifugation at 200g for 15 min. Platelets were prepared from PRP by centrifugation at 1200g for 15 min. The pellets were washed and resuspended in adhesion/aggregation buffer (145mM NaCl, 5mM KCl, 1mM MgCl₂, 2mM CaCl₂, 10mM
30 glucose, 3.5mg/ml BSA and 10mM HEPES, pH 7.35) and adjusted to a count of 3×10^8 /ml. Platelet aggregation (320 µl incubations) was induced with 10µM ADP in the presence of 1.67mg/ml fibrinogen and measured using a Payton Dual-Aggregometer linked to a chart recorder.

35

Platelet adhesion: Platelet adhesion was also measured as described to further identify RGD function as described in Lu et al (1994) *supra*. Briefly, 96 well plates were coated

overnight at 4°C with either human fibrinogen or fibronectin reconstituted in PBS (pH 7.4) at appropriate concentrations (2-10µg/ml, 100µl volumes). Platelets were treated with antagonists at appropriate concentrations for 5 3 min before addition of 90µl of the mixtures to the microtitre plates which were pre-loaded with 10µl of 500µM ADP giving a final concentration of 50µM and the number of adherent platelets was determined by measuring endogenous acid phosphatase using 130µl/well of developing buffer 10 (sodium acetate, 10mM p-nitrophenyl phosphate, 0.1% Triton X-100, pH 5.5) and read at 410/630nm on an automated plate reader.

Iodination studies of ligands and ligand binding assays:
15 Iodination of all variants of dendroaspin was performed using Enzymobead Radioiodination Reagent (Biorad Laboratories) according to the manufacturer's instructions. The binding of ¹²⁵I-labelled disintegrins, dendroaspin and modified dendroaspins to washed platelets was performed 20 under equilibrium conditions essentially as described previously in Lu et al (1994) *supra*. The incubation mixture contained 300µl of washed platelets (3×10^8 /ml), 10µl agonist (1.75mM ADP giving a final concentration 50µM), 10µl ¹²⁵I-labelled protein samples, 5-20µl 25 resuspension buffer and was made up to a final volume of 350µl. In antibody inhibition studies, platelet suspensions were treated with antibody for 30 min prior to exposure to ADP and then the ¹²⁵I-protein samples added and the mixture was incubated at RT for a further 60 min. 30 Incubations were terminated by loading the mixture onto a 25% (w/v) sucrose, 1% BSA cushion and centrifugation at 12,000g for 10 min. Both platelet pellets and supernatants were counted to determine the levels of bound and free ligand. Background binding levels were determined in the 35 presence of a 50-fold excess of cold protein samples or disintegrin or 10mM EDTA.

Identification of grafted loops: Identification of the functions of a grafted loop is dependent on the function of the parent proteins of the grafted loop.

5 Example 4 - Assays for effects of PDGF Loop I in dendroaspin

Competition ELISAs (CELIA): The relevant quantity of Den-PDGF, or PDGF as competitors were added simultaneously with
10 appropriately diluted rabbit anti-PDGF anti-serum and incubated for 2h. Each rabbit anti-serum was used at a dilution within the linear part of the standard direct ELISA curve and gave an OD of 1.2 to 1.5 at 405nm, after a 30 min incubation with substrate, when assayed against the
15 appropriate RSA-peptide conjugate.

Cell culture and [³H]-thymidine incorporation assay: Human dermal foreskin fibroblasts (used between passage number 7-10) were maintained in complete DMEM containing 10% FBS.
20 Sub-confluent monolayers from 75 cm³ tissue culture flasks were trypsinized, counted and seeded into 24 well (Costar) or 48 well (Falcon) flat bottom tissue culture plates at cell densities of 10,000 cells/well, and grown until approximately 65% confluent (2 days). Cells were rendered
25 quiescent by replacing the medium with complete DMEM containing 0.2% or 0.5% FBS for 48-72h. All cell cultures were performed at 37°C in a humidified atmosphere containing 8% CO₂ in air.

30 Inhibition of growth factor induced cellular proliferation was brought about by replacing the medium, at time zero, with complete DMEM containing varying concentrations of the Den-PDGF and peptide P1 (linear sequence of PDGF domain), respectively. The PDGF concentration for induction was
35 used at a range of 10-20ng/ml.

[³H]-thymidine (0.3 mCi/100μl/well) was added after 20h-22h and incubated until hours 27.5-28. Medium was aspirated, cells were washed twice with cold PBS and fixed by the

addition of 500µl 10% TCA and incubated for 30 min at 4°C. Cells were next washed once with 0.5 ml 70% ethanol and stored at -20 °C. Cells were then solubilised by addition of 500µl of 0.1 N NaOH to each well for 30 min at room temperature. Cellular incorporated [³H]-thymidine was quantified by scintillation counting of a 400µl sample from each well. The percentage of inhibition of cellular proliferation was worked out as:

$$10 \quad \frac{([^3\text{H}]\text{-thymidine incorporated with PDGF}) - ([^3\text{H}]\text{-thymidine incorporated with PDGF+M* or P1})}{([^3\text{H}]\text{-thymidine incorporated with PDGF}) - ([^3\text{H}]\text{-thymidine incorporated with 0.2\% FCS})} \times 100$$

M* : denotes modified dendroaspins

15

Figure 8 shows that the results of tests in which PDGF-dendroaspin inhibits PDGF-induced proliferation by 10-34% (there was no inhibition which has been found when using wild-type dendroaspin as a control) in the range 6.5-60µM.

20

Example 5 - Modified dendroaspin containing a sequence derived from the fifth EGF-like domain of thrombomodulin

Thrombomodulin serves as a receptor of thrombin. Thrombin is a trypsin-like serine protease fulfilling a central role in both haemostasis and thrombosis. In the coagulation cascade, thrombin is the final key enzyme, proteolytically cleaving fibrinogen to release fibrinopeptides A and B and generating fibrin monomers which can then polymerize to form a haemostatic plug. In addition to fibrinogen cleavage, thrombin exerts a positive feedback on its own production by generating coagulation factors Va and VIIIa which act as cofactors of thrombin activation. Factor XIII is also activated by thrombin and cross-links and stabilises the fibrin polymer. Natural anticoagulant mechanisms limit these processes through inhibition by the serpin, antithrombin III and through activation of protein C by thrombin/thrombomodulin complex. Thrombomodulin is a

cell receptor located on the endothelial cell surface and it binds and alters the molecular specificity of thrombin by decreasing its ability to catalyze clot formation while converting thrombin into a potent protein C activator.

5 Activated protein C destroys factor Va and VIIIa terminating the clotting cascade. Thus, the balance between pro- and anti-coagulant mechanisms maintains the normal physiological conditions and allows the local generation of thrombin, whilst preventing it from becoming

10 a systemic or potentially dangerous process. Moreover, thrombin also activates platelets and endothelial cells. Upon platelet activation by thrombin, platelets undergo shape change, aggregation and release their storage granule contents (eg platelet factor-4, ADP, 5-hydroxytryptamine).

15 Thrombin also increases the synthesis and secretion of thromboxane A₂ and platelet activating factor. The interaction of thrombin with endothelial cells also results in the secretion of various agents (eg tissue plasminogen activator, PDGF, endothelin) as well as the acceleration of

20 protein C activation binding to thrombomodulin which initiates protein C anticoagulant pathway.

As examples, a sequence derived from the fifth epidermal growth factor (EGF) like domain of thrombomodulin was

25 grafted into dendroaspin. The thrombin binding affinity of the novel protein was determined on the basis of inhibition of fibrinogen clotting.

An appropriate plasmid expression vector was made according

30 to the procedures of Example 1 except that a thrombomodulin domain was inserted into dendroaspin as shown in Figure 3A where the sequence grafted into loop II of dendroaspin is shown.

35 Expression, isolation and purification of the TM-modified dendroaspin was also performed in accordance with Example 1 and the RGD function of the modified proteins tested as described in Example 2.

Human thrombomodulin has Mr about 100 kDa and consists of an N-terminal domain that is homologous to the family of C-type Lectins, six tandemly repeated epidermal growth factor (EGF)-like domains, a Ser/Thr rich domain, a transmembrane domain, and a short cytoplasmic tail. In a particular example shown in Figure 3C, we have inserted a sequence derived from the fifth EGF-like domain of thrombomodulin into dendroaspin and made additional modifications of appropriate parts of the dendroaspin to prevent the steric hindrance which causes loss of its biological functions.

As indicated in Figure 3D the modified dendroaspin molecules have ADP- and thrombin-induced platelet aggregation activities and they also prolongs the thrombin clotting time.

Example 6 - Assays for effects of thrombomodulin domain in dendroaspin

Thrombin-induced fibrinogen clotting time: Thrombin-induced fibrinogen clotting time was determined using an Amelung KC-10 instrument as follows. 50µl of thrombin (3.3µg/ml) in 50mM Tris-HCL, pH7.5, containing 150mM NaCl and 5mM CaCl₂ was mixed with 10µl of various concentrations of both modified (Den-TM) and wild type dendroaspin (as a control for comparison). After a 2 min incubation at 37°C, 100µl of fibrinogen (160µg/ml) in the same buffer was added to determine the clotting time.

Protein C activation: To check whether the Den-TM has any influence on protein C activation a two stage assay was used as described by Tsiang et al (1990) Biochemistry 29: 10602-10612. In the first stage, thrombin and protein C were added to suitable final concentrations in a reaction volume of 110µl, with or without wild-type or mutant dendroaspins with either 2mM CaCl₂ or 1mM Na₂ EDTA. Reaction mixtures were incubated for 30 min and stopped by addition of antithrombin III and heparin. The active

protein C generated was assayed by hydrolysis of substrate S-2366.

Thrombin-induced platelet aggregation: Thrombin-induced
5 platelet aggregation in washed platelets for both wild-type
and mutant dendroaspins were determined as described in
Example 3 above, except thrombin was used as an agonist.

Thrombomodulin binding: Thrombomodulin binding was
10 performed as described by Tsiang et al (1990) supra.

Example 7 - Modified dendroaspin containing a sequence
derived from glycoprotein (GP) IB

15 Glycoprotein (GP) IB α is required for expression of the
high affinity α -thrombin binding site on platelets (Marco
et al (1994) J Biol Chem 269: 6478-6484). This function
may be of crucial importance in the initiation of
haemostasis and thrombosis and may play a role in the
20 development of pathological vascular occlusion. A modified
dendroaspin was engineered which contains a sequence
derived from GP IB to create both thrombin and integrin
antagonist activities.

25 An appropriate plasmid expression vector was made as
described in Example 1, except that a glycoprotein IB α
domain was inserted into loop I of the dendroaspin molecule
as shown in Figures 3A and 3C where the amino acid residues
of the grafted domain are aligned with the full amino acid
30 sequence of dendroaspin.

As summarised in Figure 3D, the modified dendroaspin
molecules inhibit thrombin activity and platelet
aggregation.

35

Assembly and cloning of the gene of dendroaspin containing
glycoprotein IB (GP IB) domain: The gene of dendroaspin
containing GP IB domain was assembled from the fragments

(81 mer, 82 mer, 42 mer and 44 mer) of wild-type gene, after digestion with Bam HI, EcoR I, Hinf I and Hpa II, and a pair of phosphorylated mutagenesis oligos (83 mer and 82 mer). The experimental procedures of annealing, ligation, PCR and assembling were similar as described previously for Den-PDGF.

Expression, isolation and purification of the GP IB α -modified dendroaspin was performed as described in Example I and the RGD function of the molecule assayed in accordance with Example 3.

Example 8 - Assays for effects of the GP IB domain in dendroaspin

Measurement of thrombin binding to platelets: Thrombin binding to washed platelets was measured in a calcium-free aggregation/adhesion buffer (see measurement of platelet aggregation in example 3). Washed platelets were equilibrated at 37°C for 10 min before the assay and then incubated with ^{125}I - α -thrombin for 10 min at 25°C. Binding as a function of ligand concentration was determined with a constant concentration (0.1nM) of ^{125}I - α -thrombin mixed with increasing concentrations (between 1 and 200nM) of unlabeled α -thrombin. The binding was initiated by the addition of platelets to the thrombin mixture. Platelets and bound thrombin were separated from unbound thrombin after 10 min incubation by centrifugation through a layer of 20% sucrose at 12000g for 4 min (Lu et al (1994) supra).

The effect of dendroaspin containing GP IB domain on thrombin binding to platelets was evaluated by mixing varying concentrations of the mutants with the washed platelets and adding a constant concentration of ^{125}I - α -thrombin. The results were analysed as described previously (Lu et al (1996) supra).

Measurement of platelet aggregation and secretion: The release of ATP from the dense granules of platelets was measured by the luciferin-luciferase assay. Washed platelets were resuspended in 0.4ml calcium-free aggregation/adhesion buffer at a count of 2.5×10^8 /ml, aliquots of 0.4ml were measured using a lumiaggregometer (Chrono-Log Corp). 50 μ l of luciferin-luciferase reagent was then added, followed by α -thrombin at the final concentration of 0.26nM; the ensuing release of ATP was determined by recording the change in luminescence, using a lumiaggregometer (Chrono-Log Corp). Platelet aggregation was measured as described in Example 3 above. To test the inhibitory effect of mutant dendroaspin containing GP IB domain on ATP release and platelet aggregation, mutant proteins were added and mixed with platelets for 5 min at 37°C before addition of luciferin/luciferase and thrombin.

Amidolytic activity of thrombin and clotting of fibrinogen: Amidolytic activity of thrombin and clotting of fibrinogen was determined as described in Example 10 below.

Example 9 - Modified dendroaspin containing a domain of hirudin

Hirudin, a potent thrombin inhibitor from the bloodsucking leech *Hirudo medicinalis*, is a single polypeptide chain protein containing 65 amino acid residues (Maraganore et al (1989) J Biol Chem 264: 8692-8698). We produced a modified dendroaspin which includes amino acid residues Asn⁵²-Leu⁶⁴, or Phe⁴⁵-Gln⁶⁵ from hirudin. The new construct contains both anti-thrombin binding and platelet anti-adhesive domains.

The plasmid expression vector was made as described in Example 7, except that further mutation site has been made for a minor change corresponding to the amino acid residues PRP in loop II of dendroaspin. For the minor changes, the Transformer™ Site-Directed mutagenesis kit from Clontech

Laboratories was used. The experimental procedures were performed according to manufacturer's instructions except that the hirudin domain was grafted into loops I and II of dendroaspin. The modified molecules are shown in Figures 3A and 3C where the hirudin-derived amino acid residues are aligned with the native dendroaspin sequence. Figure 3D indicates how the molecule of Figure 3C delays thrombin clotting time and inhibits ADP or thrombin induced platelet aggregation.

10

Expression, isolation and purification of the Den-HR was essentially as described in Example 1. The RGD function of the molecule was established according to the assay methods of Example 3.

15

Example 10 - Assays for effects of hirudin domain in dendroaspin

Amidolytic activity of thrombin and clotting of fibrinogen:

The amidolytic activity of α -thrombin was performed using the chromogenic substrate S-2238 (Chromogenix) and α -thrombin at a final concentration of 0.06 unit/ml. Thrombin releases p-nitroaniline from the substrate and the rate of this reaction was monitored in microtiter plates at 405 nm using an automated spectrophotometer (Autoreader III, Ortho Diagnostic Systems). The inhibitory effect of Den-HR on thrombin cleavage of the chromogenic substrate was measured at final concentration between nM-mM. Thrombin and Den-HR were premixed and reactions were initiated by the addition of substrate. To evaluate the fibrinogen clotting activity 200 μ l of α -thrombin (final concentration, 1nM) in 0.05 M Na phosphate, pH 6.5 (BSA, final concentration, 1%) was incubated for 5 min at RT and then 200 μ l of normal plasma was added containing 0.011 M trisodium citrate as anticoagulant. The time to observe the start of fibrinogen clotting (thrombin time) was measured using an automated coagulometer at 37°C. The effect of mutants on fibrinogen clotting by α -thrombin was

determined by substituting the HEPES buffer in the first mixture with mutants at a range of concentrations.

5 Example 11 - Modified dendroaspin containing a thrombin-based peptide which blocks the procoagulant activity of thrombin

The binding site for thrombomodulin within human thrombin has been localised to a region in the B-chain of thrombin.
10 Its sequence was introduced into dendroaspin as shown in Figure 3B to generate a bifunctional molecule which blocks thrombin procoagulant activities and also inhibit ADP/thrombin-induced platelet aggregation. Functional characterisation of this mutant includes the measurement of
15 thrombin-induced fibrinogen clotting time as described in Example 6 and the measurement of platelet aggregation induced by ADP/thrombin as described in Examples 3 and 6 above. Figure 3D summarises the properties of the bifunctional molecule as being able to delay thrombin
20 clotting and inhibits platelet aggregation induced either by ADP or thrombin.

In the case of the molecules of Figure 3C, modification of the loops has become necessary due to a steric effect
25 caused by the introduction of "foreign" sequence, e.g. when a sequence derived from PDGF introduced into dendroaspin, anti-PDGF activity was generated, but the anti-platelet activity was lost. This steric effect was designed out by the introduction of a similar RGD-loop as the loop III of
30 dendroaspin into loop I. The sequences of engineered molecules based on dendroaspin scaffold and their functions are summarised in following Table 1 and Figure 1 respectively.

35 Example 12 - Site directed mutagenesis of modified dendroaspins

Each of the modified dendroaspins described above can be modified around the RGD loop or any other portion of the

molecule as is desired by way of site directed mutagenesis. Procedures used are as described in Lu et al (1996) *supra*. Possible RGD flanking region modifications are shown in Figure 3B together with the results of dendroaspin activity
5 assays. Some modifications increase activity whereas other modifications negate activity.

Example 13 - Antithrombotic activity of modified dendroaspins in a guinea pig arterial thrombosis model

10

The four modified dendroaspins produced in the above Examples can be tested for antithrombotic activity *in vivo* in a guinea pig model for arterial thrombosis as described in detail by Carteaux J P et al (1995) *Circulation* 91:
15 1568-1574. A range of doses of each modified dendroaspin in the range 0.1mg/kg of body weight to 1mg/kg can be administered separately to animals by intravenous infusion. Control tests of 50-150IU/kg heparin and placebo can be carried out in parallel.

20

Example 14 - Activity of modified dendroaspins on cellular proliferation following arterial injury in a rabbit atherosclerosis model

25 Den-PDGF, Den-TM, Den-GP and Den-HR can be tested in the rabbit in an *in vivo* model system as described by Ragosla M et al (1996) *Circulation* 93: 1194-1200. After induction of atherosclerosis, rabbits can be infused intravenously with a dosage of 0.1mg-0.5mg/kg of these modified dendroaspins. A
30 first set of control animals were treated intra-arterially with a single bolus of heparin (150IU/kg). A second control set of animals can be set up to receive saline instead of modified dendroaspins. Balloon angioplasty is then performed on test and control animals and followed by
35 quantitative angiography, measurement of activated partial thromboplastin time (aPTT), ³H-thymidine incorporation into the injured artery and follow up studies of luminal narrowing.

Example 15 - Thrombolytic activity of modified dendroaspins
tested in an in vivo pig model system

The model system of Mruk J S et al (1995) Circulation 93:
5 792-799 can be employed. Pigs with occlusive thrombi may
be administered intravenously with 0.1mg-1mg/kg of the
modified dendroaspins mentioned in Example 12 above. A
dose of heparin or hirudin (bolus of 100IU/kg followed
infusion of 20IU/kg) can be administered to control
10 animals. Animals receiving only saline serve as controls.



Claims:

1. A hybrid polypeptide comprising a first amino acid sequence including the RGD motif and conferring dendroaspin activity and a further amino acid sequence conferring activity other than that of dendroaspin activity.
2. A hybrid polypeptide having integrin binding activity comprising a dendroaspin scaffold and a further non-dendroaspin amino acid sequence.
3. A polypeptide as claimed in claim 1 or claim 2, comprising at least two said further amino acid sequences, optionally said further sequences are the same.
4. A polypeptide as claimed in any one of claims 1 to 3 wherein the said further amino acid sequence comprises two or more amino acid sequence portions separated by at least one amino acid residue of dendroaspin.
5. A polypeptide as claimed in any one of claims 1 to 4, wherein said further sequence selected from: platelet derived growth factor (PDGF), glycoprotein IB α , hirudin, thrombomodulin, vascular epidermal growth factor, transforming growth factor- β 1, basic fibroblast growth factor, angiotensin II, factor VIII and von Willebrand factor.
6. A polypeptide as claimed in any preceding claim comprising an amino acid sequence as shown in Figure 3A 3B or 3C.
7. A polypeptide as claimed in any preceding claim, wherein said further sequence is incorporated into (a) loop I and/or loop II; (b) loop I and/or loop III; (c) loop II and/or loop III; or (d) loop I, loop II and loop III of the dendroaspin scaffold.

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8. A polypeptide as claimed in claim 7, wherein said further sequence is incorporated into either loop I or loop II.
- 5 9. A polypeptide as claimed in any preceding claim, wherein the RGD-containing loop is modified by insertion, deletion or substitution of one or more amino acid residues, preferably a maximum of 8 and a minimum of 1 amino acids can be modified within loop III of dendroaspin.
- 10 10. A polypeptide as claimed in claim 9, wherein the RGD loop has an amino acid sequence as shown in table I.
- 15 11. A polypeptide as claimed in any preceding claim, wherein loop I and/or loop II are modified by insertion, deletion or substitution of one or more amino acid residues, preferably a number of amino acid residues in the range 14 to 36.
- 20 12. A polypeptide as claimed in any preceding claim, wherein the said further sequence is inserted into the dendroaspin scaffold between amino acid residues selected from one or more of 2-16, 21-36, 21-31, 28-32, 9-13, 21-33, or at the end of the dendroaspin scaffold after residue 50.
- 25 13. A nucleic acid molecule encoding a polypeptide of any one of claims 1 to 12.
- 30 14. A nucleic acid as claimed in claim 13, operatively linked to a promoter and optionally to a nucleic acid sequence encoding a heterologous protein or peptide thereby to encode a fusion product.
- 35 15. A nucleic acid as claimed in claim 14, wherein the promoter is IPTG inducible and optionally the heterologous protein or peptide is glutathione S-transferase.
16. A plasmid comprising a nucleic acid of any one of claims 13 to 15.





17. Plasmid pGEX-3X comprising the nucleic acid of claim 13.
- 5 18. A host cell transformed with a plasmid as claimed in claim 16 or 17.
19. A host cell as claimed in claim 18 being *E coli*.
- 10 20. A cell culture comprising host cells as claimed in claims 18 or 19.
21. A method of producing a polypeptide as defined in any of claims 1 to 12 comprising culturing a host cell of claim 15 18 or claim 19 so as to express said polypeptide, extracting the polypeptide from the culture and purifying the same.
22. A method of producing a multifunctional anticoagulant 20 comprising:
- a) constructing an expression vector comprising a nucleic acid sequence encoding a dendroaspin scaffold operatively linked to a promoter and optionally linked 25 to nucleic acid encoding an heterologous protein for co-expression therewith.
 - b) modifying at least a portion of the nucleic acid sequence of the vector encoding the dendroaspin scaffold, excluding the RGD motif, by one or more of 30 insertion, deletion or substitution of nucleic acid residues so that on expression the dendroaspin scaffold comprises a further amino acid sequence of activity other than that of dendroaspin activity.
 - 35 c) transforming a host cell with the vector and causing the host cell to express the modified dendroaspin nucleic acid sequence.

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23. A method as claimed in claim 22, further comprising the steps of:

5 d) extracting the modified dendroaspin from a host cell culture,

10 e) purifying the modified dendroaspin from the cell culture extract, optionally including the step of cleaving the dendroaspin from a co-expressed heterologous protein.

24. A method as claimed in claim 23 wherein the heterologous protein is glutathione S-transferase (GST) and the purification involves GST affinity chromatography
15 followed by cleavage of the modified dendroaspin from GST.

25. A polypeptide as claimed in any of claims 1 to 12 obtainable by the method of any one of claims 21 to 24.

20 26. A pharmaceutical composition comprising a therapeutically effective amount of a polypeptide of any one of claims 1 to 12 or 25.

25 27. A composition as claimed in claim 26, further comprising a pharmaceutically acceptable excipient or carrier.

28. A polypeptide as claimed in any one of claims 1 to 12 or 5 for use as a pharmaceutical.
30

29. The use of a polypeptide as claimed in any one of claims 1 to 12 or 25 for the manufacture of a medicament for the treatment or prophylaxis of disease associated with thrombosis.

35

30. The use as claimed in claim 29, wherein said disease is one of more of thrombosis, myocardial infarction, retinal neovascularization, endothelial injury, dysregulated apoptosis, abnormal cell migration, leukocyte





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recruitment, immune system activation, tissue fibrosis and tumorigenesis.

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