(19) DANMARK

(10) DK/EP 3524617 T5

(12) Rettet oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: *C 07 K 16/00 (2006.01)* *C 12 P 21/08 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2024-09-02**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2023-04-12**

(86) Europæisk ansøgning nr.: **19160280.4**

(86) Europæisk indleveringsdag: **2014-03-13**

(87) Den europæiske ansøgnings publiceringsdag: **2019-08-14**

(30) Prioritet: **2013-03-15 US 201361791537 P** **2013-03-15 US 201361787753 P**

(62) Stamansøgningsnr: **14769812.0**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **GLAdiator Biosciences, Inc., 305 East Strawberry Drive, Mill Valley CA 94941, USA**

(72) Opfinder: **BAUZON, Maxine, 1033 Park Street, Hercules, CA 94547, USA**
HERMISTON, Terry, GLAdiator Biosciences, Inc., 305 East Strawberry Drive, Mill Valley, CA 94941, USA

(74) Fuldmægtig i Danmark: **Cosmovici Intellectual Property, Paul Cosmovici, Rue du Commerce 4, 1204 Geneva, Schweiz**

(54) Benævnelse: **GLA-DOMÆNER SOM TERAPEUTISKE MIDLER**

(56) Fremdragne publikationer:
US-A1- 2003 220 490
US-A1- 2009 098 103
US-A1- 2011 159 571
US-B1- 6 312 694
OKADA HIROMI ET AL: "A novel splice site mutation in intron C of PROS1 leads to markedly reduced mutant mRNA level, absence of thrombin-sensitive region, and impaired secretion and cofactor activity of mutant protein S", THROMBOSIS RESEARCH, vol. 125, no. 5, May 2010 (2010-05), pages E246-E250, XP0027027180, ISSN: 0049-3848
MEREL VAN WIJNEN ET AL: "Characterization of mini-protein S, a recombinant variant of protein S that lacks the sex hormone binding globulin-like domain", BIOCHEM. J., vol. 330, 1 January 1998 (1998-01-01), pages 389-396, XP055016149, DOI: 10.1042/bj3300389
KRISTOF SCHUTTERS ET AL: "Phosphatidylserine targeting for diagnosis and treatment of human diseases", APOPTOSIS ; AN INTERNATIONAL JOURNAL ON PROGRAMMED CELL DEATH, KLUWER ACADEMIC PUBLISHERS, BO, vol. 15, no. 9, 4 May 2010 (2010-05-04), pages 1072-1082, XP019812780, ISSN: 1573-675X
BLANKENBERG F G: "Imaging the molecular signatures of apoptosis and injury with radiolabeled annexin V", AMERICAN THORACIC SOCIETY. PROCEEDINGS, AMERICAN THORACIC SOCIETY, NEW YORK, US, vol. 6, no. 5, 15 August 2009 (2009-08-15) , pages 469-476, XP002760074, ISSN: 1546-3222, DOI: 10.1513/PATS.200901-001AW
COHEN AVI ET AL: "From the Gla domain to a novel small-molecule detector of apoptosis", CELL RESEARCH -

Fortsættes ...

XIBAO YANJIU, NATURE PUBLISHING GROUP, GB, CN, vol. 19, no. 5, 1 May 2009 (2009-05-01), pages 625-637, XP002607697, ISSN: 1001-0602, DOI: 10.1038/CR.2009.17 [retrieved on 2009-02-17]
MINGDONG HUANG ET AL: "Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins", NATURE STRUCTURAL BIOLOGY, vol. 10, no. 9, 1 September 2003 (2003-09-01), pages 751-756, XP055092113, ISSN: 1072-8368, DOI: 10.1038/nsb971
M. JACOBS ET AL: "Membrane Binding Properties of the Factor IX y-Carboxyglutamic Acid-rich Domain Prepared by Chemical Synthesis*", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 41, 14 October 1994 (1994-10-14), pages 25494-25501, XP055234798,
STENFLO J: "CONTRIBUTIONS OF GLA AND EGF-LIKE DOMAINS TO THE FUNCTION OF VITAMIN K-DEPENDENT COAGULATION FACTORS", CRITICAL REVIEWS IN EUKARYOTIC GENE EXPRESS, BEGELL HOUSE, NEW YORK, NY, US, vol. 9, no. 1, 1 January 1999 (1999-01-01) , pages 59-88, XP009076199, ISSN: 1045-4403
MILLE-BAKER BLANDINE ET AL: "Deletion or replacement of the second EGF-like domain of protein S results in loss of APC cofactor activity.", BLOOD 15 FEB 2003, vol. 101, no. 4, 15 February 2003 (2003-02-15), pages 1416-1418, XP002792040, ISSN: 0006-4971

DESCRIPTION

BACKGROUND

[0001] This application claims benefit of priority to U.S. Provisional Application Serial No. 61/791,537 filed March 15, 2013, and U.S. Provisional Application Serial No. 61/787,753, filed March 15, 2013.

1. Field

[0002] This disclosure relates to the targeting of phosphatidylserine (PtdS) on cell membranes using Gla domain peptides and polypeptides. The use of these peptides and polypeptides as therapeutic agents is disclosed.

2. Related Art

[0003] Phosphatidlyserine (PtdS) is a negatively charged phospholipid component usually localized to the inner-leaflet (the cytoplasmic side) of the cell membrane. However, PtdS can be transported by scramblase (a member of the flippase family) from the inner-leaflet to the outer-leaflet and exposed on the cell surface. With very few exceptions, this active externalization of PtdS is a response to cellular damage (van den Eijnde *et al.,* 2001; Erwig and Henson, 2008). For example, tissue injury signals platelets, leukocytes, and endothelial cells to rapidly and reversibly redistribute PtdS which leads to the promotion of coagulation and complement activation on cell surfaces. Similarly, apoptotic signals result in the externalization of PtdS however in a more gradual and sustained manner. This external PtdS provides a key recognition marker that enables macrophages to ingest dying cells from surrounding tissue while suppressing a full and detrimental immune response (Erwig and Henson, 2008). This removal process is essential for tissue homeostasis and in a "healthy" environment it is extremely efficient. In fact, despite the loss of $>10^9$ cells per day, the histological detection of apoptotic cells is a rare event in normal tissues (Elltiot and Ravichandran, 2010; Elltiot *et al.,* 2009). However, there is evidence that in many pathological conditions the process of apoptotic cell removal is overwhelmed, delayed or absent (Elltiot and Ravichandran, 2010; Lahorte *et al.,* 2004). For example several oncology studies suggest that a high apoptotic index is associated with higher grade tumors, increased rate of metastasis and a poor prognosis for the patient (Naresh *et al.,* 2001; Loose *et al.,* 2007; Kurihara *et al.,* 2008; Kietselaer *et al.,* 2002). These studies, and others like them, suggest that apoptosis and external PtdS expression can be a powerful marker of disease (Elltiot and Ravichandran, 2010).

There are several proteins with a high affinity for anionic phospholipid surfaces with Annexin-V

being the most widely utilized as a PtdS targeting probe (Lahorte *et al.,* 2004). With a high affinity for PtdS containing vesicles (K_d = 0.5-7 nM) and a molecular weight (37 kDa) that falls below the threshold for kidney filtration (approx. 60 kDa) Annexin-V has shown promise in the clinic as an apoptosis-probe (Lin *et al.,* 2010; Tait and Gibson, 1992). Moreover, it has been utilized for a wide range of indications including those in oncology, neurology and cardiology (Lahorte *et al.,* 2004; Boersma *et al.,* 2005; Reutelingsperger *et al.,* 2002). The use of biologic probes which target PtdS cell-surface expression has been shown both *in vitro* and *in vivo:* Blankenberg, 2009 discloses imaging the molecular signatures of apoptosis injury with radiolabeled annexin V. While their utility in the clinic is promising, they have, for the most part, not yet been exploited.

[0004] Okada *et al,* 2010 discusses a novel splice site mutation in intron C of PROS1 that leads to markedly reduced mutant mRNA level, absence of thrombin-sensitive region, and impaired secretion and cofactor activity of mutant protein S.

[0005] Wijnen *et al,* 1998 discusses the characterization of mini-protein S which is a recombinant variant of protein S that lacks the sex hormone binding globulin-like domain.

[0006] Schutters and Reutelingsperger, 2010 discusses phosphatidylserine targeting for diagnosis and treatment of human disease.
US6,312,694 describes methods of treating cancer using therapeutic conjugates that bind to aminophospholipids.

SUMMARY

[0007] Thus, in accordance with the present disclosure, there is provided a probe and/or therapeutic polypeptide suitable for targeting phosphatidylserine cell-surface expression both *in vitro* and *in vivo* said polypeptide comprising:

a protein S gamma-carboxyglutamic-acid (Gla) domain, and

an EGF domain,

wherein said polypeptide is fused to a proteinaceous sequence; and

wherein said polypeptide lacks a protease domain and also lacks a hormone-binding domain
The molecules according to the disclosure targe cell membrane phosphatidylserine.e . The cell membrane may be a cardiac cell membrane, a neuronal cell membrane, an endothelial cell membrane, a virus-infected cell membrane, an apoptotic cell membrane, a platelet membrane, a plasma membrane-derived veriscle (PMV) or a cancer cell membrane. The polypeptide may further comprise a Kringle domain, and/or an aromatic amino acid stack domain. The polypeptide may comprise a detectable label, such as a fluorescent label, a chemilluminescent label, a radiolabel, an enzyme, a dye or a ligand.

[0008] The polypeptide may be 300 residues or less, 200 residues or less, or 100 residues or less, including ragnes of 100-200 and 100-300 residues. The polypeptide may comprise 5-15 Gla residues, 9-13 Gla residues, including 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 Gla residues. The polypeptide may comprise more than 13 Gla residues, but less than 30% total Gla residues. The polypeptide may be between about 4.5 and 30 kD in size. The polypeptide may comprise at least one disulfide bond, or 2-5 disulfide bonds. The polypeptide may comprise a protein S EGF domain. The polypeptide may further comprise an antibody Fc region. Any of the foregoing may contain conservative substitutions of the native sequences for the foregoing proteins, and/or exhibit a percentage homology to the native domains set forth.

[0009] The polypeptide described herein is useful in treating cancer in a subject. The cancer may be breast cancer, brain cancer, stomach cancer, lung cancer, prostate cancer, ovarian cancer, testicular cancer, colon cancer, skin cancer, rectal cancer, cervical cancer, uterine cancer, liver cancer, pancreatic cancer, head & neck cancer or esophageal cancer. The subject may be treated with a second cancer therapy, such as an immunotherapy, a radiotherapy, a chemotherapy, a toxin therapy, a cytokine therapy or a hormone therapy.

[0010] The polypeptide described herein is useful intreating an autoimmune disease. The autoimmune disease may be spondyloarthropathy, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, enteropathic arthritis, ulcerative colitis, Crohn's disease, irritable bowel disease, inflammatory bowel disease, rheumatoid arthritis, juvenile rheumatoid arthritis, familial Mediterranean fever, amyotrophic lateral sclerosis, Sjogren's syndrome, early arthritis, viral arthritis, multiple sclerosis, systemic lupus erythematosus, psoriasis, vasculitis, Wegener's granulomatosis, Addison's disease, alopecia, antiphospholipid syndrome, Behcet's disease, celiac disease, chronic fatigue syndrome, ulcerative colitis, type I diabetes, fibromyalgia, autoimmune gastritis, Goodpasture syndrome, Graves' disease, idiopathic thrombocytopenic purpura (ITP), myasthenia gravis, pemphigus vulgaris, primary biliary cirrhosis, rheumatic fever, sarcoidosis, scleroderma, vitiligo, vasculitis, small vessel vasculitis, hepatitis, primary biliary cirrhosis, sarcoidosis, scleroderma, graft versus host disease (acute and chronic), aplastic anemia, or cyclic neutropenia. The subject may also be treated with a second autoimmune disease therapy, such as prednisone, methylprednisone, Venipred, Celestone, hydrocortisone, triamcinoclone, Aristonpan Intra-Articular injection, Methapred, Rayos oral, betamethasone, or etanercept.

[0011] The polypeptide described herein is useful intreating a viral disease in a subject. The viral disease may be influenza, human immunodeficiency virus, dengue virus, West Nile virus, smallpox virus, respiratory syncytial virus, Korean hemorrhagic fever virus, chickenpox, varicella zoster virus, herpes simplex virus 1 or 2, Epstein-Barr virus, Marburg virus, hantavirus, yellow fever virus, hepatitis A, B, C or E, Ebola virus, human papilloma virus, rhinovirus, Coxsackie virus, polio virus, measles virus, rubella virus, rabies virus, Newcastle disease virus, rotavirus, HTLV-1 and -2.

[0012] The subject may be further treated with a second anti-viral therapy, such as Abacavir, Aciclovir, Acyclovir, Adefovir, Amantadine, Amprenavir, Ampligen, Arbidol, Atazanavir, Atripla,

Boceprevirertet, Cidofovir, Combivir, Darunavir, Delavirdine, Didanosine, Docosanol, Edoxudine, Efavirenz, Emtricitabine, Enfuvirtide, Entecavir, Entry inhibitors, Famciclovir, Fomivirsen, Fosamprenavir, Foscarnet, Fosfonet, Ganciclovir, Ibacitabine, Imunovir, Idoxuridine, Imiquimod, Indinavir, Inosine, Integrase inhibitor, Interferon type III, Interferon type II, Interferon type I, Interferon, Lamivudine, Lopinavir, Loviride, Maraviroc, Moroxydine, Methisazone, Nelfinavir, Nevirapine, Nexavir, Nucleoside analogues, Oseltamivir, Peginterferon alfa-2a, Penciclovir, Peramivir, Pleconaril, Podophyllotoxin, Protease inhibitor, Raltegravir, Reverse transcriptase inhibitor, Ribavirin, Rimantadine, Ritonavir, Pyramidine, Saquinavir, Stavudine, Synergistic enhancer (antiretroviral), Tea tree oil, Telaprevir, Tenofovir, Tenofovir disoproxil, Tipranavir, Trifluridine, Trizivir, Tromantadine, Truvada, Valaciclovir, Valganciclovir, Vicriviroc, Vidarabine, Viramidine, Zalcitabine, Zanamivir or Zidovudine.

[0013] The polypeptide described herein is useful intreating a hypercoagulation disorder. The subject may be further treated with one or more additional anti-coagulants.

[0014] The polypeptide described herein is useful in modulating clotting in a subj ect. The subj ect may be further administered a clotting factor.

[0015] The polypeptide described herein is useful in treating sepsis in a subject. The polypeptide described herein is useful in treating vaso-occlusive crisis in a sickle cell subject.

[0016] Finally, the polypeptide described herein is useful in treating a disorder characterized by pathologic expression of phosphatidylserine on the surface of a cell.

[0017] Other objects, features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the disclosure, are given by way of illustration only.

BRIEF DESCRIPTION OF THE FIGURES

[0018] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure. The disclosure may be better understood by reference to one or more of these drawings in combination with the detailed.

FIG. 1- Construction of a panel of Gla and Gla-EGF/Kringle domain proteins.

FIG. 2 - Testing of Gla domain protein constructs for expression. Transient transfection into 293 cells using 293cellFectin. 10% gels with reduced samples, 23.3 µl of media loaded.

FIG. 3 - Testing of Gla domain protein constructs for expression. Transient transfection in BHK21 cells. 10% gels with reduced samples, 20 µl (1/100 total cell pellet) loaded.

FIG. 4 - Changing signal sequence alter secretion. Transient transfection in BHK21 cells. 10%

gels with reduced samples, 13.3 µl loaded.

FIG. 5 - Protein S Gla + EGF sequence.

FIG. 6 - Purification of Protein S Gla + EGF. F1-F4 are column chromatography fractions. 10% gels, non-reducing conditions.

FIG. 7 - Apoptosis Assays for Protein S Gla + EGF. Top and bottom panels represent identical duplicate procedures except that amounts of Protein S Gla + EGF was reduced, and the amount of anti-His domain antibody was reduced.

FIG. 8 - Apoptosis Assays for Protein S Gla + EGF. Top and bottom panels represent identical duplicate procedures except for amounts of Annexin V used, which are double in the bottom panels.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0019] Like annexin, gamma-carboxyglutamic-acid (Gla)-domain proteins such as Factors II, VII, IX, X, protein C, and protein S bind anionic membranes. In fact, the Gla-domain has been used as a model for a small molecule that was rationally designed to be an apoptosis-specific probe (Cohen *et al.,* 2009). The use of these naturally-occurring and targeted proteins may lead to enhanced specificity relative to current probes with the added advantage of a smaller size (<30 kDa). Even in larger embodiments, which would include EGF and/or Kringle domains, these proteins can still be smaller than Annexin V (37 kDa), and potentially as small as <5 kDa. These biologic probes can target PtdS cell-surface expression both *in vitro* and *in vivo.* Thus, it is possible to develop an apoptosis/disease targeting probe that is superior to Annexin V in affinity, specificity and size with the added potential for use as a therapeutic. These and other aspects of the disclosure are described in greater detail below.

[0020] Whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any definition set forth below conflicts with the usage of that word in any other document, the definition set forth below shall always control for purposes of interpreting this specification and its associated claims unless a contrary meaning is clearly intended (for example in the document where the term is originally used). The use of "or" means "and/or" unless stated otherwise. The use of "a" herein means "one or more" unless stated otherwise or where the use of "one or more" is clearly inappropriate. The use of "comprise," "comprises," "comprising," "include," "includes," and "including" are interchangeable and are not limiting. For example, the term "including" shall mean "including, but not limited to." The word "about" means plus or minus 5% of the stated number.

[0021] An "isolated peptide or polypeptide," as used herein, is intended to refer to a peptide or polypeptide which is substantially free of other biological molecules, including peptides or

polypeptides having distinct sequences. In some embodiments, the isolated peptide or polypeptide is at least about 75%, about 80%, about 90%, about 95%, about 97%, about 99%, about 99.9% or about 100% pure by dry weight. In some embodiments, purity can be measured by a method such as column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

[0022] As used herein, "conservative substitutions" refers to modifications of a polypeptide that involve the substitution of one or more amino acids for amino acids having similar biochemical properties that do not result in loss of a biological or biochemical function of the polypeptide. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.,* glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), β-branched side chains (*e.g.*, threonine, valine, isoleucine), and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Antibodies of the present disclosure can have one or more conservative amino acid substitutions yet retain antigen binding activity.

[0023] For nucleic acids and polypeptides, the term "substantial homology" indicates that two nucleic acids or two polypeptides, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide or amino acid insertions or deletions, in at least about 80% of the nucleotides or amino acids, usually at least about 85%, in some embodiments about 90%, 91%, 92%, 93%, 94%, or 95%, in at least one embodiment at least about 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, or 99.5% of the nucleotides or amino acids. Alternatively, substantial homology for nucleic acids exists when the segments will hybridize under selective hybridization conditions to the complement of the strand. Also included are nucleic acid sequences and polypeptide sequences having substantial homology to the specific nucleic acid sequences and amino acid sequences recited herein.

[0024] The percent identity between two sequences is a function of the number of identical positions shared by the sequences (*i.e.,* % homology = # of identical positions / total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. Comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, such as without limitation the AlignX™ module of VectorNTI™ (Invitrogen Corp., Carlsbad, CA). For AlignX™, the default parameters of multiple alignment are: gap opening penalty: 10; gap extension penalty: 0.05; gap separation penalty range: 8; % identity for alignment delay: 40. (further details at world-wide-web at invitrogen.com/site/us/en/home/LINNEA-Online-Guides/LINNEA-Communities/Vector-NTI-Community/Sequence-analysis-and-data-management-software-for-PCs/AlignX-Module-for-Vector-NTI-Advance.reg.us.html).

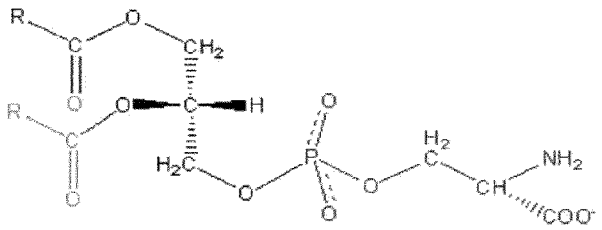
[0025] Another method for determining the best overall match between a query sequence (a sequence of the present disclosure) and a subject sequence, also referred to as a global sequence alignment, can be determined using the CLUSTALW computer program (Thompson et al., Nucleic Acids Res, 1994, 2(22): 4673-4680), which is based on the algorithm of Higgins et al., Computer Applications in the Biosciences (CABIOS), 1992, 8(2): 189-191). In a sequence alignment the query and subject sequences are both DNA sequences. The result of the global sequence alignment is in percent identity. Parameters that can be used in a CLUSTALW alignment of DNA sequences to calculate percent identity via pairwise alignments are: Matrix = IUB, k-tuple = 1, Number of Top Diagonals = 5, Gap Penalty = 3, Gap Open Penalty = 10, Gap Extension Penalty = 0.1. For multiple alignments, the following CLUSTALW parameters can be used: Gap Opening Penalty = 10, Gap Extension Parameter = 0.05; Gap Separation Penalty Range = 8; % Identity for Alignment Delay = 40.

[0026] The nucleic acids can be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components with which it is normally associated in the natural environment. To isolate a nucleic acid, standard techniques such as the following can be used: alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art.

I. Phosphatidylserine (PtdS)

A. Structure and Synthesis

[0027] Phosphatidylserine (abbreviated PtdS, Ptd-L-Ser or PS) is a phospholipid component, usually kept on the inner-leaflet (the cytosolic side) of cell membranes by an enzyme called flippase. When a cell undergoes apoptosis, phosphatidylserine is no longer restricted to the cytosolic part of the membrane, but becomes exposed on the surface of the cell. The chemical formula of PtdS is $C_{13}H_{24}NO_{10}P$ and has a molecular mass of 385.304. The structure is shown below:



[0028] Phosphatidylserine is biosynthesized in bacteria by condensing the amino acid serine with CDP (cytidine diphosphate)-activated phosphatidic acid. In mammals, phosphatidylserine is produced by base-exchange reactions with phosphatidylcholine and phosphatidylethanolamine. Conversely, phosphatidylserine can also give rise to

phosphatidylethanolamine and phosphatidylcholine, although in animals the pathway to generate phosphatidylcholine from phosphatidylserine only operates in the liver.

B. Function

[0029] Early studies of phosphatidylserine distilled the chemical from bovine brain. Modem studies and commercially available products are made from soybeans, because of concerns about mad cow disease. The fatty acids attached to the serine in the soy product are not identical to those in the bovine product and is also impure. Preliminary studies in rats indicate that the soy product is at least as potent as that of bovine origin.

[0030] The U.S. FDA has given "qualified health claim" status to phosphatidylserine, stating that, "Consumption of phosphatidylserine may reduce the risk of dementia in the elderly" and "Consumption of phosphatidylserine may reduce the risk of cognitive dysfunction in the elderly."

[0031] Phosphatidylserine has been demonstrated to speed up recovery, prevent muscle soreness, improve well-being, and might possess ergogenic properties in athletes involved in cycling, weight training and endurance running. Soy-PtdS, in a dose dependent manner (400 mg), has been reported to be an effective supplement for combating exercise-induced stress by blunting the exercise-induced increase in cortisol levels. PtdS supplementation promotes a desirable hormonal balance for athletes and might attenuate the physiological deterioration that accompanies overtraining and/or overstretching. In recent studies, PtdS has been shown to enhance mood in a cohort of young people during mental stress and to improve accuracy during tee-off by increasing the stress resistance of golfers. First pilot studies indicate that PtdS supplementation might be beneficial for children with attention-deficit hyperactivity disorder.

[0032] Traditionally, PtdS supplements were derived from bovine cortex (BC-PS); however, due to the potential transfer of infectious diseases, soy-derived PS (S-PS) has been established as a potential safe alternative. Soy-derived PS is Generally Recognized As Safe (GRAS) and is a safe nutritional supplement for older persons if taken up to a dosage of 200 mg three times daily. Phosphatidylserine has been shown to reduce specific immune response in mice.

[0033] PtdS can be found in meat, but is most abundant in the brain and in innards such as liver and kidney. Only small amounts of PS can be found in dairy products or in vegetables, with the exception of white beans.

[0034] Annexin-A5 is a naturally-occurring protein with avid binding affinity for PtdS. Labeled-annexin-A5 enables visualization of cells in the early- to mid-apoptotic state *in vitro* or *in vivo.* Another PtdS binding protein is Mfge8. Technetium-labeled annexin-A5 enables distinction between malignant and benign tumors whose pathology includes a high rate of cell division and apoptosis in malignant compared with a low rate of apoptosis in benign tumors.

II. Gla Domain Proteins

A. Gla Domains

[0035] The general structure for the Gla-domain proteins is that of a Gla domain followed by EGF domains and then a C terminal serine protease domain. The exceptions are prothrombin, which contains Kringle domains in place of EGF domains, and protein S, which does not have a serine protease domain but rather sex hormone-binding globulin-like (SHBG) domains (Hansson and Stenflo, 2005). The affinities of Gla-domain proteins to anionic membranes vary. Roughly, they fall into 3 categories 1) high affinity binders with a Kd of 30-50 nM, 2) mid-affinity binders with a K_d of 100-200 nM and 3) low affinity binders with a Kd of 1000-2000 nM. The high affinity Gla domain proteins have been shown to bind anionic membranes with Protein S specifically demonstrating binding to apoptotic cells via its interaction with PtdS (Webb *et al.,* 2002). The low affinity Gla domain proteins use a secondary receptor to bind to the cell membrane. For example, FVII utilizes Tissue Factor (TF). The Gla domain/1st EGF domain is believed to constitute the high affinity TF binding domain of FVII. Importantly for this approach, there are many studies that have shown TF up-regulation on the surface of cancer cells including colorectal cancer, NSCL carcinoma, and breast cancer and these high TF levels have been associated with a poor prognosis (Yu *et al.,* 2004). Although the affinity for anionic membranes is relatively low for FVII, the addition of the high affinity TF interaction along with the documented up-regulation of TF in cancer makes it a potentially interesting cancer specific probe.

B. Gla Domain Containing Proteins

1. Factor II

[0036] Prothrombin, also known as coagulation factor II, is proteolytically cleaved to form thrombin in the coagulation cascade, which ultimately results in the stemming of blood loss.

2. Factor VII

[0037] Factor VII (formerly known as proconvertin) is one of the proteins that causes blood to clot in the coagulation cascade.

3. Factor IX

[0038] Factor IX (or Christmas factor) is one of the serine proteases of the coagulation system; it belongs to peptidase family S1.

[0039] Factors VII, IX, and X all play key roles in blood coagulation and also share a common domain architecture. The factor IX protein is composed of four protein domains. These are the Gla domain, two tandem copies of the EGF domain and a C-terminal trypsin-like peptidase domain which carries out the catalytic cleavage. The N-terminal EGF domain has been shown to at least in part be responsible for binding Tissue factor. Wilkinson *et al.* conclude that residues 88 to 109 of the second EGF domain mediate binding to platelets and assembly of the Factor X activating complex. The structures of all four domains have been solved. A structure of the two EGF domains and trypsin like domain was determined for the pig protein. The structure of the Gla domain, which is responsible for Ca(II)-dependent phospholipid binding, was also determined by NMR. Several structures of "super active" mutants have been solved which reveal the nature of Factor IX activation by other proteins in the clotting cascade.

4. Factor X

[0040] Factor X (Stuart-Prower factor; prothrombinase) is an enzyme of the coagulation cascade.

5. Protein S

[0041] Protein S is a vitamin K-dependent plasma glycoprotein synthesized in the endothelium. In the circulation, Protein S exists in two forms: a free form and a complex form bound to complement protein C4b-binding protein (C4BP). In humans, Protein S is encoded by the *PROS1* gene. The best characterized function of Protein S is its role in the anti coagulation pathway, where it functions as a cofactor to Protein C in the inactivation of Factors Va and Villa. Only the free form has cofactor activity.

[0042] Protein S can bind to negatively charged phospholipids via the carboxylated GLA domain. This property allows Protein S to function in the removal of cells which are undergoing apoptosis. Apoptosis is a form of cell death that is used by the body to remove unwanted or damaged cells from tissues. Cells which are apoptotic (*i.e.,* in the process of apoptosis) no longer actively manage the distribution of phospholipids in their outer membrane and hence begin to display negatively charged phospholipids, such as phosphatidyl serine, on the cell surface. In healthy cells, an ATP (Adenosine triphosphate)-dependent enzyme removes these from the outer leaflet of the cell membrane. These negatively charged phospholipids are recognized by phagocytes such as macrophages. Protein S can bind to the negatively charged phospholipids and function as a bridging molecule between the apoptotic cell and the

phagocyte. The bridging property of Protein S enhances the phagocytosis of the apoptotic cell, allowing it to be removed 'cleanly' without any symptoms of tissue damage such as inflammation occurring.

[0043] Mutations in the PROS1 gene can lead to Protein S deficiency which is a rare blood disorder which can lead to an increased risk of thrombosis. Protein S has been shown to interact with Factor V.

6. Protein C

[0044] Protein C, also known as autoprothrombin IIA and blood coagulation factor XIV, is a zymogenic (inactive) protein, the activated form of which plays an important role in regulating blood clotting, inflammation, cell death, and maintaining the permeability of blood vessel walls in humans and other animals. Activated protein C (APC) performs these operations primarily by proteolytically inactivating proteins Factor V_a and Factor $VIII_a$. APC is classified as a serine protease as it contains a residue of serine in its active site.

[0045] Human protein C is a vitamin K-dependent glycoprotein structurally similar to other vitamin K-dependent proteins affecting blood clotting, such as prothrombin, Factor VII, Factor IX and Factor X. Protein C synthesis occurs in the liver and begins with a single-chain precursor molecule: a 32 amino acid N-terminus signal peptide preceding a propeptide. Protein C is formed when a dipeptide of Lys^{198} and Arg^{199} is removed; this causes the transformation into a heterodimer with N-linked carbohydrates on each chain. The protein has one light chain (21 kDa) and one heavy chain (41 kDa) connected by a disulfide bond between Cys^{183} and Cys^{319}.

[0046] Inactive protein C comprises 419 amino acids in multiple domains: one Gla domain (residues 43-88); a helical aromatic segment (89-96); two epidermal growth factor (EGF)-like domains (97-132 and 136-176); an activation peptide (200-211); and a trypsin-like serine protease domain (212-450). The light chain contains the Gla- and EGF-like domains and the aromatic segment. The heavy chain contains the protease domain and the activation petide. It is in this form that 85-90% of protein C circulates in the plasma as a zymogen, waiting to be activated. The remaining protein C zymogen comprises slightly modified forms of the protein. Activation of the enzyme occurs when a thrombin molecule cleaves away the activation peptide from the N-terminus of the heavy chain. The active site contains a catalytic triad typical of serine proteases (His^{253}, Asp^{299} and Ser^{402}).

C. Gla Domain Peptides and Polypeptide

[0047] The present disclosure contemplates the design, production and use of various Gla

domain-containing peptides and polypeptides. The structural features of these molecules are as follows. First, the peptides or polypeptides have a Gla domain containing about 30-45 consecutive residues comprising a Gla domain. Thus, the term "a peptide having no more than "X" consecutive residues," even when including the term "comprising," cannot be understood to comprise a greater number of consecutive residues. Second, the peptides and polypeptides may contain additional non-Gla domain residues, such as EGF domains, Kringle domains, Fc domains, *etc.*.

[0048] In general, the peptides and polypeptides will be 300 residues or less, again, comprising 30-45 consecutive residues of Gla domain. The overall length may be 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 225, 250, 275 and up to 300 residues. Ranges of peptide length of 50-300 residues, 100-300 residues, 150-300 residues 200-300, residues, 50-200 residues, 100-200 residues, and 150-300 residues, and 150-200 residues are contemplated. The number of consecutive Gla residues may be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15.

[0049] The present disclosure may utilize L-configuration amino acids, D-configuration amino acids, or a mixture thereof. While L-amino acids represent the vast majority of amino acids found in proteins, D-amino acids are found in some proteins produced by exotic sea-dwelling organisms, such as cone snails. They are also abundant components of the peptidoglycan cell walls of bacteria. D-serine may act as a neurotransmitter in the brain. The L and D convention for amino acid configuration refers not to the optical activity of the amino acid itself, but rather to the optical activity of the isomer of glyceraldehyde from which that amino acid can theoretically be synthesized (D-glyceraldehyde is dextrorotary; L-glyceraldehyde is levorotary).

[0050] One form of an "all-D" peptide is a retro-inverso peptide. Retro-inverso modification of naturally occurring polypeptides involves the synthetic assemblage of amino acids with α-carbon stereochemistry opposite to that of the corresponding L-amino acids, *i.e.,* D-amino acids in reverse order with respect to the native peptide sequence. A retro-inverso analogue thus has reversed termini and reversed direction of peptide bonds (NH-CO rather than CO-NH) while approximately maintaining the topology of the side chains as in the native peptide sequence. See U.S. Patent 6,261,569.

D. Synthesis

[0051] It will be advantageous to produce peptides and polypeptides using the solid-phase synthetic techniques (Merrifield, 1963). Other peptide synthesis techniques are well known to those of skill in the art (Bodanszky *et al.,* 1976; Peptide Synthesis, 1985; Solid Phase Peptide Synthelia, 1984). Appropriate protective groups for use in such syntheses will be found in the above texts, as well as in Protective Groups in Organic Chemistry (1973). These synthetic methods involve the sequential addition of one or more amino acid residues or suitable protected amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable

protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group, such as lysine.

[0052] Using solid phase synthesis as an example, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to provide the final peptide. The peptides and polypeptides of the disclosure are preferably devoid of benzylated or methylbenzylated amino acids. Such protecting group moieties may be used in the course of synthesis, but they are removed before the peptides and polypeptides are used. Additional reactions may be necessary, as described elsewhere, to form intramolecular linkages to restrain conformation.

[0053] Aside from the twenty standard amino acids can be used, there are a vast number of "non-standard" amino acids. Two of these can be specified by the genetic code, but are rather rare in proteins. Selenocysteine is incorporated into some proteins at a UGA codon, which is normally a stop codon. Pyrrolysine is used by some methanogenic archaea in enzymes that they use to produce methane. It is coded for with the codon UAG. Examples of non-standard amino acids that are not found in proteins include lanthionine, 2-aminoisobutyric acid, dehydroalanine and the neurotransmitter gamma-aminobutyric acid. Non-standard amino acids often occur as intermediates in the metabolic pathways for standard amino acids - for example ornithine and citrulline occur in the urea cycle, part of amino acid catabolism. Non-standard amino acids are usually formed through modifications to standard amino acids. For example, homocysteine is formed through the transsulfuration pathway or by the demethylation of methionine via the intermediate metabolite S-adenosyl methionine, while hydroxyproline is made by a posttranslational modification of proline.

E. Linkers

[0054] Linkers or cross-linking agents may be used to fuse Gla domain peptides or polypeptides to other proteinaceous sequences (*e.g.*, antibody Fc domains). Bifunctional cross-linking reagents have been extensively used for a variety of purposes including preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites, and structural studies. Homobifunctional reagents that carry two identical functional groups proved to be highly efficient in inducing cross-linking between identical and different macromolecules or subunits of a macromolecule, and linking of polypeptide ligands to their specific binding sites. Heterobifunctional reagents contain two different functional groups. By taking advantage of the differential reactivities of the

two different functional groups, cross-linking can be controlled both selectively and sequentially. The bifunctional cross-linking reagents can be divided according to the specificity of their functional groups, *e.g.*, amino-, sulfhydryl-, guanidino-, indole-, or carboxyl-specific groups. Of these, reagents directed to free amino groups have become especially popular because of their commercial availability, ease of synthesis and the mild reaction conditions under which they can be applied. A majority of heterobifunctional cross-linking reagents contains a primary amine-reactive group and a thiol-reactive group.

[0055] In another example, heterobifunctional cross-linking reagents and methods of using the cross-linking reagents are described in US5,889,155. The cross-linking reagents combine a nucleophilic hydrazide residue with an electrophilic maleimide residue, allowing coupling in one example, of aldehydes to free thiols. The cross-linking reagent can be modified to cross-link various functional groups and is thus useful for cross-linking polypeptides. In instances where a particular peptide does not contain a residue amenable for a given cross-linking reagent in its native sequence, conservative genetic or synthetic amino acid changes in the primary sequence can be utilized.

F. Additional Peptide/Polypeptide Sequences

[0056] One factor drug development is to achieve adequate circulating half-lives, which impact dosing, drug administration and efficacy, and this has particular important to biotherapeutics. Small proteins below 60 kD are cleared rapidly by the kidney and therefore do not reach their target. This means that high doses are needed to reach efficacy. The modifications currently used to increase the half-life of proteins in circulation include: PEGylation; conjugation or genetic fusion with proteins, e.g., transferrin (WO2006/096515), albumin, growth hormone (US2003/104578); conjugation with cellulose (Levy and Shoseyov, 2002); conjugation or fusion with Fc fragments; glycosylation and mutagenesis approaches (Carter, 2006).

[0057] In the case of PEGylation, polyethylene glycol (PEG) is conjugated to the protein, which can be for example a plasma protein, antibody or antibody fragment. The first studies regarding the effect of PEGylation of antibodies were performed in the 1980s. The conjugation can be done either enzymatically or chemically and is well established in the art (Chapman, 2002; Veronese and Pasut, 2005). With PEGylation the total size can be increased, which reduces the chance of renal filtration. PEGylation further protects from proteolytic degradation and slows the clearance from the blood. Further, it has been reported that PEGylation can reduce immunogenicity and increase solubility. The improved pharmacokinetics by the addition of PEG is due to several different mechanisms: increase in size of the molecule, protection from proteolysis, reduced antigenicity, and the masking of specific sequences from cellular receptors. In the case of antibody fragments (Fab), a 20-fold increase in plasma half-life has been achieved by PEGylation (Chapman, 2002).

[0058] To date there are several approved PEGylated drugs, *e.g.*, PEG-interferon alpha2b (PEG-INTRON) marketed in 2000 and alpha2a (Pegasys) marketed in 2002. A PEGylated

antibody fragment against TNF alpha, called Cimzia or Certolizumab Pegol, was filed for FDA approval for the treatment of Crohn's disease in 2007 and has been approved on Apr. 22, 2008. A limitation of PEGylation is the difficulty in synthesizing long monodisperse species, especially when PEG chains over 1000 kD are needed. For many applications, polydisperse PEG with a chain length over 10000 kD is used, resulting in a population of conj ugates having different length PEG chains, which need extensive analytics to ensure equivalent batches between productions. The different length of the PEG chains may result in different biological activities and therefore different pharmacokinetics. Another limitation of PEGylation is a decrease in affinity or activity as it has been observed with alpha-interferon Pegasys, which has only 7% of the antiviral activity of the native protein, but has improved pharmacokinetics due to the enhanced plasma half-life.

[0059] Another approach is to conjugate the drug with a long lived protein, *e.g.*, albumin, which is 67 kD and has plasma half-life of 19 days in human. Albumin is the most abundant protein in plasma and is involved in plasma pH regulation, but also serves as a carrier of substances in plasma. In the case of CD4, increased plasma half-life has been achieved after fusing it to human serum albumin (Yeh *et al.,* 1992). Other examples for fusion proteins are insulin, human growth hormone, transferrin and cytokines (Duttaroy *et al.,* 2005; Melder *et al.,* 2005; Osborn *et al.,* 2002a; Osborn *et al.,* 2002b; Sung *et al.,* 2003) and see (US 2003/104578, WO2006/096515, and WO2007/047504).

[0060] The effect of glycosylation on plasma half-life and protein activity has also been extensively studied. In the case of tissue plasminogen activator (tPA), the addition of new glycosylation sites decreased the plasma clearance, and improved the potency (Keyt *et al.,* 1994). Glycoengineering has been successfully applied for a number of recombinant proteins and immunoglobulins (Elliott *et al.,* 2003; Raju and Scallon, 2007; Sinclair and Elliott, 2005; Umana *et al.,* 1999). Further, glycosylation influences the stability of immunoglobulins (Mimura *et al.,* 2000; Raju and Scallon, 2006).

[0061] Another molecule used for fusion proteins is the Fc fragment of an IgG (Ashkenazi and Chamow, 1997). The Fc fusion approach has been utilized, for example in the Trap Technology developed by Regeneron (*e.g.*, IL1 trap and VEGF trap). The use of albumin to extend the half-life of peptides has been described in US2004/001827. Positive effects of albumin have also been reported for Fab fragments and scFv-HSA fusion protein. It has been demonstrated that the prolonged serum half-life of albumin is due to a recycling process mediated by the FcRn (Anderson *et al.,* 2006; Chaudhury *et al.,* 2003).

[0062] Another strategy is to use directed mutagenesis techniques targeting the interaction of immunoglobulins to their receptor to improve binding properties, *i.e.,* affinity maturation in the Fc region. With an increased affinity to FcRn a prolonged half-life can be achieved *in vivo* (Ghetie *et al.,* 1997; Hinton *et al.,* 2006; Jain *et al.,* 2007; Petkova *et al.,* 2006a; Vaccaro *et al.,* 2005). However, affinity maturation strategies require several rounds of mutagenesis and testing. This takes time, is costly and is limited by the number of amino acids that when mutated result in prolonged half-lives. Therefore, simple alternative approaches are needed to

improve the *in vivo* half-life of biotherapeutics. Therapeutics with extended half-lives *in vivo* are especially important for the treatment of chronic diseases, autoimmune disorders, inflammatory, metabolic, infectious, and eye diseases, and cancer, especially when therapy is required over a long time period. Accordingly, a need still exists for the development of therapeutic agents (*e.g.*, antibodies and Fc fusion proteins) with enhanced persistence and half-lives in circulation, in order to reduce the dosage and/or the frequency of injections of a variety of therapeutic agents.

G. Labels

[0063] The peptides and polypeptides of the present disclosure may be conjugated to labels for diagnostic purposes. A label in accordance with the present disclosure is defined as any moiety which may be detected using an assay. Non-limiting examples of reporter molecules include enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, photoaffinity molecules, colored particles or ligands, such as biotin.

[0064] Label conjugates are generally preferred for use as diagnostic agents. Diagnostic agents generally fall within two classes, those for use in *in vitro* diagnostics, and those for use *in vivo* diagnostic protocols, generally known as "directed imaging." Many appropriate imaging agents are known in the art, as are methods for their attachment to peptides and polypeptides (see, for e.g., US5,021,236, US4,938,948, and US4,472,509). The imaging moieties used can be paramagnetic ions, radioactive isotopes, fluorochromes, NMR-detectable substances, and X-ray imaging agents.

[0065] In the case of paramagnetic ions, one might mention by way of example ions such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and/or erbium (III), with gadolinium being particularly preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III).

[0066] In the case of radioactive isotopes for therapeutic and/or diagnostic application, one might mention astatine^{211}, ^{14}carbon, ^{51}chromium, ^{36}chlorine, ^{57}cobalt, ^{58}cobalt, copper^{67}, ^{152}Eu, gallium^{67}, ^{3}hydrogen, iodine^{123}, iodine^{125}, iodine^{131}, indium^{111}, ^{59}iron, ^{32}phosphorus, rhenium^{186}, rhenium^{188}, ^{75}selenium, ^{35}sulphur, technicium^{99m} and/or yttrium^{90}. ^{125}I is often being preferred for use in certain embodiments, and technicium^{99m} and/or indium^{111} are also often preferred due to their low energy and suitability for long range detection. Radioactively labeled peptides and polypeptides may be produced according to well-known methods in the art. For instance, peptides and polypeptides can be iodinated by contact with sodium and/or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Petides may be labeled with technetium^{99m} by ligand

exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the peptide to this column. Alternatively, direct labeling techniques may be used, *e.g.*, by incubating pertechnate, a reducing agent such as $SNCl_2$, a buffer solution such as sodium-potassium phthalate solution, and the peptide. Intermediary functional groups which are often used to bind radioisotopes which exist as metallic ions to peptide are diethylenetriaminepentaacetic acid (DTPA) or ethylene diaminetetracetic acid (EDTA).

[0067] Among the fluorescent labels contemplated for use as conjugates include Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, Cy3, Cy5,6-FAM, Fluorescein Isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, TAMRA, TET, Tetramethylrhodamine, and/or Texas Red.

[0068] Another type of conjugate contemplated is that intended primarily for use *in vitro,* where the peptide is linked to a secondary binding ligand and/or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of suitable enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase or glucose oxidase. Preferred secondary binding ligands are biotin and avidin and streptavidin compounds. The use of such labels is well known to those of skill in the art and is described, for example, in US3,817,837, US3,850,752, US3,939,350, US3,996,345, US4,277,437, US4,275,149 and US4,366,241.

[0069] Other methods are known in the art for the attachment or conjugation of a peptide to its conjugate moiety. Some attachment methods involve the use of a metal chelate complex employing, for example, an organic chelating agent such a diethylenetriaminepentaacetic acid anhydride (DTPA); ethylenetriaminetetraacetic acid; N-chloro-p-toluenesulfonamide; and/or tetrachloro-3α-6α-diphenylglycouril-3 attached to the antibody (US4,472,509 and US4,938,948). Polypeptides may also be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate.

IV. Therapies

A. Pharmaceutical Formulations and Routes of Administration

[0070] Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

[0071] One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present disclosure comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes 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 vectors or cells of the present disclosure, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

[0072] The active compositions of the present disclosure may include classic pharmaceutical preparations. Administration of these compositions according to the present disclosure will be via any common route so long as the target tissue is available via that route. Such routes include oral, nasal, buccal, rectal, vaginal or topical route. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intratumoral, intraperitoneal, or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra.*

[0073] The active compounds may also be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0074] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It 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 proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, 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.

[0075] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various other ingredients as enumerated above, as required, followed by filtered sterilization. Generally, dispersions are 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, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0076] As used herein, "pharmaceutically acceptable carrier" includes 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 pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[0077] For oral administration the peptides and polypeptides of the present disclosure may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

[0078] The compositions of the present disclosure may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

[0079] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic

with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences," 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

B. Disease States and Conditions

1. Cancer

[0080] Background. Cancer results from the outgrowth of a clonal population of cells from tissue. The development of cancer, referred to as carcinogenesis, can be modeled and characterized in a number of ways. An association between the development of cancer and inflammation has long-been appreciated. The inflammatory response is involved in the host defense against microbial infection, and also drives tissue repair and regeneration. Considerable evidence points to a connection between inflammation and a risk of developing cancer, *i.e.,* chronic inflammation can lead to dysplasia. There are hundreds of different forms of human cancers, and with an increasing understanding of the underlying genetics and biology of cancer, these forms are being further subdivided and reclassified.

[0081] Determining what causes cancer is complex. Many things are known to increase the risk of cancer, including tobacco use, certain infections, radiation, lack of physical activity, obesity, and environmental pollutants. These can directly damage genes or combine with existing genetic faults within cells to cause the disease. Approximately five to ten percent of cancers are entirely hereditary.

[0082] Cancer can be detected in a number of ways, including the presence of certain signs and symptoms, screening tests, or medical imaging. Once a possible cancer is detected it is diagnosed by microscopic examination of a tissue sample. Cancer is usually treated with chemotherapy, radiation therapy and surgery. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age. In 2007, cancer caused about 13% of all human deaths worldwide (7.9 million). Rates are rising as more people live to an old age and as mass lifestyle changes occur in the developing world.

[0083] Treatments fall in to five general categories: surgery, chemotherapy, radiation, alternative medicine and palliative care. Surgery is the primary method of treatment of most isolated solid cancers and may play a role in palliation and prolongation of survival. It is typically an important part of making the definitive diagnosis and staging the tumor as biopsies are usually required. In localized cancer surgery typically attempts to remove the entire mass along with, in certain cases, the lymph nodes in the area. For some types of cancer this is all that is needed to eliminate the cancer.

[0084] Chemotherapy in addition to surgery has proven useful in a number of different cancer types including: breast cancer, colorectal cancer, pancreatic cancer, osteogenic sarcoma, testicular cancer, ovarian cancer, and certain lung cancers. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body.

[0085] Radiation therapy involves the use of ionizing radiation in an attempt to either cure or improve the symptoms of cancer. It is used in about half of all cases and the radiation can be from either internal sources in the form of brachytherapy or external sources. Radiation is typically used in addition to surgery and or chemotherapy but for certain types of cancer such as early head and neck cancer may be used alone. For painful bone metastasis it has been found to be effective in about 70% of people.

[0086] Alternative and complementary treatments include a diverse group of health care systems, practices, and products that are not part of conventional medicine "Complementary medicine" refers to methods and substances used along with conventional medicine, while "alternative medicine" refers to compounds used instead of conventional medicine. Most complementary and alternative medicines for cancer have not been rigorously studied or tested. Some alternative treatments have been investigated and shown to be ineffective but still continue to be marketed and promoted.

[0087] Finally, palliative care refers to treatment which attempts to make the patient feel better and may or may not be combined with an attempt to attack the cancer. Palliative care includes action to reduce the physical, emotional, spiritual, and psycho-social distress experienced by people with cancer. Unlike treatment that is aimed at directly killing cancer cells, the primary goal of palliative care is to improve the patient's quality of life.

[0088] Gla domain activity. In the context of the present disclosure, it is contemplated that the engineered Gla proteins may be used as anti-cancer agents in several different fashions. First, cancer patients often suffer from a hypercoagulable state which can lead to embolism and death. In this embodiment, the Gla domain proteins (optionally linked to an Fc region) are administered into the vasculature of the subject in amounts sufficient to reduce emboli formation and pathological coagulation. Such Gla domain proteins disclosed herein are useful in reducing, blocking and/or inhibiting pathologic coagulation in such patients.

[0089] Also, it is known that PtdS plays a role in suppressing the immune response to cancer. Thus, in another embodiment, the inventors envision the use of Gla domain proteins to bind

and block/mask PtdS on cancer cells. This serves to prevent tolerogenic signaling through PtdS that induces anti-inflammatory cytokines and suppresses pro-inflammatory cytokines, thereby facilitating immune escape of the cancer cells. This may prove a particularly useful approach when combined with other "standard" cancer therapeutics, as discussed in detail below.

2. Autoimmune/Inflammatory Disease

[0090] Background. The present disclosure contemplates the treatment of a variety of autoimmune and/or inflammatory disease states such as spondyloarthropathy, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, enteropathic arthritis, ulcerative colitis, Crohn's disease, irritable bowel disease, inflammatory bowel disease, rheumatoid arthritis, juvenile rheumatoid arthritis, familial Mediterranean fever, amyotrophic lateral sclerosis, Sjogren's syndrome, early arthritis, viral arthritis, multiple sclerosis, systemic lupus erythematosus, psoriasis, Wegener's granulomatosis, Addison's disease, alopecia, antiphospholipid syndrome, Bechet's disease, celiac disease, chronic fatigue syndrome, ulcerative colitis, type I diabetes, fibromyalgia, autoimmune gastritis, Goodpasture syndrome, Graves' disease, idiopathic thrombocytopenic purpura (ITP), myasthenia gravis, pemphigus vulgaris, primary biliary cirrhosis, rheumatic fever, sarcoidosis, scleroderma, vitiligo, vasculitis, small vessel vasculitis, hepatitis, primary biliary cirrhosis, sarcoidosis, scleroderma, graft versus host disease (acute and chronic), aplastic anemia, or cyclic neutropenia. The diagnosis and treatment of these diseases are well documented in the literature.

[0091] Gla domain activity. It is known that certain immune disorders are characterized by deficient phagocytosis of apoptotic cells, leading to secondary necrosis, pro-inflammatory signaling and autoantibody production. Further, plasma membrane-derived vesicles (PMVs) containing PtdS on their surface are shed from apoptotic cells and are thought to bind to immune cells, such as macrophages (DeRose *et al.,* 2011). Gla domain proteins can be used to mask the PtdS found on the surface of PMVs, thereby reducing the "decoy" effect of the PMVs and permitting phagocytosis of apoptotic cells. This should break the cycle of continued inflammatory signaling and reduce immune hyperactivation.

3. Hypercoagulation Disorders

[0092] Background. Hypercoagulation disorders give rise to an increased tendency for clotting of the blood, which put a patient at risk for obstruction of veins and arteries. In normal hemostasis, or the stoppage of bleeding, clots form at the site of the blood vessel's injury. In hypercoagulation, clots develop in circulating blood. When this occurs throughout the body's blood vessels, a condition known as thrombosis can arise. Thrombosis can lead to infarction, or death of tissue. Hypercoagulation disorders include hyperhomocystinemia, antithrombin III deficiency, factor V leyden, and protein C or protein S deficiency.

[0093] The diagnosis of hypercoagulation disorders is completed with a combination of physical examination, medical history, and blood tests. When found, coumadin and heparin anticoagulants may be administered to reduce the clotting effects and maintain fluidity in the blood. The prognosis for patients with hypercoagulation disorders varies depending on the severity of the clotting and thrombosis, but if undetected and untreated, thrombosis could lead to pulmonary embolism and death.

[0094] Sickle cell disease (SCD), though not a clotting disorder, has some of the same manifestations. Interestingly, an increased level of surface expressed PtdS on a subpopulation of erythrocytes is a universal feature of this disease (Wood et al., Blood, Vol 88, No 5 (September 1), 1996). SCD is an autosomal recessive genetic blood disorder with overdominance, characterized by red blood cells that assume an abnormal, rigid, sickle shape. Sickling decreases the cells' flexibility and results in a risk of various complications. The sickling occurs because of a mutation in the hemoglobin gene. Life expectancy is shortened. Sickle-cell disease occurs more commonly in people (or their descendants) from parts of tropical and sub-tropical sub-Saharan regions where malaria is or was common. In areas where malaria is common, there is a fitness benefit in carrying only a single sickle-cell gene (sickle cell trait). Those with only one of the two alleles of the sickle-cell disease, while not totally resistant, are more tolerant to the infection and thus show less severe symptoms when infected.

[0095] Sickle-cell anaemia is the name of a specific form of sickle-cell disease in which there is homozygosity for the mutation that causes HbS. Sickle-cell anaemia is also referred to as "HbSS," "SS disease," "hemoglobin S" or permutations thereof. In heterozygous people, who have only one sickle gene and one normal adult haemoglobin gene, it is referred to as "HbAS" or "sickle cell trait." Other, rarer forms of sickle-cell disease include sickle-hemoglobin C disease (HbSC), sickle beta-plus-thalassemia (HbS/β^+) and sickle beta-zero-thalassaemia (HbS/β^0). These other forms of sickle-cell disease are compound heterozygous states in which the person has only one copy of the mutation that causes HbS and one copy of another abnormal hemoglobin allele.

[0096] In particular aspects, SCD can relate to blood flow. The vaso-occlusive crisis is caused by sickle-shaped red blood cells that obstruct capillaries and restrict blood flow to an organ, resulting in ischemia, pain, necrosis and often organ damage. The frequency, severity, and duration of these crises vary considerably. Painful crises are treated with hydration, analgesics, and blood transfusion; pain management requires opioid administration at regular intervals until the crisis has settled. For milder crises, a subgroup of patients manage on NSAIDs (such as diclofenac or naproxen). For more severe crises, most patients require inpatient management for intravenous opioids; patient-controlled analgesia (PCA) devices are commonly used in this setting. Vaso-occlusive crisis involving organs such as the penis or lungs are considered an emergency and treated with red-blood cell transfusions. Diphenhydramine is sometimes effective for the itching associated with the opioid use. Incentive spirometry, a technique to encourage deep breathing to minimize the development of atelectasis, is recommended.

[0097] Gla domain activity. The present invention contemplates the use of Gla domains to control or modulate the pro-coagulant actions of PtdS in hypercoagulation. PtdS surface expression is a potent stimulator of the coagulation cascade. Masking exposed PtdS via the use of Gla domain polypeptides could inhibit or dampen this cascade. This treatment could be particularly beneficial for SCA individuals where it is believed that only a small population of RBCs may be responsible for the hypercoagulability associated with the disease

4. Viral Infection

[0098] Background. A virus is a small infectious agent that can replicate only inside the living cells of an organism. Viruses can infect all types of organisms, from animals and plants to bacteria and archaea. About 5,000 viruses have been described in detail, although there are millions of different types. Viruses are found in almost every ecosystem on Earth and are the most abundant type of biological entity.

[0099] Virus particles (known as virions) consist of two or three parts: i) the genetic material made from either DNA or RNA, long molecules that carry genetic information; ii) a protein coat that protects these genes; and in some cases iii) an envelope of lipids that surrounds the protein coat when they are outside a cell. The shapes of viruses range from simple helical and icosahedral forms to more complex structures. The average virus is about one one-hundredth the size of the average bacterium. Most viruses are too small to be seen directly with an optical microscope.

[0100] Viruses spread in many ways; viruses in plants are often transmitted from plant to plant by insects that feed on plant sap, such as aphids; viruses in animals can be carried by blood-sucking insects. These disease-bearing organisms are known as vectors. Influenza viruses are spread by coughing and sneezing. Norovirus and rotavirus, common causes of viral gastroenteritis, are transmitted by the fecal-oral route and are passed from person to person by contact, entering the body in food or water. HIV is one of several viruses transmitted through sexual contact and by exposure to infected blood. The range of host cells that a virus can infect is called its "host range." This can be narrow or, as when a virus is capable of infecting many species, broad.

[0101] Viral infections in animals provoke an immune response that usually eliminates the infecting virus. Immune responses can also be produced by vaccines, which confer an artificially acquired immunity to the specific viral infection. However, some viruses including those that cause AIDS and viral hepatitis evade these immune responses and result in chronic infections. Antibiotics have no effect on viruses, but several antiviral drugs have been developed.

[0102] A variety of diseases are fostered by virus infections, including influenza, human immunodeficiency virus, dengue virus, West Nile virus, smallpox virus, respiratory syncytial

virus, Korean hemorrhagic fever virus, chickenpox, varicella zoster virus, herpes simplex virus 1 or 2, Epstein-Barr virus, Marburg virus, hantavirus, yellow fever virus, hepatitis A, B, C or E, Ebola virus, human papilloma virus, rhinovirus, Coxsackie virus, polio virus, measles virus, rubella virus, rabies virus, Newcastle disease virus, rotavirus, HTLV-1 and -2.

[0103] Gla domain activity. PtdS is segregated to the inner leaflet of the plasma membrane of resting mammalian cells. Loss of phosphatidylserine asymmetry occurs during apoptosis, cell injury and cell activation such as observed during viral infection. This results from inhibition of the translocases or activation of phosphatidylserine exporters, or lipid-scrambling enzymes, such as scramblases (Soares *et al.,* 2008). Viruses activate host cells to replicate efficiently, and virus-induced cell activation may lead to rises in intracellular Ca^{2+} that, in turn, cause phosphatidylserine externalization by activating phosphatidylserine exporters and by inhibiting phosphatidylserine import by translocases. Moreover, phosphatidylserine translocation to the external host cell surface could be an early event associated with virus-induced apoptosis (Soares *et al.,* 2008).

[0104] PtdS on the surface of infected cells, budding virions or microvesicles derived from pre-apoptotic cells leads to immune evasion. Using the Gla domain polypeptides of the present disclosure to mask the PtdS on these membranes, normal immune function can more effectively address the viral challenge, thus reducing viral load, limiting infection, and destroying virions and infected cells.

5. Sepsis

[0105] Background. In sepsis, inflammation and thrombosis are both cause and result of interactions between circulating (for example, leukocytes and platelets), endothelial and smooth muscle cells. Microparticles are pro-inflammatory and procoagulant fragments originating from plasma membrane generated after cellular activation and released in body fluids. In the vessel, they constitute a pool of bioactive effectors pulled from diverse cellular origins and may act as intercellular messengers. Microparticles expose phosphatidylserine, a procoagulant phospholipid made accessible after membrane remodeling, and tissue factor (TF), the initiator of blood coagulation at the endothelial and leukocyte surface. They constitute a secretion pathway for IL-1β and upregulate the pro-inflammatory response of target cells. Microparticles circulate at low levels in healthy individuals, but undergo phenotypic and quantitative changes that could play a pathophysiological role in inflammatory diseases. Microparticles (MPs) may participate in the pathogenesis of sepsis through multiple ways. They are able to regulate vascular tone and are potent vascular pro-inflammatory and pro-coagulant mediators. Microparticles' abilities are of increasing interest in deciphering the mechanisms underlying the multiple organ dysfunction of septic shock.

[0106] The plasma membrane of endothelial cells and monocytes is reorganized with externalisation of phosphatidylserine and encrypted tissue factor TF expression, allowing factor

VII (FVIIa) activation and thrombin (FIIa) generation at the cell surface. Blebbing occurs, with release of microparticles bearing TF, resulting in an increased surface for procoagulant reactions. Platelet adhesion and aggregation also occur with the release of MPs; platelets and MPs bear GPIbα, a cofactor for factor XI activation by thrombin, leading to the propagation phase with high levels of thrombin generation and fibrin formation. Endothelial TF-bearing MPs allow transfer of TF to PMNs, increasing TF dissemination and thrombotic microangiopathy or disseminated intravascular coagulopathy. TF initiation of blood coagulation is quickly down-regulated by tissue factor pathway inhibitor (TFPI) on endothelial and monocytic cell surfaces, as on MPs. Endothelial protein C receptor (EPCR)-bound protein C is activated by the thrombin-thrombomodulin complex and activated protein C (APC) inhibits factor Va and factor VIIIa, limiting the propagation phase of thrombin generation. EPCR-bound APC also regulates NF-KB, with cytoprotective effects on endothelial cells and monocytes. APC induces blebbing, with emission of EPCR-bearing MPs able to activate protein C, resulting in the dissemination of anticoagulant and antiapoptotic activities.

[0107] Gla domain activity. The present invention contemplates the use of Gla domains to control or modulate the pro-coagulant actions of microparticles containing PtdS and TF on their surface. They can also down-regulate the related pro-inflammatory signaling that occurs in sepsis, for example, through IL-1β, as well as modulating the effects of PtdS-bearing microparticles on vascular tone.

C. Treatment Methods

[0108] Peptides and polypeptides can be administered to mammalian subjects (*e.g.*, human patients) alone or in conjunction with other drugs that treat the diseases set forth above. The dosage required depends on the choice of the route of administration; the nature of the formulation, including additional agents attached to the polypeptide; the nature of the patient's illness; the subject's size, weight, surface area, age, and sex; further combination therapies; and the judgment of the attending physician. Suitable dosages are in the range of 0.0001-100 mg/kg. Wide variations in the needed dosage are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art. Administrations can be single or multiple (*e.g.*, 2-, 3-, 4-, 6-, 8-, 10-, 20-, 50-,100-, 150-, or more times). Encapsulation of the polypeptide in a suitable delivery vehicle (*e.g.,* polymeric microparticles or implantable devices) may increase the efficiency of delivery, particularly for oral delivery.

D. Combination Therapies

[0109] It is common in many fields of medicine to treat a disease with multiple therapeutic

modalities, often called "combination therapies." To treat disorders using the methods and compositions of the present disclosure, one would generally contact a target cell or subject with a Gla domain polypeptide and at least one other therapy. These therapies would be provided in a combined amount effective to achieve a reduction in one or more disease parameter. This process may involve contacting the cells/subjects with the both agents/therapies at the same time, *e.g.*, using a single composition or pharmacological formulation that includes both agents, or by contacting the cell/subject with two distinct compositions or formulations, at the same time, wherein one composition includes the Gla domain polypeptide and the other includes the other agent.

[0110] Alternatively, the Gla domain polypeptide may precede or follow the other treatment by intervals ranging from minutes to weeks. One would generally ensure that a significant period of time did not expire between the time of each delivery, such that the therapies would still be able to exert an advantageously combined effect on the cell/subject. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other, within about 6-12 hours of each other, or with a delay time of only about 12 hours. In some situations, it may be desirable to extend the time period for treatment significantly; however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

[0111] It also is conceivable that more than one administration of either the Gla domain protein or the other therapy/agent will be desired. Various combinations may be employed, where the Gla domain protein is "A," and the other therapy is "B," as exemplified below:

A/B/A	B/A/B	B/B/A	A/A/B	B/A/A	A/B/B	B/B/B/A	B/B/A/B
A/A/B/B	A/B/A/B		A/B/B/A	B/B/A/A	B/A/B/A	B/A/A/B	B/B/B/A
A/A/A/B	B/A/A/A		A/B/A/A	A/A/B/A	A/B/B/B	B/A/B/B	B/B/A/B

Other combinations are contemplated.

[0112] Agents or factors suitable for use in a combined therapy against a cancer include radionuclides, chemotherapeutic agents or toxins. Specific chemotherapeutics include temozolomide, epothilones, melphalan, carmustine, busulfan, lomustine, cyclophosphamide, dacarbazine, polifeprosan, ifosfamide, chlorambucil, mechlorethamine, busulfan, cyclophosphamide, carboplatin, cisplatin, thiotepa, capecitabine, streptozocin, bicalutamide, flutamide, nilutamide, leuprolide acetate, doxorubicin hydrochloride, bleomycin sulfate, daunorubicin hydrochloride, dactinomycin, liposomal daunorubicin citrate, liposomal doxorubicin hydrochloride, epirubicin hydrochloride, idarubicin hydrochloride, mitomycin, doxorubicin, valrubicin, anastrozole, toremifene citrate, cytarabine, fluorouracil, fludarabine, floxuridine, interferon α-2b, plicamycin, mercaptopurine, methotrexate, interferon α-2a, medroxyprogersterone acetate, estramustine phosphate sodium, estradiol, leuprolide acetate, megestrol acetate, octreotide acetate, deithylstilbestrol diphosphate, testolactone, goserelin acetate, etoposide phosphate, vincristine sulfate, etoposide, vinblastine, etoposide, vincristine sulfate, teniposide, trastuzumab, gemtuzumab ozogamicin, rituximab, exemestane, irinotecan hydrocholride, asparaginase, gemcitabine hydrochloride, altretamine, topotecan hydrochloride,

hydroxyurea, cladribine, mitotane, procarbazine hydrochloride, vinorelbine tartrate, pentrostatin sodium, mitoxantrone, pegaspargase, denileukin diftitix, altretinoin, porfimer, bexarotene, paclitaxel, docetaxel, arsenic trioxide, or tretinoin. Toxins include *Pseudomonas* exotoxin (PE38), ricin A chain, diphtheria toxin, Besides PE and RT, Pokeweed antiviral protein (PAP), saporin and gelonin. Radionuclides for cancer therapy include Y-90, P-32, I-131, In-111, Sr-89, Re-186, Sm-153, and Sn-117m.

[0113] Agents or factors suitable for use in a combined therapy against an autoimmune disorder include anti-inflammatories and immune-modulating compositions. These include prednisone, methylprednisone, Venipred, Celestone, hydrocortisone, triamcinoclone, Aristonpan Intra-Articular injection, Methapred, Rayos oral, betamethasone, and etanercept.

[0114] Agents or factors suitable for use in a combined therapy against cardiovascular disease include beta blockers, anti-hypertensives, cardiotonics, anti-thrombotics, vasodilators, hormone antagonists, endothelin antagonists, cytokine inhibitors/blockers, calcium channel blockers, phosphodiesterase inhibitors and angiotensin type 2 antagonists.

[0115] Agents or factors suitable for use in a combined therapy against a hypercoagulation disorder include anti-coagulants such as heparin and warfarin.

[0116] Agents or factors suitable for use in a combined therapy against a viral infections include Abacavir, Aciclovir, Acyclovir, Adefovir, Amantadine, Amprenavir, Ampligen, Arbidol, Atazanavir, Atripla, Boceprevirertet, Cidofovir, Combivir, Darunavir, Delavirdine, Didanosine, Docosanol, Edoxudine, Efavirenz, Emtricitabine, Enfuvirtide, Entecavir, Entry inhibitors, Famciclovir, Fomivirsen, Fosamprenavir, Foscarnet, Fosfonet, Ganciclovir, Ibacitabine, Imunovir, Idoxuridine, Imiquimod, Indinavir, Inosine, Integrase inhibitor, Interferon type III, Interferon type II, Interferon type I, Interferon, Lamivudine, Lopinavir, Loviride, Maraviroc, Moroxydine, Methisazone, Nelfinavir, Nevirapine, Nexavir, Nucleoside analogues, Oseltamivir, Peginterferon alfa-2a, Penciclovir, Peramivir, Pleconaril, Podophyllotoxin, Protease inhibitor, Raltegravir, Reverse transcriptase inhibitor, Ribavirin, Rimantadine, Ritonavir, Pyramidine, Saquinavir, Stavudine, Synergistic enhancer (antiretroviral), Tea tree oil, Telaprevir, Tenofovir, Tenofovir disoproxil, Tipranavir, Trifluridine, Trizivir, Tromantadine, Truvada, Valaciclovir, Valganciclovir, Vicriviroc, Vidarabine, Viramidine, Zalcitabine, Zanamivir and Zidovudine.

[0117] The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

[0118] It also should be pointed out that any of the foregoing therapies may prove useful by themselves in treatment.

V. Examples

EXAMPLE 1

[0119] The affinities of Gla-domain proteins for cell membranes have been determined *in vitro* by using prepared phospholipid vesicles (Shah *et al.,* 1998; Nelsestuen, 1999). How these *in vitro* values translate to an *in vivo* context, however, has not been fully elucidated. The interaction of FVII with TF, for example, underscores the fact that although the Gla domains of these proteins are very homologous, additional differences in their cell membrane binding specificity and affinity may be mediated through their EGF and/or Kringle domains. Unfortunately, these interactions cannot be recapitulated by studies based solely on phospholipid vesicles and may remain unidentified.

[0120] Therefore, inventors proposed making and testing the Gla+EGF/Kringle domains as well as the Gla domain alone from the following panel of proteins: hS (high affinity binder), hZ(mid affinity binder), hPT (mid affinity-kringle containing), hFVII (low affinity-utilizes secondary "receptor" that is also up-regulated in cancer), and B0178 (hFVII with increased phospholipid affinity). These proteins potentially have varying *in vivo* binding characteristics that may be beneficial to their use as probes (and, if validated and selective, potentially as therapeutics) and that to date have gone unrecognized.

[0121] The general approach was to construct recombinant proteins and test them for expression. Assays would then be developed to assess binding. Then, expression and purification methods would be optimized, followed by quality control of gamma-carboxylation.

[0122] FIG. 1 shows sequences from a variety of Gla domain proteins including carboxylation sites. FIG. 2 shows the expression of a variety of different Gla domain proteins that were engineered and transiently expressed in 293 cells. FIG. 3 shows a similar study in BHK21 cells. Given that one of the best expressing constructs was a Protein S + EGF construct, the signal sequence from Protein S was utilized with Prothrombin Gla + Kringle and Protein Z + EGF. However, expression was only observed intracellularly (FIG. 4).

[0123] Protein S Gla + EGF was selected for further study. The sequence is shown in FIG. 5. Protein was produced in BHK21 cells using RF286 medium. 600 ml was harvested and concentrated 4X. Purification utilized three steps:

1. 1. Ni-NTA column, 10 ml, fresh packed. The medium are loaded to column and eluted with Imidazole gradient. All the fractions are subject to Gla western blot to identify the His tagged Gla protein S G+E.
2. 2. Hitrap Q with eaCh step elution. The Gla positive fractions are pooled and subject to 1 ml Hitrap Q with 10 mM eaCh elution.

3. 3. Hitrap Q with eaCh gradient (0-10 mM shadow gradient). The step purified Gla proteins were applied to Q and eluted with gradient eaCh (up to 10 mM). A total of 0.9 mg of protein at a 95% purity level was produced. FIG. 6 shows the purification fractions under both reducing and non-reducing conditions. FIGS. 7 and 8 show different FACs-based apoptosis assays. Both show that the Protein S Gla + EGF construct is specific for cells undergoing apoptosis just like Annexin V (FIG. 7), and that Annexin V can compete off the Protein S Gla + EGF binding.

[0124] In summary, Protein S Gla+EGF was expressed and purified. Analysis on the purified material suggested that it was highly gamma-carboxylated. FACs-based Apoptosis Assays demonstrated that Protein S G+E (11 Gla) could bind to "apoptotic" cells, and that this binding was to cells was via targeting of phosphatidylserine, as demonstrated by Annexin V competition assays.

VI. References

[0125]

US5,440,013, US5,446,128, US5,475,085, US5,597,457, US5,618,914, US5,670,155, US5,672,681, US5,674,976, US5,710,245, US5,790,421, US5,840,833, US5,859,184, US5,889,155, US5,929,237, US6,093,573, US6,261,569, US6,312,694, US2005/0015232, US2004/001827, US2003/104578, US2003/104578, WO2006/096515, WO2007/047504, WO200/6096515A2

Aaronson and Horvath, Science, 296(5573):1653-5, 2002.

Abe and Kufe, Cancer Res., 49(11):2834-2839, 1989.

Agata et al., Cancer Res., 68:6136-44, 2008.

Ahmad et al., Cancer Res., 68:2920-2926, 2008.

Ahmad et al., J. Biol. Chem., 281:35764-9, 2006.

Ahmad et al., Nat. Cell Biol., 9:1419-1427, 2007.

Alvarez et al., Cancer Res., 65(12):5054-62, 2005.

Alvarez et al., Cancer Res., 66(6):3162-8, 2006.

Anderson et al., Trends Immunol 27, 343-348, 2006.

Ashkenazi and Chamow, Curr Op Immunol 9, 195-200, 1997.

Baldus et al., Clin. Cancer Res., 10(8):2790-2796, 2004.

Blankenberg, Proc Am Thorac Soc, 6, p 469-476, 2009.

Bodanszky et al., J. Antibiot., 29(5):549-53, 1976.

Boersma et al., J Nuclear Med, 46 (12), p2035-2050, 2005.

Bowman et al., Oncogene, 19(21):2474-88, 2000.

Bromberg et al., Cell, 98(3):295-303, 1999.

Buerger et al., J. Biol. Chem., 278(39):37610-21, 2003.

Carter, Nature Reviews Immunol 6, 343-357, 2006.

Chapman, Advanced Drug Delivery Reviews 54, 531-545, 2002.

Chaudhury et al., J Exper Med 197, 315-322, 2003.

Chen & Greene, Mol. Cell. Biol. 5:392-401, 2004.

Cohen et al., J. Med. Chem., 33:883-894, 1990.

Cohen et al., Cell Res, 19 p625-637, 2009.

Duraisamy et al., Gene, 373:28-34, 2006.

Duttaroy et al., Diabetes 54, 251-258, 2005.

Elliott et al., Nat Biotechnol 21, 414-421, 2003.

Elltiot et al., Nature, 461, p2-286, 2009.

Elltiot and Ravichandran, J. Cell Biol, 189 (7) p1059-1070, 2010.

Erwig and Henson, Cell Death Differentiation 15, p243-250, 2008.

Fischer, Med. Res. Rev., 27(6):755-796, 2007.

Gaemers et al., J. Biol. Chem., 276:6191-6199, 2001.

Gendler et al., J. Biol. Chem., 263:12820-12823, 1988.

Germain and Frank, Clin. Cancer Res., 13(19):5665-9, 2007.

Gerondakis et al., Oncogene 25(51):6781-99, 2006.

Ghetie et al., Nature Biotechnol 15, 637-640, 1997.

Ghosh et al., Annu. Rev. Cell. Dev. Biol., 16:225-60, 1998.

Gilmore, available from NF-kB.org, 2008.

Grillot et al., J. Immunol., 158:4750-7, 1997.

Gronenborn et al., Anal. Chem., 62(1):2-15, 1990.

Hansson and Stenflo. J Thrombosis Haemostasis, 3, P2633-2648, 2005.

Hayden and Ghosh, Cell, 132:344-62, 2008.

Hinton et al., J Immunol 176, 346-356, 2006.

Hodel et al., Mol. Cell, 10(2):347-58, 2002.

Hoffman et al., Oncogene, 25:6706-16, 2006.

Huang et al., Cancer Biol Ther., 2:702-706, 2003.

Huang et al., Cancer Res., 65: 10413-10422, 2005.

Huxford et al., Cell 95(6):759-70, 1998.

Jackson, Seminars in Oncology, 24:L164-172, 1997.

Jacobs et al., Cell, 95:749-58, 1998.

Jain et al., Trends Biotechnol 25, 307-316, 2007.

Johnson et al., In: Biotechnology And Pharmacy, Pezzuto et al. (Eds.), Chapman and Hall, NY, 1993.

Jones et al., J. Med. Chem., 39:904-917, 1996.

Karin & Lin, Nat. Immunol., 3:221-7, 2002.

Kau et al., Nat. Rev. Cancer, 4(2):106-17, 2004.

Kawano et al., Cancer Res., 67:11576-84, 2007.

Keyt et al., Proc Natl Acad Sci USA 91, 3670-3674, 1994.

Kietselaer et al., Netherlands Heart J, 10(7/8), p313-317,(002.

Kinlough et al., J. Biol. Chem., 279(51):53071-53077, 2004.

Kufe et al., Hybridoma, 3:223-232, 1984.

Kurihara et al., Appl Radiat Isot, 66(9); p1175-1182, 2008.

Lagow and Carson, J. Cell. Biochem., 86:759-72, 2002.

Lahorte et al., Eur J Nuclear Medicine Mol Imaging, 31 (6), p887-919, 2004.

Lee et al., Cancer Cell, 15(4):283-293, 2009.

Leng et al., J. Biol. Chem., 282:19321-19330, 2007.

Levitan et al., J. Biol. Chem., 280:33374-33386, 2005.

Levy and Shoseyov, Biotechnol Advances 20, 191-213, 2002.

Li et al., Cancer Biol. Ther., 2:187-193, 2003b.

Li et al., J. Biol. Chem., 276:35239-35242, 2001.

Li et al., J. Biol. Chem., 276:6061-6064, 2001.

Li et al., Mol. Cancer Res., 1:765-775, 2003c.

Li et al., Mol. Cell Biol., 18:7216-7224, 1998.

Li et al., Oncogene, 22:6107-6110, 2003a.

Ligtenberg et al., J. Biol. Chem., 267, 6171-6177, 1992.

Lin et al., Amino Acids, Published online 17 March 2010.

Loose et al., Eur JNucl Med Mol Imaging, 2007.

Macao, Nat. Struct. Mol. Biol., 13, 71-76, 2006.

McPherson, J. Biol. Chem., 251:6300-6306, 1976.

Melder et al., Cancer Immunol Immunother 54, 535-5475, 2005.

Merlo et al., Cancer Res., 49, 6966-6971, 1989.

Merrifield, J. Am. Chem. Soc., 85:2149-2154, 1963.

Micheau & Tschopp, Cell, 114:181-90, 2003.

Mimura et al., Mol Immunol 37, 697-706, 2000.

Muthuswamy, Nat. Cell Biol., 3(9):785-92, 2001.

Naresh et al., Cancer, 91(3), p578-548, 2001.

Natoli et al., Nat. Immunol., 6:439-45, 2005.

Navia et al., Curr. Opin. Struct. Biol., 2:202-210, 1992.

Nelsestuen, Trends Cardiovasc Med, 9(6), p162-167, 1999.

Okada et al., Thrombosis Research 125 (2010): e246-e250.

Osborn et al., J Pharmacal Experimental Therapeutics 303, 540-548, 2002a.

Osborn et al., Eur J Pharmacol 456, 149-158, 2002b.

Pasparakis et al., Cell Death Differ. 13:861-72, 2006.

PCT Appln. PCT/US00/03745

PCT Appln.PCT/US00/14667

PCT Appln.PCT/US99/11913

PCT Appln.PCT/US99/18441

Peptide Synthesis, 1985

Percipalle et al., J. Mol. Biol., (4):722-32, 1997.

Perey et al., Cancer Res., 52(22):6365-6370, 1992.

Petkova et al., International Immunol 18, 1759-1769, 2006a.

Protective Groups in Organic Chemistry, 1973

Protein NMR Spectroscopy, Principles and Practice, J. Cavanagh et al., Academic Press, San Diego, 1996.

Raina et al., Direct targeting of the GLA DOMAIN oncoprotein blocks survival and tumorigenicity of human breast carcinoma cells. Cancer Res., 2009 (IN PRESS).

Raina et al., EMBO J., 25:3774-3783, 2006.

Raina et al., J. Biol. Chem., 279:20607-20612, 2004.

Raju and Scallon, Biotechnol Prog 23, 964-971, 2007.

Raju, and Scallon, Biochem Biophys Res Commun 341, 797-803, 2006.

Ramasamy et al., Mol. Cell, 27:992-1004, 2007.

Remington's Pharmaceutical Sciences, 15th Ed., 1035-1038 and 1570-1580, 1990.

Remington's Pharmaceutical Sciences, 15th Ed., 3:624-652, 1990.

Ren et al., Cancer Cell, 5:163-175, 2004.

Ren et al., J. Biol. Chem., 277:17616-17622, 2002.

Reutelingsperger et al., J Immunol Methods 265, 123- 132, 2002.

Ryan and Wente, Curr. Opin. Cell Biol., 12(3):361-71, 2000.

Schneider-Brachert et al., Immunity, 21:415-28, 2004.

Schroeder et al., J. Biol. Chem., 276(16):13057-13064 2001.

Schroeder et al., Oncogene, 23:5739-5747, 2004.

Schutters and Reutelingsperger, Apoptosis (2010), 15: 1072-1082.

Shah et al., Proc. Natl. Acad. Sci. USA 95, p4229-4234, 1998.

Shuai, Oncogene, 19(21):2638-44, 2000.

Siddiquee et al., Proc. Natl. Acad. Sci. USA, 104(18):7391-6, 2007.

Siddiqui et al., Proc. Natl. Acad. Sci. USA, 85:2320-2323, 1988.

Sinclair and Elliott, J Pharmaceutical Sci 94, 1626-1635, 2005.

Solid Phase Peptide Synthelia, 1984

Song et al., Proc. Natl. Acad. Sci. USA, 102(13):4700-5, 2005.

Soule et al., Cancer Res., 50(18):6075-6086, 1990.

Suh and Gumbiner, Exp. Cell Res., 290(2):447-56, 2003.

Sung et al., J Interferon Cytokine Res 23, 25-36, 2003.

Tait and Gibson. Arch Biochem Biophys. 298(1), p187-191, 1992.

Truscott et al., J Cell Biol., 163(4):707-713, 2003.

Umana et al., Nat Biotechnol 17, 176-180, 1999.

Vaccaro et al., Nature Biotechnol 23, 1283-1288, 2005.

van den Eijnde et al., J Cell Science, 114, p3631-3642, 2001.

Vermeer et al., Nature, 422(6929):322-6, 2003.

Veronese and Pasut, Drug Discovery Today 10, 1451-1458, 2005.

Webb et al., J Immunol, 169 p2580-2586, 2002.

Weber, Advances Protein Chem., 41:1-36, 1991.

Wegenka et al., Mol. Cell Biol., 14(5):3186-96, 1994.

Wei et al., Cancer Cell, 7:167-178, 2005.

Wei et al., Cancer Res., 67(4):1853-8, 2007.

Wei et al., Mol. Cell., 21:295-305, 2006.

Weis, Cell, 112(4):441-51, 2003.

Wen et al., J. Biol. Chem., 278:38029-38039, 2003.

Wider, BioTechniques, 29:1278-1294, 2000.

Wijnen et al., Biochem J. (1998), 330, 389-396.

Yamamoto et al., J. Biol. Chem., 272:12492-12494, 1997.

Yeh et al., Proc. Nat'l Acad. Sci. USA 89, 1904-1908, 1992.

Yin et al., J. Biol. Chem., 278:35458-35464, 2003.

Yin et al., J. Biol. Chem., 279:45721-45727, 2004.

Yin et al., J. Biol. Chem., 282:257-266, 2007.

Young et al., Cell. 112(1):41-50, 2003.

Yu et al., Seminars Thrombosis Hemostasis, 30(1), p21-30, 2004.

Yu and Jove, Nat. Rev. Cancer, 4(2):97-105, 2004.

Zhang et al., Mol. Cell. Biol., 19:7138-7146, 1999.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US61791537 [0001]
- US61787753 [0001]
- US6312694B [0006] [0125]
- US6261569B [0050] [0125]
- US5889155A [0055] [0125]
- WO2006096515A [0056] [0059] [0125]

- US2003104578A [0056] [0059] [0125] [0125]
- WO2007047504A [0059] [0125]
- US2004001827A [0061] [0125]
- US5021236A [0064]
- US4938948A [0064] [0069]
- US4472509A [0064] [0069]
- US3817837A [0068]
- US3850752A [0068]
- US3939350A [0068]
- US3996345A [0068]
- US4277437A [0068]
- US4275149A [0068]
- US4366241A [0068]
- US5440013A [0125]
- US5446128A [0125]
- US5475085A [0125]
- US5597457A [0125]
- US5618914A [0125]
- US5670155A [0125]
- US5672681A [0125]
- US5674976A [0125]
- US5710245A [0125]
- US5790421A [0125]
- US5840833A [0125]
- US5859184A [0125]
- US5929237A [0125]
- US6093573A [0125]
- US20050015232A [0125]
- WO2006096515A2 [0125]
- US0003745W [0125]
- US0014667W [0125]
- US9911913W [0125]
- US9918441W [0125]

Non-patent literature cited in the description

- **THOMPSON et al.**Nucleic Acids Res, 1994, vol. 2, 224673-4680 [0025]
- **HIGGINS et al.**Computer Applications in the Biosciences (CABIOS), 1992, vol. 8, 2189-191 [0025]
- Peptide Synthesis, 1985, [0051] [0125]

- Solid Phase Peptide Synthelia, 1984, [0051] [0125]
- Protective Groups in Organic Chemistry, 1973, [0051] [0125]
- Remington's Pharmaceutical Sciences1035-10381570-1580 [0079]
- **WOOD et al.**Blood, 1996, vol. 88, 5 [0094]
- Remington's Pharmaceutical Sciences624-652 [0117]
- **AARONSONHORVATH**Science, 2002, vol. 296, 55731653-5 [0125]
- **ABEKUFE**Cancer Res., 1989, vol. 49, 112834-2839 [0125]
- **AGATA et al.**Cancer Res., 2008, vol. 68, 6136-44 [0125]
- **AHMAD et al.**Cancer Res., 2008, vol. 68, 2920-2926 [0125]
- **AHMAD et al.**J. Biol. Chem., 2006, vol. 281, 35764-9 [0125]
- **AHMAD et al.**Nat. Cell Biol., 2007, vol. 9, 1419-1427 [0125]
- **ALVAREZ et al.**Cancer Res., 2005, vol. 65, 125054-62 [0125]
- **ALVAREZ et al.**Cancer Res., 2006, vol. 66, 63162-8 [0125]
- **ANDERSON et al.**Trends Immunol, 2006, vol. 27, 343-348 [0125]
- **ASHKENAZICHAMOW**Curr Op Immunol, 1997, vol. 9, 195-200 [0125]
- **BALDUS et al.**Clin. Cancer Res., 2004, vol. 10, 82790-2796 [0125]
- **BLANKENBERG**Proc Am Thorac Soc, 2009, vol. 6, 469-476 [0125]
- **BODANSZKY et al.**J. Antibiot., 1976, vol. 29, 5549-53 [0125]
- **BOERSMA et al.**J Nuclear Med, 2005, vol. 46, 122035-2050 [0125]
- **BOWMAN et al.**Oncogene, 2000, vol. 19, 212474-88 [0125]
- **BROMBERG et al.**Cell, 1999, vol. 98, 3295-303 [0125]
- **BUERGER et al.**J. Biol. Chem., 2003, vol. 278, 3937610-21 [0125]
- **CARTER**Nature Reviews Immunol, 2006, vol. 6, 343-357 [0125]
- **CHAPMAN**Advanced Drug Delivery Reviews, 2002, vol. 54, 531-545 [0125]
- **CHAUDHURY et al.**J Exper Med, 2003, vol. 197, 315-322 [0125]
- **CHENGREENE**Mol. Cell. Biol., 2004, vol. 5, 392-401 [0125]
- **COHEN et al.**J. Med. Chem., 1990, vol. 33, 883-894 [0125]
- **COHEN et al.**Cell Res, 2009, vol. 19, 625-637 [0125]
- **DURAISAMY et al.**Gene, 2006, vol. 373, 28-34 [0125]
- **DUTTAROY et al.**Diabetes, 2005, vol. 54, 251-258 [0125]
- **ELLIOTT et al.**Nat Biotechnol, 2003, vol. 21, 414-421 [0125]
- **ELLTIOT et al.**Nature, 2009, vol. 461, 2-286 [0125]
- **ELLTIOTRAVICHANDRAN**J. Cell Biol, 2010, vol. 189, 71059-1070 [0125]
- **ERWIGHENSON**Cell Death Differentiation, 2008, vol. 15, 243-250 [0125]
- **FISCHER**Med. Res. Rev., 2007, vol. 27, 6755-796 [0125]
- **GAEMERS et al.**J. Biol. Chem., 2001, vol. 276, 6191-6199 [0125]
- **GENDLER et al.**J. Biol. Chem., 1988, vol. 263, 12820-12823 [0125]
- **GERMAINFRANK**Clin. Cancer Res., 2007, vol. 13, 195665-9 [0125]
- **GERONDAKIS et al.**Oncogene, 2006, vol. 25, 516781-99 [0125]
- **GHETIE et al.**Nature Biotechnol, 1997, vol. 15, 637-640 [0125]
- **GHOSH et al.**Annu. Rev. Cell. Dev. Biol., 1998, vol. 16, 225-60 [0125]
- **GRILLOT et al.**J. Immunol., 1997, vol. 158, 4750-7 [0125]
- **GRONENBORN et al.**Anal. Chem., 1990, vol. 62, 12-15 [0125]
- **HANSSONSTENFLO**J Thrombosis Haemostasis, 2005, vol. 3, 2633-2648 [0125]

- **HAYDENGHOSH**Cell, 2008, vol. 132, 344-62 [0125]
- **HINTON et al.**J Immunol, 2006, vol. 176, 346-356 [0125]
- **HODEL et al.**Mol. Cell, 2002, vol. 10, 2347-58 [0125]
- **HOFFMAN et al.**Oncogene, 2006, vol. 25, 6706-16 [0125]
- **HUANG et al.**Cancer Biol Ther., 2003, vol. 2, 702-706 [0125]
- **HUANG et al.**Cancer Res., 2005, vol. 65, 10413-10422 [0125]
- **HUXFORD et al.**Cell, 1998, vol. 95, 6759-70 [0125]
- **JACKSON**Seminars in Oncology, 1997, vol. 24, L164-172 [0125]
- **JACOBS et al.**Cell, 1998, vol. 95, 749-58 [0125]
- **JAIN et al.**Trends Biotechnol, 2007, vol. 25, 307-316 [0125]
- **JOHNSON et al.**Biotechnology And PharmacyChapman and Hall19930000 [0125]
- **JONES et al.**J. Med. Chem., 1996, vol. 39, 904-917 [0125]
- **KARINLIN**Nat. Immunol., 2002, vol. 3, 221-7 [0125]
- **KAU et al.**Nat. Rev. Cancer, 2004, vol. 4, 2106-17 [0125]
- **KAWANO et al.**Cancer Res., 2007, vol. 67, 11576-84 [0125]
- **KEYT et al.**Proc Natl Acad Sci USA, 1994, vol. 91, 3670-3674 [0125]
- **KIETSELAER et al.**Netherlands Heart J, vol. 10, 7/8313-317 [0125]
- **KINLOUGH et al.**J. Biol. Chem., 2004, vol. 279, 5153071-53077 [0125]
- **KUFE et al.**Hybridoma, 1984, vol. 3, 223-232 [0125]
- **KURIHARA et al.**Appl Radiat Isot, 2008, vol. 66, 91175-1182 [0125]
- **LAGOWCARSON**J. Cell. Biochem., 2002, vol. 86, 759-72 [0125]
- **LAHORTE et al.**Eur J Nuclear Medicine Mol Imaging, 2004, vol. 31, 6887-919 [0125]
- **LEE et al.**Cancer Cell, 2009, vol. 15, 4283-293 [0125]
- **LENG et al.**J. Biol. Chem., 2007, vol. 282, 19321-19330 [0125]
- **LEVITAN et al.**J. Biol. Chem., 2005, vol. 280, 33374-33386 [0125]
- **LEVYSHOSEYOV**Biotechnol Advances, 2002, vol. 20, 191-213 [0125]
- **LI et al.**Cancer Biol. Ther., 2003, vol. 2, 187-193 [0125]
- **LI et al.**J. Biol. Chem., 2001, vol. 276, 35239-35242 [0125]
- **LI et al.**J. Biol. Chem., 2001, vol. 276, 6061-6064 [0125]
- **LI et al.**Mol. Cancer Res., 2003, vol. 1, 765-775 [0125]
- **LI et al.**Mol. Cell Biol., 1998, vol. 18, 7216-7224 [0125]
- **LI et al.**Oncogene, 2003, vol. 22, 6107-6110 [0125]
- **LIGTENBERG et al.**J. Biol. Chem., 1992, vol. 267, 6171-6177 [0125]
- **LIN et al.**Amino Acids, 2010, [0125]
- **LOOSE et al.**Eur JNucl Med Mol Imaging, 2007, [0125]
- **MACAO**Nat. Struct. Mol. Biol., 2006, vol. 13, 71-76 [0125]
- **MCPHERSON**J. Biol. Chem., 1976, vol. 251, 6300-6306 [0125]
- **MELDER et al.**Cancer Immunol Immunother, 2005, vol. 54, 535-5475 [0125]
- **MERLO et al.**Cancer Res., 1989, vol. 49, 6966-6971 [0125]
- **MERRIFIELD, J**Am. Chem. Soc., 1963, vol. 85, 2149-2154 [0125]
- **MICHEAUTSCHOPP**Cell, 2003, vol. 114, 181-90 [0125]
- **MIMURA et al.**Mol Immunol, 2000, vol. 37, 697-706 [0125]
- **MUTHUSWAMY**Nat. Cell Biol., 2001, vol. 3, 9785-92 [0125]
- **NARESH et al.**Cancer, 2001, vol. 91, 3578-548 [0125]

- **NATOLI et al.**Nat. Immunol., 2005, vol. 6, 439-45 [0125]
- **NAVIA et al.**Curr. Opin. Struct. Biol., 1992, vol. 2, 202-210 [0125]
- **NELSESTUEN**Trends Cardiovasc Med, 1999, vol. 9, 6162-167 [0125]
- **OKADA et al.**Thrombosis Research, 2010, vol. 125, e246-e250 [0125]
- **OSBORN et al.**J Pharmacal Experimental Therapeutics, 2002, vol. 303, 540-548 [0125]
- **OSBORN et al.**Eur J Pharmacol, 2002, vol. 456, 149-158 [0125]
- **PASPARAKIS et al.**Cell Death Differ., 2006, vol. 13, 861-72 [0125]
- **PERCIPALLE et al.**J. Mol. Biol., 1997, vol. 4, 722-32 [0125]
- **PEREY et al.**Cancer Res., 1992, vol. 52, 226365-6370 [0125]
- **PETKOVA et al.**International Immunol, 2006, vol. 18, 1759-1769 [0125]
- **J. CAVANAGH et al.**Protein NMR Spectroscopy, Principles and PracticeAcademic Press19960000 [0125]
- **RAINA et al.**Direct targeting of the GLA DOMAIN oncoprotein blocks survival and tumorigenicity of human breast carcinoma cellsCancer Res., 2009, [0125]
- **RAINA et al.**EMBO J., 2006, vol. 25, 3774-3783 [0125]
- **RAINA et al.**J. Biol. Chem., 2004, vol. 279, 20607-20612 [0125]
- **RAJUSCALLON**Biotechnol Prog, 2007, vol. 23, 964-971 [0125]
- **RAJUSCALLON**Biochem Biophys Res Commun, 2006, vol. 341, 797-803 [0125]
- **RAMASAMY et al.**Mol. Cell, 2007, vol. 27, 992-1004 [0125]
- Remington's Pharmaceutical Sciences199000001035-10381570-1580 [0125]
- Remington's Pharmaceutical Sciences19900000vol. 3, 624-652 [0125]
- **REN et al.**Cancer Cell, 2004, vol. 5, 163-175 [0125]
- **REN et al.**J. Biol. Chem., 2002, vol. 277, 17616-17622 [0125]
- **REUTELINGSPERGER et al.**J Immunol Methods, 2002, vol. 265, 123-132 [0125]
- **RYANWENTE**Curr. Opin. Cell Biol., 2000, vol. 12, 3361-71 [0125]
- **SCHNEIDER-BRACHERT et al.**Immunity, 2004, vol. 21, 415-28 [0125]
- **SCHROEDER et al.**J. Biol. Chem., 2001, vol. 276, 1613057-13064 [0125]
- **SCHROEDER et al.**Oncogene, 2004, vol. 23, 5739-5747 [0125]
- **SCHUTTERSREUTELINGSPERGER**Apoptosis, 2010, vol. 15, 1072-1082 [0125]
- **SHAH et al.**Proc. Natl. Acad. Sci. USA, 1998, vol. 95, 4229-4234 [0125]
- **SHUAI**Oncogene, 2000, vol. 19, 212638-44 [0125]
- **SIDDIQUEE et al.**Proc. Natl. Acad. Sci. USA, 2007, vol. 104, 187391-6 [0125]
- **SIDDIQUI et al.**Proc. Natl. Acad. Sci. USA, 1988, vol. 85, 2320-2323 [0125]
- **SINCLAIRELLIOTT**J Pharmaceutical Sci, 2005, vol. 94, 1626-1635 [0125]
- **SONG et al.**Proc. Natl. Acad. Sci. USA, 2005, vol. 102, 134700-5 [0125]
- **SOULE et al.**Cancer Res., 1990, vol. 50, 186075-6086 [0125]
- **SUHGUMBINER**Exp. Cell Res., 2003, vol. 290, 2447-56 [0125]
- **SUNG et al.**J Interferon Cytokine Res, 2003, vol. 23, 25-36 [0125]
- **TAITGIBSON**Arch Biochem Biophys., 1992, vol. 298, 1187-191 [0125]
- **TRUSCOTT et al.**J Cell Biol., 2003, vol. 163, 4707-713 [0125]
- **UMANA et al.**Nat Biotechnol, 1999, vol. 17, 176-180 [0125]
- **VACCARO et al.**Nature Biotechnol, 2005, vol. 23, 1283-1288 [0125]
- **VAN DEN EIJNDE et al.**J Cell Science, 2001, vol. 114, 3631-3642 [0125]
- **VERMEER et al.**Nature, 2003, vol. 422, 6929322-6 [0125]

- **VERONESEPASUT**Drug Discovery Today, 2005, vol. 10, 1451-1458 [0125]
- **WEBB et al.**J Immunol, 2002, vol. 169, 2580-2586 [0125]
- **WEBER**Advances Protein Chem., 1991, vol. 41, 1-36 [0125]
- **WEGENKA et al.**Mol. Cell Biol., 1994, vol. 14, 53186-96 [0125]
- **WEI et al.**Cancer Cell, 2005, vol. 7, 167-178 [0125]
- **WEI et al.**Cancer Res., 2007, vol. 67, 41853-8 [0125]
- **WEI et al.**Mol. Cell., 2006, vol. 21, 295-305 [0125]
- **WEIS**Cell, 2003, vol. 112, 4441-51 [0125]
- **WEN et al.**J. Biol. Chem., 2003, vol. 278, 38029-38039 [0125]
- **WIDER**BioTechniques, 2000, vol. 29, 1278-1294 [0125]
- **WIJNEN et al.**Biochem J., 1998, vol. 330, 389-396 [0125]
- **YAMAMOTO et al.**J. Biol. Chem., 1997, vol. 272, 12492-12494 [0125]
- **YEH et al.**Proc. Nat'l Acad. Sci. USA, 1992, vol. 89, 1904-1908 [0125]
- **YIN et al.**J. Biol. Chem., 2003, vol. 278, 35458-35464 [0125]
- **YIN et al.**J. Biol. Chem., 2004, vol. 279, 45721-45727 [0125]
- **YIN et al.**J. Biol. Chem., 2007, vol. 282, 257-266 [0125]
- **YOUNG et al.**Cell, 2003, vol. 112, 141-50 [0125]
- **YU et al.**Seminars Thrombosis Hemostasis, 2004, vol. 30, 121-30 [0125]
- **YUJOVE**Nat. Rev. Cancer, 2004, vol. 4, 297-105 [0125]
- **ZHANG et al.**Mol. Cell. Biol., 1999, vol. 19, 7138-7146 [0125]

PATENTKRAV

1. Sonde og/eller terapeutisk polypeptid, der er egnet til målretning af phosphatidylserin-celleoverfladeekspression både *in vitro* og *in vivo*, hvor polypeptidet omfatter:
et protein S gamma-carboxyglutamisyre- (Gla) domæne og
et EGF-domæne,
hvor polypeptidet er kondenseret til en proteinholdig sekvens; og
hvor polypeptidet er uden et proteasedomæne og ligeledes er uden et hormonbindingsdomæne.

2. Polypeptid ifølge krav 1, og som omfatter et EGF-domæne fra protein S.

3. Polypeptid ifølge krav 2, hvor GLA-domænet har sekvensen vist i SEQ ID NO: 1.

4. Polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor polypeptidet omfatter 300 aminosyrer eller færre.

5. Polypeptid ifølge et hvilket som helst af kravene 1 til 3, og som omfatter SEQ ID NO: 6.

6. Polypeptid ifølge et hvilket som helst af kravene 1 til 5, hvor polypeptidet endvidere omfatter et antistof-Fc-område.

7. Polypeptid ifølge et hvilket som helst af kravene 1 til 6 til anvendelse i behandling.

8. Polypeptid ifølge krav 7 til anvendelse i behandling i en kombinationsterapi.

9. Polypeptid ifølge et hvilket som helst af kravene 1 til 6 til anvendelse i behandling af cancer.

10. Polypeptid ifølge et hvilket som helst af kravene 1 til 6 til anvendelse i behandling af en virusinfektion.

11. Polypeptid ifølge krav 10 til anvendelse i behandling af en virusinfektion valgt fra gruppen bestående af influenza, humant immundefektvirus, denguevirus, West Nile-virus, koppevirus, respiratorisk syncytialvirus, koreansk hæmoragisk febervirus, skoldkopper, varicella zoster-virus, herpes simplex virus 1 eller 2, Epstein-Barr-virus, Marburg-virus, hantavirus, gul febervirus, hepatitis A, B, C eller E, Ebola-virus, humant papillomavirus, rhinovirus, Coxsackievirus, poliovirus, mæslingevirus, rubellavirus, rabiesvirus, Newcastle disease-virus, rotavirus, HTLV-1 og -2.

12. Polypeptid ifølge et hvilket som helst af kravene 1 til 6 til anvendelse i behandling af hyperkoaguleringsforstyrrelser lidelser.

13. Polypeptid ifølge et hvilket som helst af kravene 1 til 6 til anvendelse i behandling af autoimmun og/eller en inflammatorisk sygdomstilstand.

14. Polypeptid til anvendelse ifølge krav 13, hvor sygdomstilstanden vælges fra gruppen bestående af spondylarthritis, ankyloserende spondylitis, psoriasisartritis, reaktiv arthritis, enteropatisk arthritis, ulcerøs colitis, Crohns sygdom, irritabel tarmsygdom, inflammatorisk tarmsygdom, rheumatoid arthritis, juvenil rheumatoid arthritis, familiær middelhavsfeber, amyotrofisk lateral sklerose, Sjögrens syndrom, tidlig arthritis, viral arthritis, multipel sklerose, systemisk lupus erythematosus, psoriasis, vasculitis, Wegeners granulomatose, Addisons sygdom, alopeci, antiphospholipidsyndrom, Behcets sygdom, cøliaki, kronisk træthedssyndrom, ulcerøs colitis, type I diabetes, fibromyalgi, autoimmun gastritis, Goodpasture-syndrom, Graves' sygdom, idiopatisk thrombocytopenisk purpura (ITP), myasthenia gravis, pemphigus vulgaris, primær biliær cirrose, reumatisk feber, sarkoidose, sklerodermi, vitiligo, vasculitis, småkarsvasculitis, hepatitis, primær biliær cirrose, sarkoidose, sklerodermi, graft-versus-host-reaktion (akut og kronisk), aplastisk anæmi og cyklisk neutropeni.

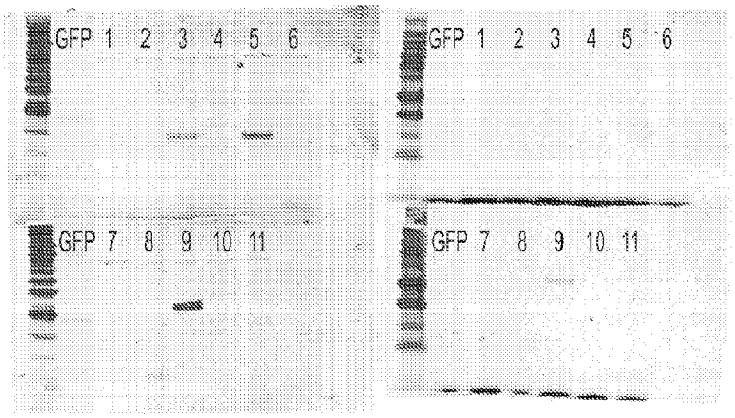
15. Polypeptid ifølge et hvilket som helst af kravene 1 til 6 til anvendelse i behandling af sepsis.

DRAWINGS

Gla PROTEIN	MEMBRANE AFFINITY	Gla RESIDUES	ADDITIONAL DOMAIN(S)	SIZE** (kD)
PROTEIN S	HIGH	11	EGF DOMAINS	4.95, 26.7
PROTEIN Z	MID	13	EGF DOMAINS	5.06, 13.9
PROTHROMBIN	MID	10	KRINGLE DOMAINS	5.06, 27.3
FVII	LOW	10	EGF DOMAIN ASSOCIATED WITH TF BINDING	4.95, 14.1
B0178	MID*	11	EGF DOMAIN ASSOCIATED WITH TF BINDING	4.95, 14.1

*AS DETERMINED BY FACs ON ACTIVATED PLATELETS IN COMPARISON TO FVII
**WITHOUT TAG

FIG. 1

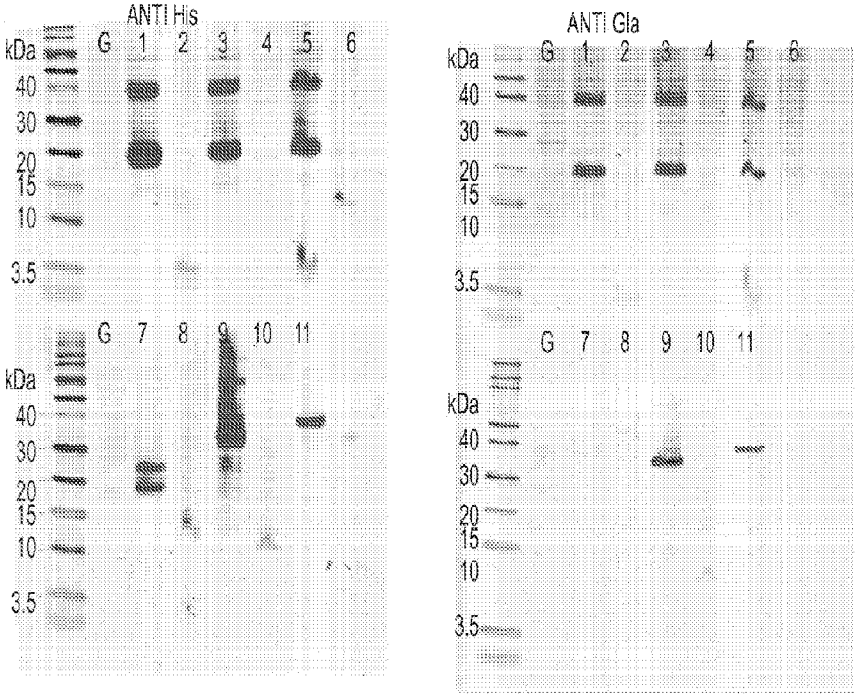


ANTI-Gla ANTI-His

PREP		DESCRIPTION
1	pEAKfl-CMV B0178 Gla+EGF	PROTHROMBIN SIGNAL SEQUENCE/PROPEPTIDE
2	pEAKfl-CMV B0178 Gla	
(3)	pEAKfl-CMV B0178 Gla+EGF	
4	pEAKfl-CMV FVII Gla	
(5)	pEAKfl-CMV FVII Gla+EGF	
6	pEAKfl-CMV ProteinZ Gla	
7	pEAKfl-CMV ProteinZ Gla+EGF	
(8)	pEAKfl-CMV ProteinS Gla	
(9)	pEAKfl-CMV ProteinS Gla+EGF	
10	pEAKfl-CMV PROTHROMBIN Ga	
11	pEAKfl-CMV PROTHROMBIN Ga+KRINGLE	

FIG. 2

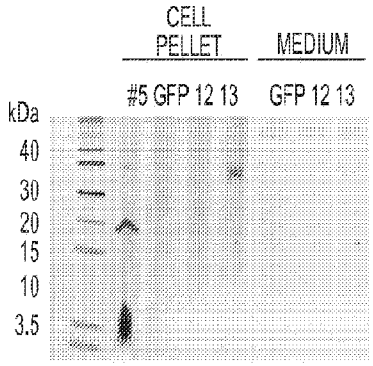
Gla PROTEIN	Gla DOMAIN ONLY (kDa)	Gla+EGF/KRINGLE (kDa)
B0178	6.5	15.4
FVII	6.5	15.4
PROTEIN Z	6.7	15.3
PROTEIN S	6.4	28
PROTHROMBIN	6.3	28.5



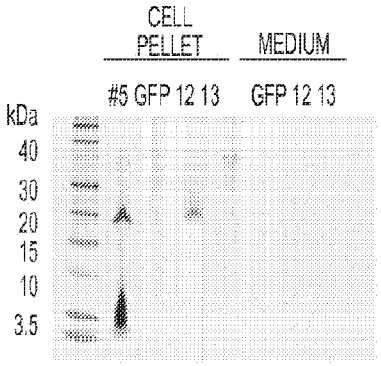
20ul OF SAMPLE (1/100 OF TOTAL CELL PELLET) LOADED

PREP		DESCRIPTION
1	pEAKfl-CMV B0178 Gla+EGF	PROTHROMBIN SIGNAL SEQUENCE/PROPEPTIDE
2	pEAKfl-CMV B0178 Gla	
3	pEAKfl-CMV B0178 Gla+EGF	
4	pEAKfl-CMV FVII Gla	
5	pEAKfl-CMV FVII Gla+EGF	
6	pEAKfl-CMV ProteinZ Gla	
7	pEAKfl-CMV ProteinZ Gla+EGF	
8	pEAKfl-CMV ProteinS Gla	
9	pEAKfl-CMV ProteinS Gla+EGF	
10	pEAKfl-CMV PROTHROMBIN Gla	
11	pEAKfl-CMV PROTHROMBIN Gla+KRINGLE	

FIG. 3



ANTI-Gla



ANTI-His

13.3ul OF EACH SAMPLE LOADED
10% BIS-TRIS GEL, REDUCING CONDITIONS

#	PROTEIN	MW (kDa)*	MW (kDa)**
5	FV11 G+E	15.4	19.6
12	PROTEIN Z G+E	15.3	19.8
13	PROTHROMBIN G+K	28.5	33.2

*PROCESSED FORM INCLUDING His TAG
**UNPROCESSED FORM INCLUDING His TAG

#5=Gla PROTEIN CONTROL (FV11 g+e FROM 3/2011 BHK21exp)
GFP=GFP TRANSFECTION CONTROL
#12=ProteinZ g+e WITH ProtS SIGNAL SEQUENCE AND PROPEPTIDE
#13=PROTHROMBIN g+k WITH ProtS SIGNAL SEQUENCE AND PROPEPTIDE

FIG. 4

1 11 21 31 41 51 61 71
ANSLLEETKQ GNLERECIEE LCNKEEAREV FENDPETDYF YPKYLVCLRS FQTGLFTAAR QSTNAYPDLR SCVNAIPDQC

81 91 101 111 121 131 141 151
SPLPCNEDGY MSCKDGKASF TCTCKPGWQG EKCEFDINEC KDPSNINGGC SQICDNTPGS YHCSCKNGFV MLSNKKDCKD

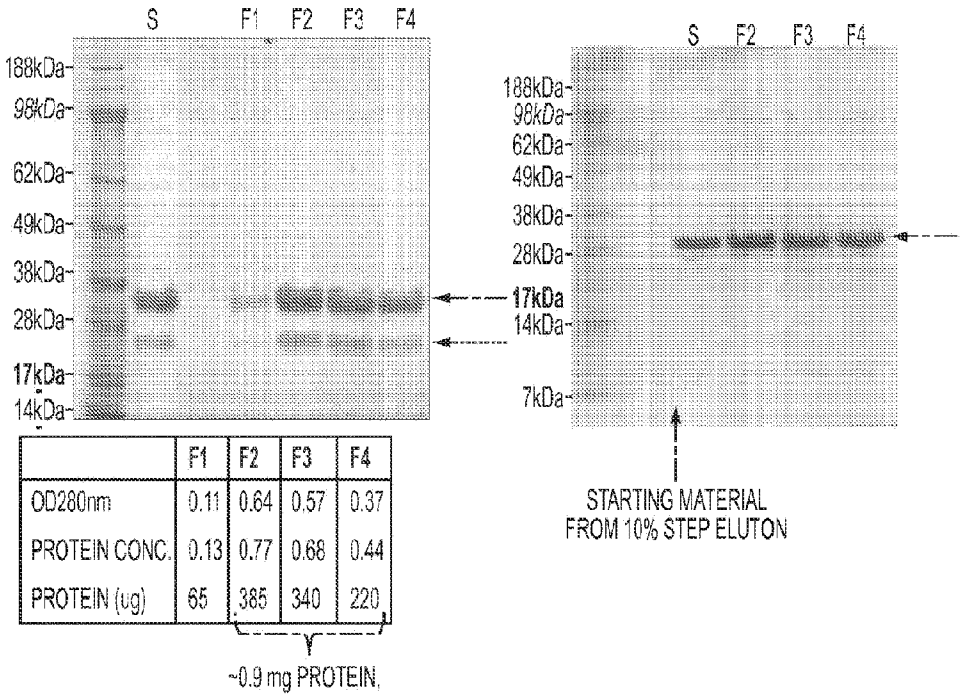
161 171 181 191 201 211 221 231
VDECSLKPSI CGTAVCKNIP GDFECECPEG YRYNLKSKSC EDIDECSENM CAQLCVNYPG GYTCYCDGKK GFKLAQDQKS

241
CESRHHHHHH

PTMs:

γ-CARBOXYLATION: 11Gla
D95: HYDROXYLATION
DISUFLIDE BOND: PSSIBLE 14, REPORTED 6
TAG: 6xHis

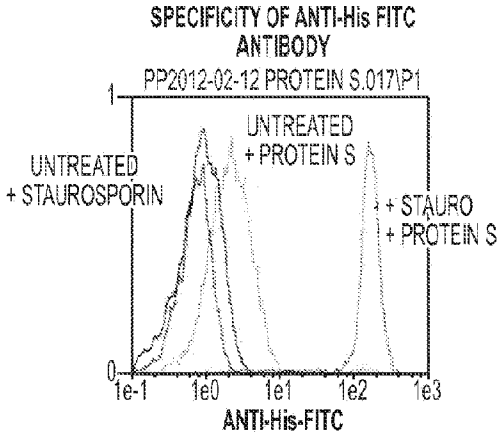
FIG. 5



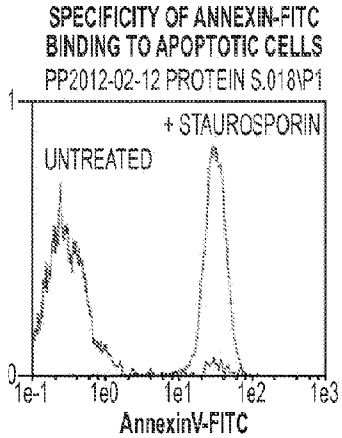
	F1	F2	F3	F4
OD280nm	0.11	0.64	0.57	0.37
PROTEIN CONC.	0.13	0.77	0.68	0.44
PROTEIN (ug)	65	385	340	220

~0.9 mg PROTEIN.

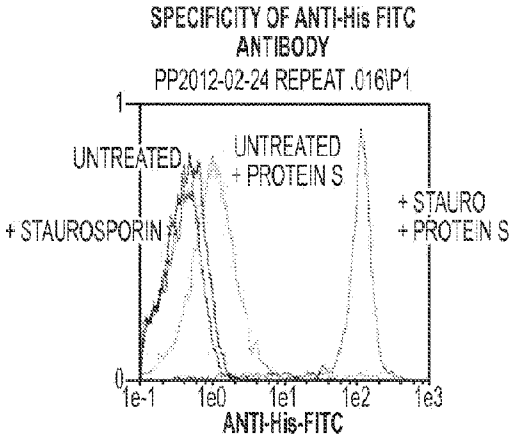
FIG. 6



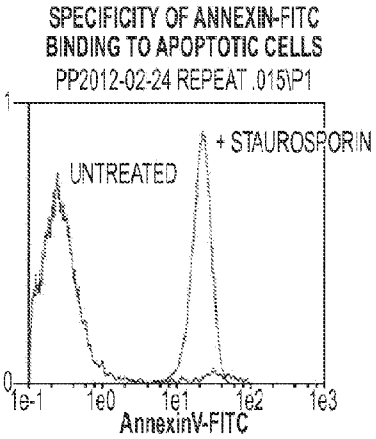
ID	UNTREATED	+ STAU	UNTREATED + PROT S	+ STAU +PROT S
MFI	0.91	0.79	4.72	159.3



ID	UNTREATED	+ STAU
MFI	1.93	32.3



ID	UNTREATED	+ STAU	UNTREATED + PROT S	+ STAU +PROT S
MFI	0.38	0.38	3.23	109.5

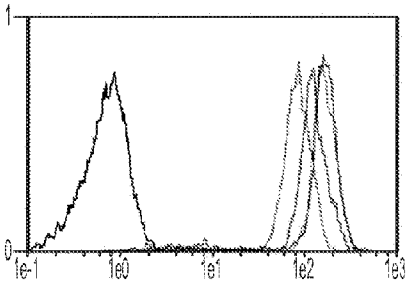


ID	UNTREATED	+ STAU
MFI	1.89	21.9

FIG. 7

ANNEXIN V COMPETES OFF PROTEIN S BINDING TO STAUROSPORIN TREATED JURKAT CELLS

PP2012-02-12 PROTEIN S.001\P1

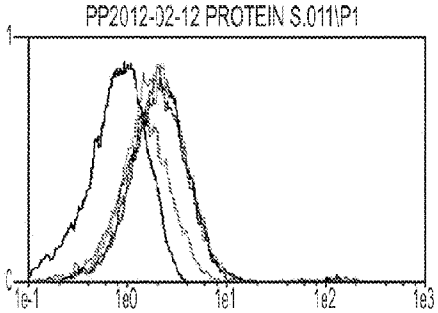


ANTI-His-FITC

ID	NO PROT S	+ PROT S	+ PROT S + 0.2X Ann-V	+ PROT S + 2.0X Ann-V	+ PROT S + 5.0X Ann-V
MFI	0.79	159.3	163.1	119.9	82.2

WEAK BINDING OF PROTEIN S TO UNTREATED JURKAT IS ALSO COMPETED OFF BY ANNEXIN V

PP2012-02-12 PROTEIN S.011\P1

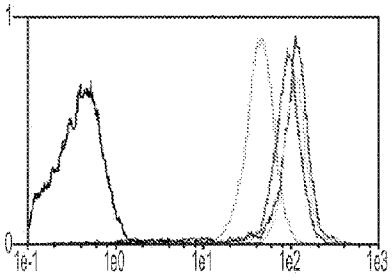


ANTI-His-FITC

ID	NO PROT S	+ PROT S	+ PROT S + 0.2X Ann-V	+ PROT S + 2.0X Ann-V	+ PROT S + 5.0X Ann-V
MFI	0.91	4.72	3.84	3.94	2.65

ANNEXIN V COMPETES OFF PROTEIN S BINDING TO STAUROSPORIN TREATED JURKAT CELLS

PP2012-02-24 REPEAT .006\P1

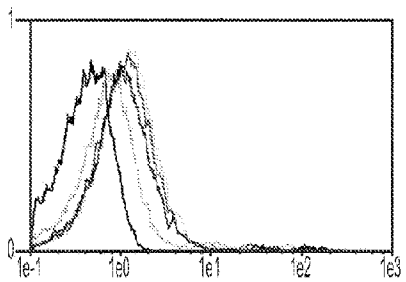


ANTI-His-FITC

ID	NO PROT S	+ PROT S	+ PROT S + 0.4X Ann-V	+ PROT S + 4.0X Ann-V	+ PROT S + 10.0X Ann-V
MFI	0.38	109.5	110.3	89.8	45.3

WEAK BINDING OF PROTEIN S TO UNTREATED JURKAT IS ALSO COMPETED OFF BY ANNEXIN V

PP2012-02-24 REPEAT .016\P1



ANTI-His-FITC

ID	NO PROT S	+ PROT S	+ PROT S -0.4X Ann-V	+ PROT S + 4.0X Ann-V	+ PROT S + 10.0X Ann-V
MFI	0.38	3.23	2.52	2.86	2.02

FIG. 8

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.