(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 August 2007 (16.08.2007)

PCT

(10) International Publication Number
WO 2007/092607 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2007/003528

(22) International Filing Date: 8 February 2007 (08.02.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/766,761 9 February 2006 (09.02.2006) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

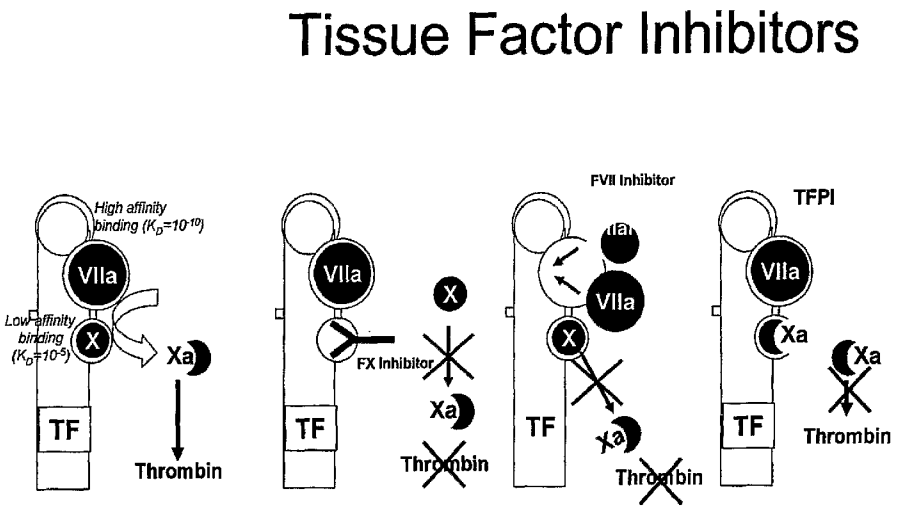
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: TREATMENT OF HEMORRHAGIC VIRAL INFECTIONS USING A TISSUE FACTOR INHIBITOR



(57) Abstract: [The present invention relates to novel methods for the treatment of hemorrhagic viral infections, such as Ebola and Marburg virus, by administering a tissue factor inhibitor.

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TREATMENT OF HEMORRHAGIC VIRAL INFECTIONS USING A TISSUE FACTOR INHIBITOR

BACKGROUND OF THE INVENTION

[Para 1] A number of viral families, such as Filoviridae, Arenaviridae, Bunyaviridae, and Flaviridae, cause lethal hemorrhagic disease in humans and certain other primates (Ruf W. Trends Immunol. (2004) 25: 461-464). In particular the Filoviridae, which include the Ebola and Marburg viruses, have resulted in significant morbidity and mortality in infected populations. Filovirus infections have reemerged in Africa over the last ten years, with outbreaks occurring more and more frequently. The present epidemic in Angola has killed more than 300 people, and the numbers are increasing (Baize, S. Nat. Med. (2005) 11:720-721).

[Para 2] Filoviruses, including the Marburg and Ebola viruses, cause sporadic epidemics of human disease characterized by systemic hemorrhage, multi-organ failure and death in most instances. In an outbreak or isolated case among humans, just how the virus is transmitted from the natural reservoir to a human is unknown, but the introduction of Ebola into the human population is often due to contact with infected non-human primates (Leroy, EM et al., Science (2004) 303:387-390). Once a human is infected, however, person-to-person transmission is the means by which further infections occur. Specifically, transmission involves close personal contact between an infected individual or their body fluids, and another person. During recorded outbreaks of hemorrhagic fever caused by filovirus infection, persons who cared for or worked very closely with infected individuals were especially at risk of becoming infected themselves. Nosocomial transmission through contact with infected body fluids, e.g., via re-use of unsterilized syringes, needles, or other medical equipment contaminated with these fluids-has also been an important factor in the spread of disease. When close contact between uninfected and infected persons is minimized, the number of new filovirus infections in humans usually declines. Although in the laboratory the viruses display some capability of infection through small-particle aerosols, airborne spread among humans has not been clearly demonstrated.

[Para 3] The onset of illness is abrupt, and initial symptoms resemble those of an influenza-like syndrome. Fever, headache, general malaise, myalgia, joint pain, and sore throat are commonly followed by diarrhea and abdominal pain. A transient morbilliform

skin rash, which subsequently desquamates, often appears at the end of the first week of illness. Other physical findings include pharyngitis, which is frequently exudative, and occasionally conjunctivitis, jaundice, and edema. After the third day of illness, hemorrhagic manifestations are common and include petechiae as well as bleeding, which can arise from any part of the gastrointestinal tract and from multiple other sites.

[Para 4] As the disease progresses, patients develop severe multifocal necroses and a syndrome resembling septic shock. In addition, activation of the fibrinolytic system coupled with the consumption of coagulation factors results in a depletion of clotting factors and degradation of platelet membrane glycoproteins.

[Para 5] The disease triggered by Ebola virus in primates is thought to involve inappropriate or maladaptive host response, and includes development of coagulation abnormalities not evident in rodents. Although the coagulopathy seen in Ebola hemorrhagic fever is probably caused by multiple factors, data suggest tissue factor plays an important part in triggering the coagulation abnormalities that characterize infections in primates (Geisbert TW, et al. J. Infect Dis. (2003) 188: 1618-1629). It has been shown that Ebola virus induces overexpression of tissue factor in primate monocytes and macrophages, and that overexpression depends on viral replication. In a recent study in monkeys infected with Ebola virus, recombinant nematode anticoagulant protein c2 (rNAPc2), a potent inhibitor of tissue factor-initiated blood coagulation, was shown to prolong survival time, attenuate blood coagulation response, and reduce systemic proinflammatory response (Geisbert TW, et al. Lancet. (2003) 362: 1953-1958).

[Para 6] There is currently no approved vaccine or direct therapy for the clinical manifestations of infection, other than general supportive measures. Interferon and ribavirin show no in vitro effect against these agents. The case-fatality rate is 40% to 90%, killing infected individuals within 1-2 weeks of disease onset. One study showed protection of monkeys infected 28 days after exposure to a vesicular stomatitis virus containing an Ebola or Marburg glycoprotein (Jones, SM et al., Nat. Med. (2005) 11:786-790). Unfortunately, when challenged with a different strain of Ebola or Marburg, the monkeys were not protected.

[Para 7] Other studies have demonstrated limited efficacy of passively transferred polyclonal antibodies in protection against Ebola challenge (Mikhailov et al, 1994, Voprosi Virusologii, 39, 82-84; Jahrling et al., 1996, Arch Virol, 11S, 135-140; Jahrling et al., 1999, J Infect Dis, 179 (Suppl 1), S224-234; Kudoyarova-Zubavichene et al., 1999, J

Infect Dis, 179(Suppl 1), S218-223). However, it is difficult to determine the effective therapeutic dose of antibodies in different preparations of polyclonal antibodies. Efforts to identify the role of antibodies in protection led to the isolation of monoclonal antibodies from mice vaccinated with Ebola GP (Wilson et al. Science 287, 1664 (2000)), and from convalescent people (Maruyama et al. J. Infect. Dis. 179 (suppl 1), S235, 1999; Maruyama et al. J. Virol. 73, 6024, 1999; Parren et al. J. Virol 76, 6408, 2002). These were tested in rodents and protected against lethal infection (Wilson et al. Science 287, 1664, 2000; Parren et al. J. Virol 76, 6408, 2002). One of these approaches may lead to an anti-viral treatment for hemorrhagic virus, but as yet none has been approved.

[Para 8] There is also a growing concern that hemorrhagic viruses may be used in biological terrorism and these viruses may be recombinantly engineered to be a more effective biological weapon. This creates the possibility of a terrorist initiated pandemic that would require an immediate response. And as mentioned, there are no satisfactory disease specific therapies or vaccines for an outbreak of Ebola or Marburg.

[Para 9] In view of the foregoing discussion, there is a clear unmet medical need for the development of treatments for these deadly disease conditions. The present invention addresses this need.

SUMMARY OF THE INVENTION

[Para 10] The present invention relates to a novel method of treating hemorrhagic viral infections, such as those caused by Filoviridae, Arenaviridae, Bunyaviridae, and Flaviridae, in particular the Filoviridae, which include the Ebola and Marburg viruses, comprising administering a tissue factor inhibitor. This approach is designed to overcome the shortcomings of the presently available treatments and prevent certain clinical outcomes including mortality and morbidity.

[Para 11] One aspect of the invention includes the treatment of hemorrhagic viral infections, including Ebola or Marburg virus infections, comprising the administration of a tissue factor inhibitor which binds tissue factor and blocks the activation of the extrinsic coagulation pathway. Tissue Factor inhibitors may include antibodies, peptide mimetics, tissue factor ligand analogs, tissue factor pathway inhibitor (TFPI), and organic molecules that inhibit tissue factor. These tissue factor inhibitors also include those that do not bind directly to tissue factor per se but to complexes of FVIIa-tissue factor, FX-tissue factor, FVIIa-FX-tissue factor, FIX-tissue factor, and FVIIa-FIX-tissue factor.

[Para 12] Another aspect of the invention is to provide other benefits such as shortened stays in the ICU, reduction in the time of hospitalization, shortened time on assisted ventilation, reduced incidence of complications, such as reduction in coagulopathy, reduction in the incidence of organ failure, reduction in multifocal necroses. Another aspect of the invention is the reduction in the mortality rates associated with these severe viral infections, and reduction in the number or severity of morbidities.

[Para 13] Other aspects of the invention include the treatment of hemorrhagic virus infections with a tissue factor inhibitor in combination with another antiviral agent such as, a vaccine comprising an attenuated virus or an anti-viral agent.

[Para 14] One aspect of the invention is the inhibition of only the extrinsic pathway, allowing some clotting to occur through the intrinsic pathway, but inhibiting the amplification of the cascade.

[Para 15] Numerous other advantages and aspects of the invention will become apparent to the skilled artisan upon consideration of the following detailed description of the invention.

BRIEF DESCRIPTION OF THE FIGURES

[Para 16] Figure 1 depicts the localization of tissue factor in the tissue of a normal blood vessel, sequestered from the lumen containing the cofactors necessary to trigger the coagulation cascade. Upon rupture of the blood vessel, the tissue factor comes in contact with its cofactors and triggers the coagulation cascade.

[Para 17] Figure 2 depicts the elements involved in the coagulation cascade, including the extrinsic pathway.

[Para 18] Figure 3 depicts the inhibitor target sites on tissue factor, including the FX binding site, the factor FVII binding site, as well as inhibitors that bind to a TF-cofactor complex.

[Para 19] Figure 4 depicts the chemical structure of various anti-TF inhibitors as disclosed in US Patent 6,608,066.

DETAILED DESCRIPTION OF THE INVENTION

[Para 20] This invention is not limited to the particular methodology, protocols, cell lines, vectors, or reagents described herein because they may vary. Further, the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention. As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless

the context clearly dictates otherwise, *e.g.*, reference to "a host cell" includes a plurality of such host cells.

[Para 21] Unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the exemplary methods, devices, and materials are described herein.

[Para 22] All patents and publications mentioned herein are incorporated herein by reference to the extent allowed by law for the purpose of describing and disclosing the proteins, enzymes, vectors, host cells, and methodologies reported therein that might be used with the present invention. However, nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

DEFINITIONS

[Para 23] The term "amino acid sequence variant" refers to polypeptides having amino acid sequences that differ to some extent from a native sequence polypeptide. Ordinarily, amino acid sequence variants will possess at least about 70% homology, or at least about 80%, or at least about 90% homology to the native polypeptide. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of the native amino acid sequence.

[Para 24] The term "identity" or "homology" is defined as the percentage of amino acid residues in the candidate sequence that are identical with the residue of a corresponding sequence to which it is compared, after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent identity for the entire sequence, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art. Sequence identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987;

. and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, 30 H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S. F. et al., J Molec. Biol. 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLASTManual, Altschul, S., et al, NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well-known Smith Waterman algorithm may also be used to determine identity.

[Para 25] The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity.

[Para 26] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. In contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al, *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991), for example. The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in

antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al, *Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984)).

[Para 27] "Antibody fragments" comprise a portion of an intact antibody comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragment(s).

[Para 28] An "intact" antibody is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, CH_1, CH_2 and CH_3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. The intact antibody may have one or more effector functions.

[Para 29] Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc.

[Para 30] Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five-major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[Para 31] The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. These hypervariable regions are also called complementarity determining regions or CDRs. The more highly conserved portions of variable domains

are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

[Para 32] The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (e.g. residues 2632 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

[Para 33] "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[Para 34] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a

review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). Anti-ErbB2 antibody scFv fragments are described in WO93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458.

[Para 35] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which comprise a variable heavy domain (V_H) connected to a variable light domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

[Para 36] A "single-domain antibody" is synonymous with "dAb" and refers to an immunoglobulin variable region polypeptide wherein antigen binding is effected by a single variable region domain. A "single-domain antibody" as used herein, includes i) an antibody comprising heavy chain variable domain (VH), or antigen binding fragment thereof, which forms an antigen binding site independently of any other variable domain, ii) an antibody comprising a light chain variable domain (VL), or antigen binding fragment thereof, which forms an antigen binding site independently of any other variable domain, iii) an antibody comprising a VH domain polypeptide linked to another VH or a VL domain polypeptide (e.g., VH-VH or VHx-VL), wherein each V domain forms an antigen binding site independently of any other variable domain, and iv) an antibody comprising VL domain polypeptide linked to another VL domain polypeptide (VL-VL), wherein each V domain forms an antigen binding site independently of any other variable domain. As used herein, the VL domain refers to both the kappa and lambda forms of the light chains.

[Para 37] "Humanized" forms of non-human (e.g., rodent) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies are human immunoglobulins wherein the hypervariable regions are replaced by residues from a hypervariable region of a non-human species, such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the human antibody or in the non-human antibody.

These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Examples of humanization technology may be found in, e.g., Queen et al. U.S. Pat. No. 5,585,089, 5,693,761; 5,693,762; and 6,180,370, which are incorporated herein by reference.

VIRAL INFECTIONS

[Para 38] Filoviruses, including the Marburg and Ebola viruses, cause sporadic epidemics of human disease characterized by systemic hemorrhage, multi-organ failure and death in most instances. In an outbreak or isolated case among humans, just how the virus is transmitted from the natural reservoir to a human is unknown. Once a human is infected, however, person-to-person transmission is the means by which further infections occur. Specifically, transmission involves close personal contact between an infected individual or their body fluids, and another person. During recorded outbreaks of hemorrhagic fever caused by filovirus infection, persons who cared for or worked very closely with infected individuals were especially at risk of becoming infected themselves. Nosocomial transmission through contact with infected body fluids, e.g., via re-use of unsterilized syringes, needles, or other medical equipment contaminated with these fluids- has also been an important factor in the spread of disease. When close contact between uninfected and infected persons is minimized, the number of new filovirus infections in humans usually declines. Although in the laboratory the viruses display some capability of infection through small-particle aerosols, airborne spread among humans has not been clearly demonstrated.

[Para 39] The onset of illness is abrupt, and initial symptoms resemble those of an influenza-like syndrome. Fever, headache, general malaise, myalgia, joint pain, and sore throat are commonly followed by diarrhea and abdominal pain. A transient morbilliform skin rash, which subsequently desquamates, often appears at the end of the first week of illness. Other physical findings include pharyngitis, which is frequently exudative, and occasionally conjunctivitis, jaundice, and edema. After the third day of illness,

hemorrhagic manifestations are common and include petechiae as well as frank bleeding, which can arise from any part of the gastrointestinal tract and from multiple other sites.

[Para 40] As the disease progresses, patients develop severe multifocal necroses and a syndrome resembling septic shock. In addition, activation of the fibrinolytic system coupled with the consumption of coagulation factors results in a depletion of clotting factors and degradation of platelet membrane glycoproteins.

[Para 41] The disease triggered by Ebola virus in primates is thought to involve inappropriate or maladaptive host response, and includes development of coagulation abnormalities not evident in rodents. Although the coagulopathy seen in Ebola haemorrhagic fever is probably caused by multiple factors, data suggest tissue factor plays an important part in triggering the coagulation abnormalities that characterize infections in primates (Geisbert Tw, et al. J. Infect Dis. (2003) 188: 1618-1629). It has been shown that Ebola virus induces overexpression of tissue factor in primate monocytes and macrophages, and that overexpression depends on viral replication.

[Para 42] There are animal models described that can be used for testing new candidate antiviral drugs for treatment. One example is a respiratory infection model has been established in cynomolgus macaques (*Macaca fascicularis*). The model is based on intratracheal inoculation of the virus as this method was reproducible and easy to standardize. FDA regulations require the use of animal models in which the animal study endpoint is clearly related to the desired human benefit: the so-called 'animal rule'. In this model has been described by Stittelaar, K. J., et. al. (Nature 2005 Dec 11; [Epub ahead of print]) for use in studies with various viral infections, such as monleypox and influenza virus. Generally, upon histopathological examination, the lungs of these animals showed acute lung injury, characterized by necrosis of the alveolar wall and flooding of the alveolar lumina with cellular and acellular components, including abundant fibrin. The association of these lesions with monkeypox virus infection was confirmed by transmission electron microscopy.

[Para 43] The present invention is designed to address the problem of treatment in individuals who have already contracted the disease and no longer can be treated with prophylactic methods. Therefore, the present invention relates to an alternative intervention strategy for the treatment of serious hemorrhagic viral infections. The present invention relates to the use of tissue factor inhibitors as a therapy to fill this unmet medical need. Another aspect of the invention is to provide other benefits such as shortened stays

in the ICU, reduction in the time of hospitalization, shortened time on assisted ventilation, reduced incidence of complications, such as reduction in coagulopathy, reduction in the incidence of organ failure, reduction in multifocal necroses. Another aspect of the invention is the reduction in the mortality rates associated with these severe viral infections, and reduction in the number or severity of morbidities.

[Para 44] One embodiment of the present invention is an anti-tissue factor antibody that binds to human TF or the TF-Factor VIIa (FVIIa) complex preventing binding and/or activation of Factor X (FX) and Factor IX (FIX), thereby inhibiting thrombin generation. By blocking the initiating events of extrinsic coagulation activation, their effects on pro-inflammatory events in the lungs and disordered fibrin deposition may be minimized and the evolution of severe structural and functional injury may be averted during the course of viral infection. Antibodies useful in the present invention may bind tissue factor, blocking or inhibiting the action of Factor VII, Factor VIIa, Factor IX or Factor X.

[Para 45] The antibody may be monoclonal and may be chimeric, humanized, or human. The antibody may also be a single-domain antibody. Examples of such antibodies of the invention that inhibit TF function by effectively blocking FX binding or access to TF molecules, include H36.D2.B7 (secreted by hybridoma ATCC HB-12255) and humanized clones of this antibody. Other anti-TF antibodies useful in the invention include those disclosed in U.S. Pat. No. 6,555,319; 5,986,065; 5,223,427; 6,677,436; 6,703,494; US20040001830; or PCT application WO2004/039842, which are incorporated by reference. Antibodies may also be directed to the cofactor itself, such as Factor VII or Factor VIIa, thereby inhibiting tissue factor by blocking the ligand necessary for activation. Examples of such antibodies have been disclosed in 5,506,134 and 6,835,817.

[Para 46] Peptide mimetics include fragments of tissue factor that bind Factor VII, Factor IX or Factor X, thereby blocking their activation. Tissue factor ligand analogs include modified Factor VII, Factor IX or Factor X, that bind tissue factor but do not allow activation.

[Para 47] Other molecules useful in the present invention include molecules such as those disclosed in WO 00/18398 and WO 01/30333.

ANTIBODY GENERATION

[Para 48] The antibodies of the present invention may be generated by any suitable method known in the art. The antibodies of the present invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the

skilled artisan (Harlow, et al., Antibodies: a Laboratory Manual, (Cold spring Harbor Laboratory Press, 2nd ed. (1988), which is hereby incorporated herein by reference in its entirety).

[Para 49] For example, antibodies may be generated by administering an immunogen comprising the antigen of interest to various host animals including, but not limited to, rabbits, mice, rats, etc., to induce the production of sera containing polyclonal antibodies specific for the antigen.

[Para 50] One method of generating such antibodies to tissue factor may be found in U.S. Pat. No. 6,555,319 and 5,986,065 (which are hereby incorporated herein by reference in their entirety).In brief, monoclonal antibodies directed to human tissue factor can be raised by immunizing rodents (e.g. mice, rats, hamsters and guinea pigs) with a purified sample of native TF, typically native human TF, or a purified recombinant human tissue factor (rhTF). Truncated recombinant human tissue factor or "rhTF" (composed of 243 amino acids and lacking the cytoplasmic domain) may be used to generate anti-TF antibodies. The antibodies also can be generated from an immunogenic peptide that comprises one or more epitopes of native TF that are not exhibited by non-native TF. References herein to "native TF" include such TF samples, including such rhTF.

[Para 51] Antibodies directed to other antigens such as Factor VII or Factor X may be generated in a similar manner.

[Para 52] The antibodies useful in the present invention include monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma technology, such as those described by Kohler and Milstein, Nature, 256:495 (1975) and U.S. Pat. No. 4,376,110, by Harlow, et al., Antibodies: A Laboratory Manual, (Cold spring Harbor Laboratory Press, 2.sup.nd ed. (1988), by Hammerling, et al., Monoclonal Antibodies and T-Cell Hybridomas (Elsevier, N.Y., (1981)), or other methods known to the artisan. Other examples of methods that may be employed for producing monoclonal antibodies include, but are not limited to, the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the antibodies of this invention may be cultivated in vitro or in vivo.

[Para 53] Using typical hybridoma techniques, a host such as a mouse, a humanized mouse, a mouse with a human immune system, hamster, rabbit, camel or any other appropriate host animal, is typically immunized with an immunogen to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the antigen of interest. Alternatively, lymphocytes may be immunized in vitro with the antigen. Hybridoma technology is well known in the art.

[Para 54] A variety of methods exist in the art for the production of monoclonal antibodies and thus, the invention is not limited to their sole production in hydridomas. For example, the monoclonal antibodies may be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567.

[Para 55] The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain cross-linking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent cross-linking.

[Para 56] Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')2 fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) and pepsin (to produce F(ab')2) fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

[Para 57] For some uses, including in vivo use of antibodies in humans and in vitro detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Gillies et al., (1989) J. Immunol. Methods 125:191-202; U.S. Pat. Nos. 5,807,715; 4,816,567; and 4,816397, which are incorporated herein by reference in their entirety.

[Para 58] Humanized antibodies are antibody molecules generated in a non-human species that bind the desired antigen having one or more complementarity determining

regions (CDRs) from the non-human species and framework (FR) regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Pat. No. 5,585,089; Riechmann et al., Nature 332:323 (1988), which are incorporated herein by reference in their entireties). Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Molecular Immunology 28(4/5):489-498 (1991); Studnicka et al., Protein Engineering 7(6):805-814 (1994); Roguska. et al., PNAS 91:969-973 (1994)), and chain shuffling (U.S. Pat. No. 5,565,332).

[Para 59] Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the methods of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possible some FR residues are substituted from analogous sites in rodent antibodies.

[Para 60] Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated

herein by reference in its entirety. The techniques of Cole et al., and Boerder et al., are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Riss, (1985); and Boerner et al., J. Immunol., 147(1):86-95, (1991)).

[Para 61] Human antibodies can also be single-domain antibodies having a VH or VL domain that functions independently of any other variable domain. These antibodies are typically selected from antibody libraries expressed in phage. These antibodies and methods for isolating such antibodies are described in U.S. Pat. No. 6,595,142; 6,248,516; and applications US20040110941 and US20030130496 all of which are incorporated herein by reference.

[Para 62] Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention.

[Para 63] Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, Int. Rev. Immunol. 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and

protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.), Genpharm (San Jose, Calif.), and Medarex, Inc. (Princeton, N.J.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[Para 64] Also human MAbs could be made by immunizing mice transplanted with human peripheral blood leukocytes, splenocytes or bone marrows (e.g., Trioma techniques of XTL). Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al., Bio/technology 12:899-903 (1988)).

[Para 65] Further, antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" polypeptides of the invention using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, FASEB J. 7(5):437-444; (1989) and Nissinoff, J. Immunol. 147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide of the invention to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligands/receptors, and thereby block its biological activity.

[Para 66] The antibodies of the present invention may be bispecific antibodies. Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present invention, one of the binding specificities may be directed towards tissue factor, the other may be for any other antigen, and preferably for a cell-surface protein, receptor, receptor subunit, tissue-specific antigen, virally derived protein, virally encoded envelope protein, bacterially

derived protein, or bacterial surface protein, etc. Bispecific antibodies may also comprise two or more single-domain antibodies.

[Para 67] Methods for making bispecific antibodies are well known. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature, 305:537-539 (1983). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published May 13, 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

[Para 68] Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It may have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transformed into a suitable host organism. For further details of generating bispecific antibodies see, for example Suresh et al., Meth. In Enzym, 121:210 (1986).

[Para 69] Heteroconjugate antibodies are also contemplated by the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980). It is contemplated that the antibodies may be prepared in vitro using known methods in synthetic protein chemistry, including those involving cross-linking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioester bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.In addition, one can generate single-domain antibodies to tissue factor. Examples of this technology have been described in WO9425591 for antibodies derived from Camelidae heavy chain Ig, as well in

US20030130496 describing the isolation of single domain fully human antibodies from phage libraries.

IDENTIFICATION OF ANTI-TISSUE FACTOR ANTIBODIES

[Para 70] Plates for the ELISA assay were coated with 100 microliters of recombinant tissue factor (0.25 μg/ml) in a carbonate based buffer. All steps were performed at room temperature. Plates were blocked with BSA, washed, and then the test samples and controls were added. Antigen/antibody binding was detected by incubating the plate with goat anti-mouse HRP conjugate (Jackson ImmunoResearch Laboratories) and then using an ABTS peroxidase substrate system (Kirkegaad and Perry Laboratories). Absorbance was read on an automatic plate reader at a wavelength of 405 nm.

PEPTIDE MIMETICS AND PEPTIDE INHIBITORS

[Para 71] Peptide mimetics useful in the present invention include FVII, Factor VIIa, Factor IX, and Factor X analogs that bind tissue factor mimicking the naturally occurring cofactor, but are non-functional and therefore inhibit the action of tissue factor. Examples of FVII and FVIIa analogs are disclosed in, e.g., 5,788,965, or US20060019893. Examples of tissue factor analogs include mutant forms of tissue factor that bind FVII or FVIIa or FX but are unable to trigger the cascade or activate FX. Such TF analogs, are described, e.g., 5,994,296, or 5,726,147.

[Para 72] Another embodiment of the present invention includes tissue factor analogs that bind FVII or FVIIa preventing the activation of FX by tissue factor.

[Para 73] Another aspect of the invention include FVII and FVIIa inhibitors, such as those disclosed in US20040087767.

PHARMACEUTICALLY ACTIVE COMPOUNDS

[Para 74] Pharmaceutically active compounds that inhibit the action of tissue factor are described in WO00/18398 and WO01/30333, which are incorporated by reference in their entirety. These compounds include Formula I: $AR\text{-}(CXY)_m\text{-}(HET)\text{-}(CX^1Y^1)_n\text{-}C(Z)_p\text{-}(PO_3)_{3\text{-}p}$, wherein Ar is optionally substituted carbocyclic aryl or optionally substituted heteroaryl; Het is optionally substituted N, O or S; each X, each Y, each X', each Y' and each Z are each independently hydrogen; halogen; hydroxyl; sulfhydryl; amino; optionally substituted alkyl preferably; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio; optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; or optionally substituted alkylamino; m

and n each is independently an integer of from 0 to 4; p is 1 or 2; and pharmaceutically acceptable salts thereof.

[Para 75] Formula II (See Figure 1), wherein X, Y, Het, X', Y', Z, m, n and p are the same as defined above; each R^1 is independently halogen; amino; hydroxy; nitro; carboxy; sulfhydryl; optionally substituted alkyl; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio; optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; optionally substituted alkylamino; optionally substituted alkanoyl; optionally substituted carbocyclic aryl; or optionally substituted aralkyl; and q is an integer of from 0 to 5; and pharmaceutically acceptable salts thereof.

[Para 76] Formula III (See Figure I), wherein X, Y, X', Y', Z, m, n and p are the same as defined above; W is hydrogen, optionally substituted alkyl; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio; optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; optionally substituted alkylamino; optionally substituted alkanoyl; optionally substituted carbocyclic aryl; or optionally substituted aralkyl; R^1 is independently halogen; amino; hydroxy; nitro; carboxy; sulfhydryl; optionally substituted alkyl; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio; optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; optionally substituted alkylamino; optionally substituted alkanoyl; optionally substituted carbocyclic aryl; or optionally substituted aralkyl; q is an integer of from 0 to 5; and pharmaceutically acceptable salts thereof.

[Para 77] Formula IIIA (See Figure 1), wherein R^1 is independently halogen; amino; hydroxy; nitro; carboxy; sulfhydryl; optionally substituted alkyl; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio; optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; optionally substituted alkylamino; optionally substituted alkanoyl; optionally substituted carbocyclic aryl; or optionally substituted aralkyl; and q is an integer of from 0 to 5; and pharmaceutically acceptable salts thereof.

[Para 78] Formula IV (See Figure 1), wherein X, Y, X', Y', Z, m, n and p are the same as defined above; R^1 is independently halogen; amino; hydroxy; nitro; carboxy; sulfhydryl; optionally substituted alkyl; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio;

optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; optionally substituted alkylamino; optionally substituted alkanoyl; optionally substituted carbocyclic aryl; or optionally substituted aralkyl; q is an integer of from 0 to 5; and pharmaceutically acceptable salts thereof; AND

[Para 79] Formula IVA (See Figure I), wherein X', Y', and n are the same as defined above; R^1 is independently halogen; amino; hydroxy; nitro; carboxy; sulfhydryl; optionally substituted alkyl; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio; optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; optionally substituted alkylamino; optionally substituted alkanoyl; optionally substituted carbocyclic aryl; or optionally substituted aralkyl; and q is an integer of from 0 to 5; and pharmaceutically acceptable salts thereof.

[Para 80] Preparation of 1-(bisphosponate)-2-amino(3-hydroxy-phenyl)ethyl, as an example, involves following the method of Degenhardt et al., *J. Org. Chem.*, 51: 3488-3490 (1986) to produce the compound. Briefly, paraformaldehyde (104.2 g, 3.47 mol) and di-ethylamine (50.8 g, 0.69 mol) are combined in 2 liters of methanol and the mixture warmed until clear. The heat is removed and $CH_2(PO_3(CH_2CH_3)_2)_2$ (200 g, 0.69 mol) is added. The mixture is refluxed for 24 hours, and then an additional 2 liters of methanol is added, and the solution concentrated under reduced pressure at 35°C. 1 liter of toluene is added to the concentrate, and the resulting solution concentrated, and the toluene addition and concentration repeated. The resulting intermediate is then dissolved in 1 liter of dry toluene, p-toluenesulfonic acid monohydrate (0.50 g) is added and the mixture is refluxed. Resulting methanol is removed, e.g. via a Dean-Stark trap or molecular sieves. After 14 hours the solution can be concentrated, diluted in chloroform, washed with water (2 x 150 ml), dried over $MgSO_4$ and concentrated. The resulting compound, $CH_2=C(PO_3(CH_2CH_3)_2)_2$, can be purified if desired such as distillation. The compound $CH_2=C(PO_3(CH_2CH_3)_2)_2$ can then be reacted as desired to provide compounds of the invention. In particular, to provide the title compound, $CH_2=C(PO_3(CH_2CH_3)_2)_2$, can be reacted with NH_2(3-hydroxyphenyl) in a Michael reaction. The phosphono di-ester can be converted to the di-acid by treatment with bromotrimethylsilane (see, e.g. Morita et al., *Bull. Chem. Soc. Jpn.*, 54:267 (1981)).

EXAMPLES

[Para 81] The invention now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention. The following detailed guidance and examples are provided to further illustrate and define preferred aspects of the invention.

EXAMPLE 1 – Fxa-SPECIFIC SUBSTRATE ASSAY

[Para 82] In general, the experiments described herein may be used to determine if a given antibody inhibits the activity of tissue factor. Experiments were conducted using rhTF lipidated with phosphatidycholine (0.07 mg/ml) and phosphatidylserine (0.03 mg/ml) at a 70/30 w/w ratio in 50 mM Tris-HCl, pH 7.5, 0.1% bovine serum albumin (BSA) for 30 minutes at 37°C. A stock solution of preformed TF:VIIa complex was made by incubating 5 nM of the lipidated rhTF and 5 nM of FVIIa for 30 minutes at 37°C. The TF:VIIa complex was aliquoted and stored at -70°C until needed. Purified human factors VII, VIIa, and FX were obtained from Enyzme Research Laboratories, Inc. The following buffer was used for all FXa and FVIIa assays: 25 mM Hepes-NaOH, 5 mM $CaCl_2$, 150 mM NaCl, 0.1% BSA, pH 7.5.

[Para 83] Mabs were tested for their capacity to block TF:VIIa-mediated activation of FX to FXa. The FX activation was determined in two discontinuous steps. In the first step (FX activation), FX conversion to FXa was assayed in the presence of Ca^{+2}. In the second step (FXa activity assay), FX activation was quenched by EDTA and the formation of FXa was determined using a FXa-specific chromogenic substrate (S-2222). The S-2222 and S-2288 (see below) chromogens were obtained from Chromogenix (distributed by Pharmacia Hepar Inc.). FX activation was conducted in 1.5 ml microfuge tubes by incubating the reaction with 0.08 nM TF:VIIa, either pre-incubated with an anti-rhTF antibody or a buffer control. The reaction was subsequently incubated for 30 minutes at 37°C, then 30 nM FX was added followed by an additional incubation for 10 minutes at 37°C. FXa activity was determined in 96-well titre plates. Twenty microlitres of sample was withdrawn from step one and admixed with an equal volume of EDTA (500 mM) in each well, followed by addition of 0.144 ml of buffer and 0.016 ml of 5 mM S-2222 substrate. The reaction was allowed to incubate for an additional 15-30 minutes at 37°C. Reactions were then quenched with 0.05 ml of 50% acetic acid, after which, absorbance at 405 nm was recorded for each reaction. The inhibition of TF:VIIa activity was calculated

from OD_{405nm} values in the experimental (plus antibody) and control (no antibody) samples. In some experiments, an anti-hTF antibody, TF/VIIa, and FX were each added simultaneously to detect binding competition. H36.D2 MAb inhibited TF:/VIIa activity toward FX to a significantly greater extent (95%) than other anti-rHTF Mabs tested.

EXAMPLE 2 – FVIIa SPECIFIC SUBSTRATE ASSAY

[Para 84] Mabs may be further screened by an FVIIa specific assay. In this assay, 5 nM lipidated rhTF was first incubated with buffer (control) or 50 nM antibody (experimental) in a 96-well titre plate for 30 minutes at 37°C, then admixed with 5 nM purified human FVIIa (V_T= 0.192 ml), followed by 30 minutes incubation at 37°C. Eight microliters of a 20 mM stock solution of the FVIIa specific substrate S-2288 was then added to each well (final concentration, 0.8 mM). Subsequently, the reaction was incubated for one hour at 37°C. Absorbance at 405 nm was then measured after quenching with 0.06 ml of 50% acetic acid. Percent inhibition of TF/VIIa activity was calculated from OD_{405nm} values from the experimental and control samples.

[Para 85] H36 antibody did not significantly block TF/VIIa activity toward the S-2288 substrate when the antibody was either pre-incubated with TF (prior to VIIa addition) or added to TF pre-incubated with VIIa (prior to adding the antibody). This indicates that H36 does not interfere with the interaction (binding) between TF and FVIIa, and that H36 also does not inhibit TF:VIIa activity toward a peptide substrate.

EXAMPLE 3 – PROTHROMBIN TIME (PT) ASSAY

[Para 86] Calcified blood plasma will clot within a few seconds after addition of thromplastin (TF); a phenomenon called the "prothrombin time" (PT). A prolonged PT is typically a useful indicator of anti-coagulation activity (see e.g., Gilman et al. *supra*).

[Para 87] The H36.D2 antibody was investigated for capacity to affect PT according to standard methods using commercially available human plasma (Ci-Trol Control, Level I obtained from Baxter Diagnostics Inc.). Clot reactions were initiated by addition of lipidated rhTF in the presence of Ca^{++}. Clot time was monitored by an automated coagulation timer (MLA Electra 800). PT assays were initiated by injecting 0.2 ml of lipidated rhTF (in a buffer of 50 mM Tris-HCl, pH 7.5, containing 0.1% BSA, 14.6 mM $CaCl_2$, 0.07 mg/ml of phosphatidylcholine, and 0.03 mg/ml of phosphatidylserine) into plastic twin-well cuvettes. The cuvettes each contained 0.1 ml of the plasma preincubated with either 0.01 ml of buffer (control sample) or antibody (experimental sample) for 1-2 minutes. The inhibition of TF-mediated coagulation by the H36.D2 antibody was

calculated using a TF standard curve in which the log [TF] was plotted against log clot time.

[Para 88] H36.D2 antibody substantially inhibits TF-initiated coagulation in human plasma. The H36.D2 antibody increased PT times significantly, showing that the antibody is an effective inhibitor of TF-initiated coagulation (up to approximately 99% inhibition).

EXAMPLE 4 – BINDING OF H36 TO rhTF

[Para 89] Evaluation of the binding of an antibody to tissue factor may be assayed according to the following protocol. H36.D2 binding to native and non-native rhTF was performed by a simplified dot blot assay. Specifically, rhTF was diluted to 30 μg/ml in each of the following three buffers: 10 mM Tris-HCl, pH 8.0; 10 mM Tris-HCl, pH 8.0 and 8 M urea; and 10 mM Tris-HCl, pH 8.0, 8 M urea and 5 mM dithiothreitol. Incubation in the Tris buffer maintains rhTF in native form, whereas treatment with 8M urea and 5nM dithiothreitol produces non-native (denatured) rhTF. Each sample was incubated for 24 hours at room temperature. After the incubation, a Millipore Immobilon (7x7cm section) membrane was pre-wetted with methanol, followed by 25 mM Tris, pH 10.4, including 20% methanol. After the membranes were air-dried, approximately 0.5 μl, 1 μl, and 2 μl of each sample (30 μg/ml) was applied to the membrane and air-dried. After blocking the membrane by PBS containing 5% (w/v) skim milk and 5% (v/v) NP-40, the membrane was probed with H36.D2 antibody, followed by incubation with a goat anti-mouse IgG peroxidase conjugate (obtained from Jackson ImmunoResearch Laboratories, Inc.). After incubation with ECL Western Blotting reagents in accordance with the manufacturer's instructions (Amersham), the membrane was wrapped with plastic film (Saran Wrap) and exposed to X-ray film for various times.

[Para 90] H36.D2 Mab binds a conformational epitope on native TF in the presence of Tris buffer or Tris buffer with 8M urea. (See U.S. Pat. No. 6,555,319) The autoradiogram was exposed for 40 seconds.

EXAMPLE 5 – VIRAL INFECTION MODEL

[Para 91] The following study is used to assess the effect of an exemplary tissue factor inhibitor, an anti-TF antibody, on the coagulation cascade, inflammatory response, viral dynamics, and lung damage due to hemorrhagic viral infection in macaques, as a model for the disease in humans.

[Para 92] A lethal intratracheal infection model in cynomolgus monkeys (*Macaca fascicularis*) with Ebola virus is used. This model is appropriate to study the possible

efficacy of an anti-TF antibody to decrease the severity of virus infection. This model complies with the FDA regulations requiring the use of animal models in which the animal study endpoint is clearly related to the desired human benefit: the so-called 'animal rule'. The intratracheal viral infection in macaques is currently the main method of studying respiratory infection in human primates.

[Para 93] The cynomolgus macaque provides unique advantages as a model due to the close similarity to humans of its pulmonary anatomy and gas exchange, the ability to use human reagents, and the availability of specific reagents including macaque microarrays. The intratracheal route of infection was chosen for this model system as this method is highly reproducible and easy to standardize.

[Para 94] The animals are placed in enhanced biosafety level 3 glove boxes and inoculated intratracheally with virus. Anti-TF antibody is given intravenously in a loading dose before virus inoculation at 5 mg/kg body weight, with repeated doses at regular intervals after infection to maintain the anti-coagulation effect. Antibody levels are monitored, as well as plasma fibrinogen, complete blood counts, PT and PTT in all macaques. There are three experiments employed in this system.

[Para 95] Experiment 1 is used to determine the effect of an agent on a lethal hemorrhagic virus infection in cynomolgus monkeys. In this experiment, Group 1 consists of 6 macaques administered antibody buffer and Ebola virus. Group 2 consists of 6 macaques administered an Irrelevant IgG4 and Ebola virus. Group 3 consists of 6 macaques administered anti-TF antibody and Ebola virus.

[Para 96] Group 3 receives an intravenous injection of anti-TF antibody (5 mg/kg body weight) at 12 hours before virus inoculation, and lower doses (0.5 mg/kg body weight) at 1 and 2 dpi (days after inoculation of virus). This dose of anti-TF antibody is chosen based on the effective dose (5 mg/kg over 34 hrs in the baboon ALI study) and the half life of the antibody (3-7 days) obtained from the preclinical safety studies in normal cynomolgus monkeys. Group 1 serves as a challenge control, whereas Group 2 receives a control/irrelevant human IgG4 antibody. The irrelevant human IgG4 is tested for negative reactivity and neutralization activity against Ebola virus by immunochemical assays and in vitro neutralization assay. At 0 dpi, Groups 1, 2 and 3 are inoculated intratracheally with a lethal dose of Ebola virus. The macaques are euthanized when they are moribund, which is 12.6 dpi for untreated animals. At 28 dpi all animals are euthanized. This first experiment

is designed to show prophylactic use of an anti-TF molecule with a "saturating" dosage schedule.

[Para 97] Experiment 2 shows the effect of an anti-TF antibody on the coagulation cascade, inflammatory response, viral dynamics, and lung damage during the course of Ebola virus infection in cynomolgus monkeys.

[Para 98] Group 1 consists of 4 macaques per time point (3 dpi , 7 dpi, and 13 dpi) administered an irrelevant IgG4 and Ebola virus. Group 2 consists of 4 macaques per time point(3 dpi , 7 dpi, and 13 dpi) administered anti-TF antibody and Ebola virus.

[Para 99] The treatment of Groups 1 and 2 of this experiment corresponds to that of Groups 2 and 3 in Experiment #1. However, the macaques are euthanized at 3, 7, and 14 dpi, in order to determine the effect of anti-TF antibody during the course of Ebola virus infection. The animals are inoculated either with a lethal dose of Ebola virus or a non-lethal dose of Ebola virus depending on the outcome of Experiment #1. A lethal dose of Ebola virus may cause injury that is too severe to observe meaningful changes. In that case a lower, non-lethal dose of Ebola virus should be used.

[Para 100] Experiment 3 shows the effect of anti-TF antibody on post-exposure antiviral treatment on the coagulation cascade, inflammatory response, viral dynamics, lung injury, and disease severity and survival during the course of Ebola virus infection in cynomolgus monkeys. Group 1 consists of 4 macaques administered Ebola virus. Group 2 consists of 6 macaques administered an antiviral and Ebola virus. Group 3 consists of 6 macaques administered an irrelevant IgG4 antibody and Ebola virus. Group 4 consists of 6 macaques administered anti-TF antibody plus Ebola virus. Group 5 consists of 6 macaques administered anti-TF antibody plus an antiviral and Ebola virus.

[Para 101] A lethal dose of Ebola virus is used. In Groups 4 and 5, anti-TF therapy is initiated 24 hours after infection with Ebola virus, as is the control antibody in Group 3. The antiviral is administered 24 hours after infection in Groups 2 and 5.

EXPERIMENTAL ENDPOINTS, DATA ANALYSIS AND INTERPRETATION

[Para 102] The efficacy of treatment with anti-TF antibody, or with the combination of anti-TF antibody and the antiviral, is assessed by statistical comparison of drug-treated and sham-treated animals using the following endpoints as described below.

[Para 103] ***Histopathology endpoints*** for lung injury are based on histological evaluation of postmortem lung tissue. Per macaque, one lung is inflated with 10% neutral-buffered formalin and samples are selected in a standard manner from cranial, medial, and

caudal parts of the lung. Influenza virus antigen expression in the lung is determined by immunohistochemistry (Kuiken T, et al. Veterinary Pathology. (2003) 40:304-310; Rimmelzwaan et al., J Virol (2001) 75; 6687-6691), and scored per animal as the number of positive fields per 100 fields (Haagmans BL, et al. Nat Med. (2004) 10:290-293). Inflammatory lesions are scored in a semiquantitative manner, based on the number and size of inflammatory foci and the severity of inflammation. The presence of polymerized fibrin and collagen within these foci are assessed by use of phosphotungstic acid-hematoxylin stain and Masson's trichrome stain, respectively.

[Para 104] ***Virology endpoints*** for virus replication and excretion are based on virological examination of swabs collected during the experiment and lung tissue collected at necropsy. Nasal swabs and pharyngeal swabs are collected under ketamine anesthesia at 0, 1, 2, 3, 5, 7, 10, and 14 dpi. Lung specimens for virological examination are collected at necropsy. Both lung specimens and swabs are tested for the presence and quantity of influenza virus RNA by use of a quantitative real time PCR assay.

[Para 105] ***Biochemical endpoints*** for inflammation and the coagulation cascade are measured in broncho-alveolar lavage fluid (BALF) collected at necropsy, and in serum collected under ketamine anesthesia at 0, 1, 2, 3, 5, 7, 10, and 13 dpi. BALF is not collected during the course of infection, because it is known to influence the course of viral infection in the lung. Cytokines (TNF-rl, IL-1β, IL-6, IL-8, TGF-β, and VEGF), which are implicated in the pathogenesis of acute lung injury, are measured in BALF by commercial ELISA kits.

[Para 106] ***Anti-TFantibody levels and anti-coagulant activities*** are measured by established assays. Sensitive ELISAs are used to measure TF and anti-TF antibody. Procoagulant activity in plasma and BALF are determined by prothrombin time (PT), and by ELISAs for fibrinogen, FDP, and thrombin-antithrombin (TAT) complexes. Anti-TF antibody levels are compared statistically to pro-coagulant and fibrinolytic activity in plasma and BALF at the end of the experiments.

[Para 107] ***Determination of gene and protein expression by gene-chip microarrays and proteomics*** are performed on whole blood and lung tissues obtained from the monkeys (Experiment #2). Gene and protein expression are analyzed, focusing on biomarkers related to inflammation and coagulation/fibrinolytic systems using, e.g., Affymetrix's gene chips (for cynomolgus monkeys). Gene expression using microarrays is now used across diverse biological applications and is playing an increasingly important

role in the study of virus-host interactions (Kato-Maeda, M et al., Cell Microbiol. (2001) 3: 713-719; Manger ID, et al., Curr Opin Immunol. (2000) 12: 215-218). Such studies are yielding many new insights into how viruses interact with the cell and mechanisms of disease pathogenesis.

[Para 108] By proteomics, a search for proteins and peptides that are differentially expressed in the lung tissue of different experimental groups is done. Because of the enormous complexity of the proteome and the dynamic range of proteins, samples may be pre-fractionated by, e.g., nano liquid chromatography techniques. The resulting fractions are compared by, e.g., Fourier transform mass spectrometry. The resulting peptides that are differentially expressed can be identified by MS/MS approaches.

[Para 109] ***Statistical power and analysis.*** The minimum sample size to employ is six animals per group. For two normally distributed samples with equal variances (max s 0.25) the estimated sample size needed to detect a mean difference of 40% is 5.6 for a<0.05 and b 0.8. For a mean difference of 33%, sample size is 7.8. Thus group sizes of 6-8 provide adequate power for measurement endpoints to detect biologically meaningful differences. We generally prefer to use the lower n=6 in primates to conserve animals and to demonstrate robust, physiologically-relevant lung protection. Data was analyzed using SPSS version 11. The outcome variables viral load at certain time points and AUC of viral load over a defined time period will be analyzed after logarithmic (base 10) transformation using one way ANOVA. We assume that, viral loads after log transformation have a normal distribution in the separate groups as well as in the combined groups. This assumption will be verified by inspection of the distribution (mainly concerning symmetry) and by using the Kolmogorov-Smirnov test, yielding p-values around 0.70. For AUC analysis we will first test homogeneity of the within-group variances across the groups. Then, depending on the appropriate assumption of either homogeneous or heterogeneous variances, differences in means between the groups will be tested. If and only if the overall group effect (i.e., comparing all groups simultaneously) turn out to be significant ($p < 0.05$), the relevant pairwise comparisons will be tested. Differences in survival between the groups will be tested using the log rank test. Again, pairwise comparisons will only be tested if the overall p-value from comparing all groups is significant ($p < 0.05$).

We Claim:

1. A method of treating a patient suffering from a hemorrhagic viral infection comprising administering a tissue factor inhibitor.
2. The method of claim 1, wherein the tissue factor inhibitor inhibits the extrinsic coagulation pathway.
3. The method of claim1, wherein the viral infection is caused by Ebola or Marburg virus.
4. The method of claim 1, wherein the tissue factor inhibitor comprises an antibody, a protein or peptide mimetic, a tissue factor ligand analog or an organic molecule.
5. The method of claim 1, wherein the mortality associated with the viral infection is reduced.
6. The method of claim 1, wherein the number or severity of morbidity is reduced.
7. The method of claim 1, wherein lung damage associated with viral infection is reduced.
8. The method of claim 1, wherein coagulopathy associated with viral infection is reduced.
9. The method of claim 1, wherein time of hospitalization is reduced, time spent in ICU is reduced, there is a shortened time on assisted ventilation, reduction in the incidence of organ failure, and/or a reduction in multifocal necroses associated with viral infection.
10. The method of claim 1, wherein the inhibitor binds tissue factor and inhibits binding of Factor X to tissue factor.
11. The method of claim 1, wherein the inhibitor binds tissue factor and inhibits the activation of Factor X to Xa.
12. The method of claim 1, wherein the inhibitor binds a tissue factor complex comprising TF:VII, TF:VIIa, TF:IX, or TF:X and inhibits activation of Factor X to Xa.
13. The method of claim 1, wherein the inhibitor binds tissue factor and inhibits binding of Factor VII to tissue factor.
14. The method of claim 1, wherein the inhibitor binds tissue factor and inhibits the activation of Factor VII to VIIa.
15. The method of claim 1, wherein the inhibitor binds Factor VII, Factor VIIa, Factor IX, Factor Xa, or Factor X.

16. The method of claim 1, wherein the inhibitor comprises a single domain antibody, a monoclonal antibody, a human antibody, a humanized antibody, a single chain antibody or a binding fragment thereof.

17. The method of claim 16, wherein the inhibitor comprises an antibody fragment comprising a Fab, a Fab', a $F(ab')_2$, or an Fv.

18. The method of claim 1, wherein the inhibitor comprises the Factor X binding site of tissue factor and binds Factor X.

19. The method of claim 1, wherein the inhibitor comprises the Factor VII binding site of tissue factor and binds Factor VII.

20. The method of claim 1, wherein the inhibitor is a compound of the Formula I $AR-(CXY)_m-(HET)_{0 \text{ or } 1}-(CX^1Y^1)_n-C(Z)_p-(PO_3)_{3-p}$,

wherein Ar is optionally substituted carbocyclic aryl or optionally substituted heteroaryl;

HET is optionally substituted N, O or S;

each X, each Y, each X', each Y' and each Z are each independently hydrogen; halogen; hydroxyl; sulfhydryl; amino; optionally substituted alkyl preferably; optionally substituted alkenyl; optionally substituted alkynyl; optionally substituted alkoxy; optionally substituted alkylthio; optionally substituted alkylsulfinyl; optionally substituted alkylsulfonyl; or optionally substituted alkylamino;

m and n each is independently an integer of from 0 to 4; p is 1 or 2;

or a physiologically acceptable salt thereof.

21. The method of claim 1, further comprising the administration of an anti-viral agent.

Figure 1 – Tissue Factor Localization

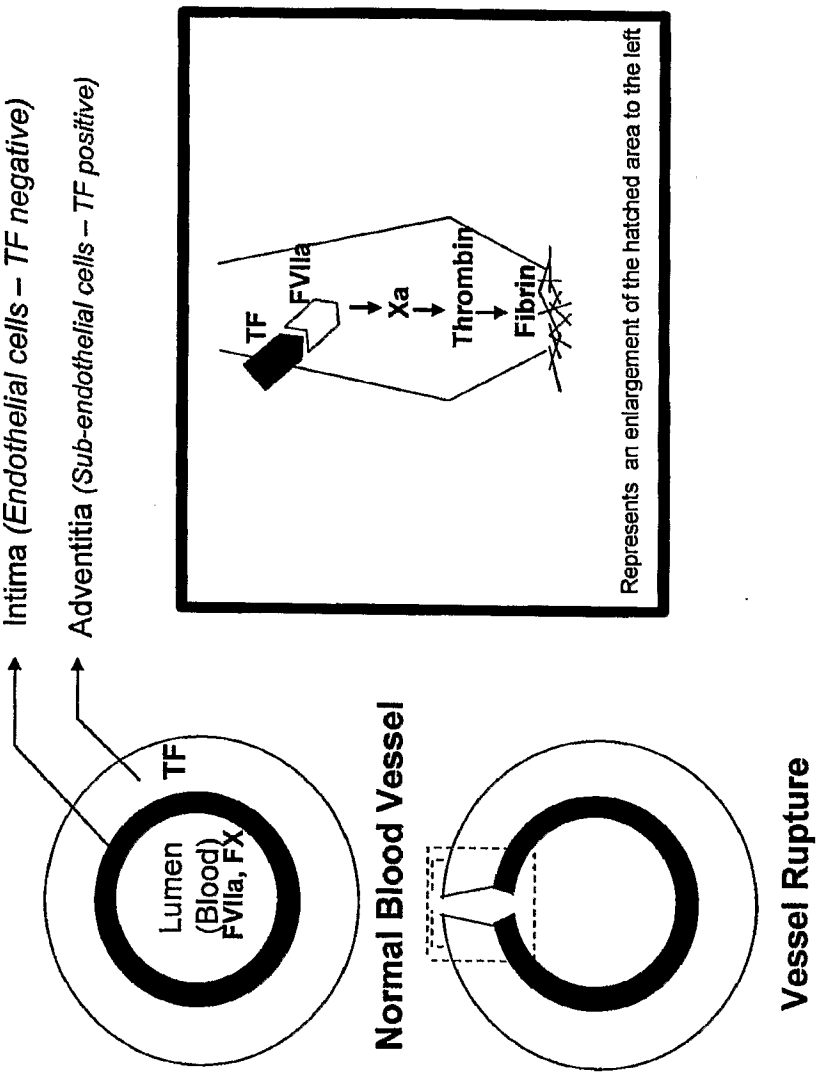


Figure 2 - Coagulation Cascade

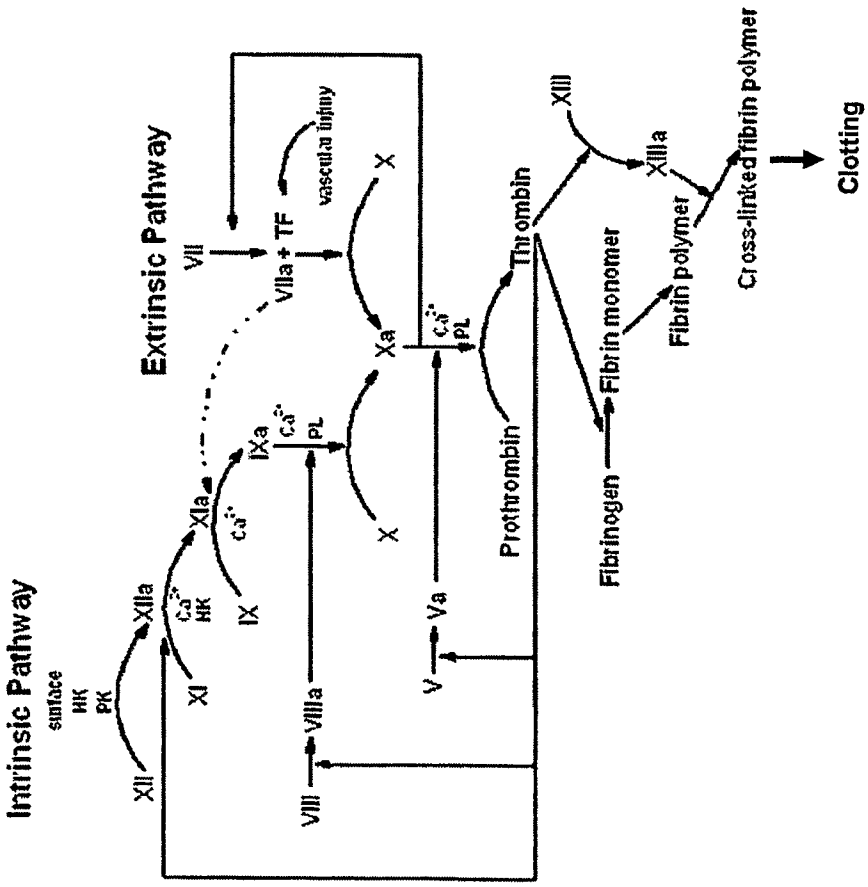


Figure 3 - Tissue Factor Inhibitors

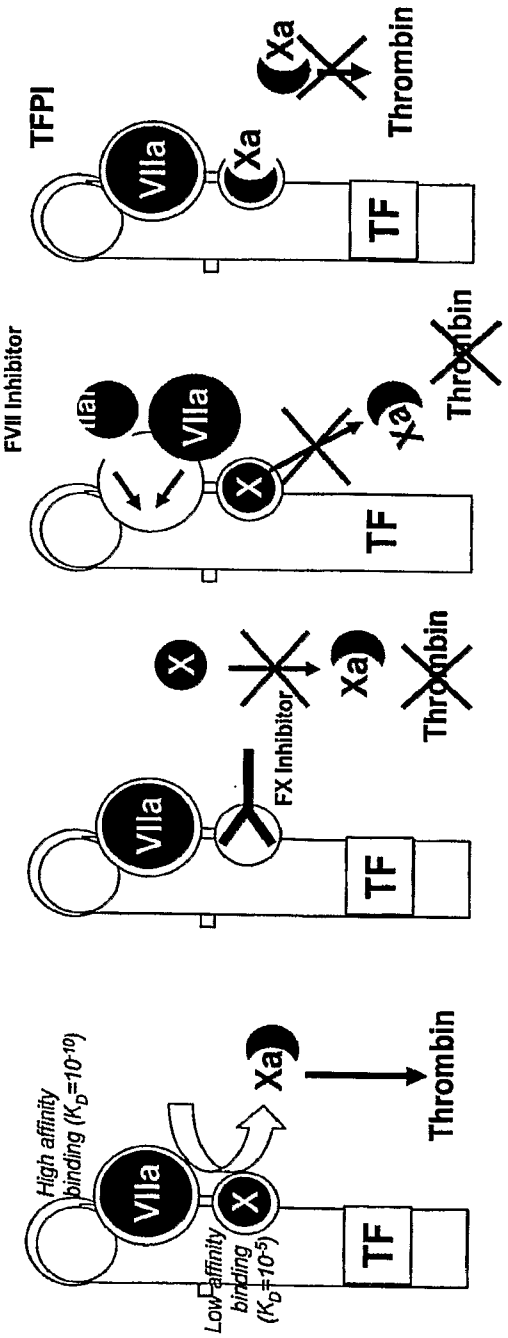
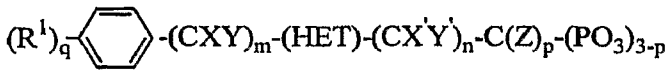
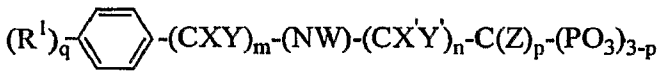


FIGURE 4

FORMULA I

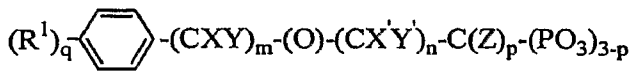


FORMULA II

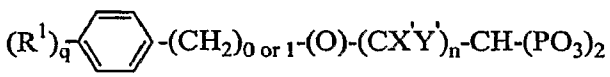


FORMULA III

FORMULA IIIA



FORMULA IV



FORMULA IVA