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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 December 2004 (09.12.2004)

PCT

(10) International Publication Number
WO 2004/106491 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number:
PCT/US2004/015781

(22) International Filing Date: 19 May 2004 (19.05.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/472,965 22 May 2003 (22.05.2003) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/472,965 (CON)
Filed on 22 May 2003 (22.05.2003)

(71) Applicant *(for all designated States except US)*: **THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES** [US/US]; 6011 Executive Boulevard, Suite 325, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHEARER, Gene, M.** [US/US]; 5512 Glenwood Road, Bethesda, MD 20817 (US). **RUGELES, Maria, T.** [CO/CO]; Medellin (CO). **RYBACK, Susanna, M.** [US/US]; 7411B Round Hill Road, Frederick, MD 21202 (US). **OPPENHEIM, Joost, J.** [US/US]; 7601 Winterberry Place, Bethesda, Md Maryland 20817 (US).

(74) Agents: **PATTON, Stephana, E.** et al.; Edwards & Angell, LLP, 101 Federal Street, P.O. Box 55874, Boston, MA 02205-5874 (US).

(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, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, 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, IT, LU, 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).

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: ANGIOGENIN-BASED HIV-1 THERAPIES

(57) Abstract: Methods for treatment of highly mutating viruses and viruses which establish chronic or latent infections in an individual are provided. In particular, it is shown that angiogenin is a potent inhibitor of viral replication, such as HIV-1. This has several advantages over other known ribonucleases that are used to inhibit HIV-1 replication as angiogenin inhibits HIV-1 replication in primary activated T lymphocyte cultures as well as chronically infected cell lines. Inhibition of HIV-1 replication in primary activated T lymphocytes would decrease the risk of HIV spreading to other susceptible cells.

WO 2004/106491 A2

ANGIOGENIN-BASED HIV-1 THERAPIES

This application claims priority from U.S. Provisional Application No. 60/472,965, filed May 22, 2003, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The invention provides methods and compositions to inhibit replication of RNA viruses. In a particular aspect, methods and compositions are provided to inhibit replication of human immunodeficiency virus-1 (HIV-1) with use of recombinant angiogenin, including in both primary activated lymphocytes and chronically infected lymphocytes.

BACKGROUND

Angiogenin has 34% sequence identity to bovine pancreatic RNAse A (Strydom *et al.*, 1985, *Biochem.*, 24:5486-5494; Kurachi *et al.*, 1985, *Biochem.*, 24:5494-5499). Angiogenin's tertiary structure has relation to that of RNAse A, based on: conservation of three of four disulfide bonds; extremely tight binding to placental ribonuclease inhibitor; and, a computer generated three dimensional structure (Shapiro and Vallee, 1987, *Proc. Natl. Acad. Sci.* USA 84:2238-2241; Lee *et al.*, 1989b, *Biochem.* 28:225-230; Palmer *et al.*, *Proc. Natl. Acad. Sci.* USA 83:1965-1969). Nevertheless, despite such relationships, angiogenin exhibits differences in activities as compared to the other ribonucleases.

Two distinct activities have been demonstrated for human-tumor derived angiogenin (Vallee, *et al.*, U.S. Patent No.: 4,727,137). First, it was reported to behave as a very potent angiogenic factor *in vivo* (Fett *et al.*, 1985, *Biochem.*, 24:5480-5486). Second, angiogenin has been found to exhibit a characteristic ribonucleolytic activity towards 28S and 18S rRNA that differs from pancreatic RNAse in that it requires up to 105 times as much angiogenin to obtain the same degree of rRNA degradation as with RNAse; the degradation products are much larger (100 to 500 nucleotides); and

angiogenin, is essentially inactive toward classic RNAse A substrates (Rybak *et al.*, 1988, *Biochem.*, 27:2288-2294; Shapiro *et al.*, 1986, *Biochem.*, 25:3527-3532; St. Clair *et al.*, 1987, *Proc. Natl. Acad. Sci.* USA. 84:8330-8334). The angiogenic and ribonucleolytic activities of angiogenin, whether tumor-derived, plasma-derived or recombinant DNA-derived, are still exhibited.

HIV-1 is a member of the lentivirus family of retroviruses (Teich, N. *et al.*, 1984 In RNA Tumor Viruses ed. R. Weiss, N. Teich, H. Varmus, J. Coffin CSH Press, pp. 949-56). The life cycle of HIV-1 is characterized by a period of proviral latency followed by active replication of the virus. The primary cellular target for the infectious HIV-1 virus is the $CD4^+$ subset of human T-lymphocytes. Targeting of the virus to the $CD4^+$ subset of cells is due to the fact that the $CD4^+$ cell surface protein acts as the cellular receptor for the HIV-1 virus (Dalgleish, A. *et al.*, 1984, *Nature* 312:763-67; Klatzmann *et al.* 1984, *Nature* 312:767-68; Maddon *et al.* 1986 *Cell* 47:333-48).

In more detail, HIV-1 infection of susceptible cells is initiated via interactions between the virus envelope glycoprotein (gp120) and the $CD4^+$ cell surface receptor. Fusion of the viral and cell membranes then proceeds through subsequent interaction of this complex with a specific chemokine receptor, primarily the CCR5 or the CXCR4 chemokine receptor (Bieniasz & Cullen, 1998, *Front. Biosci.* 3:D44-D58; Moore *et al.*, 1997, *Curr. Opin. Immunol.* 9:551-562). HIV-1 isolates that can infect T-cell lines and induce syncytia (SI) use the CXCR4 receptor and are termed X4 HIV-1. Such isolates are typically recovered late in the course of HIV progression and differ from the non-syncytia inducing (NSI) strains which predominate in the early stages of HIV infection. NSI strains gain entry to target cells through use of the CCR5 receptor and are referred to as R5 HV-1.

After binding to the cell surface and fusion of the virus and cell membrane, the HIV-1 virion becomes internalized and the virus's RNA genome is converted into linear double-stranded DNA molecules. This process is dependent on the action of the virally encoded reverse transcriptase. Following replication of the viral genome, the linear DNA

molecule integrates into the host genome through the action of the viral integrase protein, thus establishing the proviral form of HIV-1. During the early phase of proviral expression, transcription of the viral genome results in expression of regulatory proteins such as Tat, Nef and Rev. Transcriptional activation of the proviral DNA is mediated through the viral 5' LTR sequences (long terminal repeats). The initial low level of viral transcription is dramatically increased by the HIV encoded transactivator protein termed tat (transactivator protein) (Cullen, B. R. *et al.* 1989, *Cell* 58:423-26). The Rev protein promotes the transition from the early phase expression of regulatory proteins to late phase expression of structural proteins. Assembly of newly synthesized viral particles is followed by budding of virus particles from the cell membrane allowing the virus to infect new cells.

The HIV-1 virus is capable of establishing a latent state of infection for prolonged periods of time. Individuals infected with the human immunodeficiency virus may remain clinically healthy for long periods of time, with the estimated average length of the asymptomatic period between primary HIV infection and the progression to AIDS and increase in viral replication being approximately 8 to 10 years. It is generally believed that the humoral immune response to HIV-1 is not sufficiently protective against progression of the disease. Therefore, attention has turned to the possibility that the T-lymphocyte population of cells may maintain the period of latency by directly inhibiting HIV-1 replication.

Retroviral drugs, such as reverse transcriptase inhibitors, viral protease inhibitors, and viral entry inhibitors have been used to treat HIV infection. (Caliendo *et al.*, 1994, *Clin. Infect. Dis.*, 18:516-24). These treatments can effectively suppress viral production when used in combinations known as HAART (highly active anti-retroviral therapy). However, they are mainly effective in preventing new infection of uninfected cells. They are generally far less effective in eliminating latent virus from infected cells. Even after two years on HAART, HIV-1 can still be induced, and viral production resume when HAART is stopped (Finzi *et al.*, 1997, *Science*, 278:1295-1300; Wong *et al.*, 1997, *Science*, 278:1291-1295). Hence, HAART likely needs to be continued indefinitely.

This poses significant difficulties. HAART regimens have many side effects, are difficult to comply with, and are expensive. Moreover, prolonged treatment with these drugs often leads to the emergence of drug resistant viral strains (Larder *et al.*, 1989, *Science*, 246:1155-8; Kellam *et al.*, 1992, *Proc. Nat'l. Acad. Sci.* USA, 89:1934-8; St. Clair *et al.*, 1991, *Science*, 253:1557-9). Combination therapies entailing treatment with two or more drugs which attack different points in the HIV replication cycle delay the emergence of resistant HIV strains. (D'Aquila, 1994, *Clin. Lab. Med.*, 14:393:422). However, recent data suggest that HIV strains having multi-drug resistance may eventually develop in a significant portion of patients treated with combination therapy. (Schinazi *et al.*, 1994, *Int. Antiviral News*, 2:72-5).

HIV-2 can cause immunodeficiency similar to HIV-1. HTLV-1 has been shown to cause T cell lymphoma. EBV may cause lymphoma and other lymphoproliferative diseases. CMV may cause retinitis, hepatitis, pneumonitis, and other systemic illness, especially in immunocompromised host. HSV-1 and HSV-2, and Herpes Zoster (HZV) can cause painful vesicles at the area of infection and occasional meningitis. HHV-6 has been demonstrated to be present in and may contribute to the pathogenesis in certain subgroups of patients with multiple sclerosis and chronic fatigue syndrome. Nucleoside analogs such as ganiclovir, famciclovir, lamivudine, and ribavirin have been shown to be effective against many of these infections. These drugs interfere with viral replication, but they generally cannot attack latent virus. Hence, viral replication often resumes when the drugs are withdrawn.

There is, thus, an urgent need in the art, for antiviral therapies which are effective against mutating viruses and chronic or latent virus infections.

SUMMARY OF THE INVENTION

We now provide methods and compositions for inhibiting replication of RNA viruses. In particular, methods and compositions of the invention can provide inhibition of RNA viral replication in both primary activated T cells and chronically infected cells.

In certain preferred methods of the invention, cells infected with an RNA virus are treated with angiogenin, or a fragment or variant thereof, or a nucleic acid sequence that encodes angiogenin, or a fragment or variant thereof. This has several advantages over other known ribonucleases that are used to inhibit, for example, HIV-1 replication: angiogenin inhibits HIV-1 replication in primary activated T lymphocyte cultures as well as chronically infected cell lines. Inhibition of HIV-1 replication in primary activated T lymphocytes would decrease the risk of HIV spreading to other T cells.

Preferred methods include administering to an infected lymphocyte and/or monocyte an effective amount of a pancreatic-like ribonuclease, fragments or variants thereof. Preferably the ribonuclease is angiogenin or a variant or fragment thereof.

Also preferred is where the treated infected lymphocyte is an activated primary T lymphocyte. Preferably, the activated primary T lymphocyte is a $CD4^+$ lymphocyte.

The lymphocyte infected with an RNA virus, such as HIV, may be e.g. a chronically infected lymphocyte. Preferably, angiogenin inhibits the replication of an RNA virus in a chronically infected cell by about 100%, at least about 99.9%, 80%, 75%, 60%, 50%, or 40%. The chronically infected lymphocyte preferably may be a $CD4^+$ T lymphocyte.

The infecting RNA virus may be any number of RNA viruses, such as for example Retroviridae, Cystoviridae, Birnaviridae, Reoviridae, Coronaviridae, Flaviviridae, Togaviridae , "Arterivirus", Astroviridae, Caliciviridae, Picornaviridae, Potyviridae, Orthomyxoviridae, Filoviridae, Paramyxoviridae, Rhabdoviridae, Arenaviridae, and Bunyaviridae.

The infecting RNA virus may be a human immunodeficiency virus. The human immunodeficiency virus may be lymphocyte-tropic and/or macrophage-tropic virus.

Preferred methods for treating an individual suffering from or susceptible to infection by an RNA virus, comprise administering an effective amount of a pancreatic-like ribonuclease, suitably, in a pharmaceutically acceptable carrier. Preferably the ribonuclease is angiogenin or a variant or fragment thereof.

Preferably, the angiogenin, variants or fragments thereof, inhibit the replication of the human immunodeficiency virus in activated primary T lymphocytes and/or in chronically infected T lymphocytes.

In a preferred embodiment the virus load in a chronically infected individual is decreased by at least about 50% during a course of treatment with a therapeutically effective dose of angiogenin, variants or fragments thereof.

In another preferred embodiment, the angiogenin inhibits the replication of more than one variant of HIV and preferably the strain of HIV is a highly mutating strain as compared to different HIV strains. Other mutating viruses include the rapidly mutating coronavirus which is the etiological agent for Severe Acute Respiratory Syndrome. Other viruses include influenza such as Influenza A and B.

The invention also provides methods of treatment of other diseases caused by or otherwise associated with a virus such as viruses of the herpes family, e.g., herpes simplex viruses (HSV) including herpes simplex 1 and 2 viruses (HSV 1, HSV 2), varicella zoster virus (VZV; shingles), human herpes virus 6, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and other herpes virus infections such as feline herpes virus infections, and diseases associated with hepatitis viruses including hepatitis B viruses (HBV) B virus. Examples of clinical conditions which are caused by such viruses include herpetic keratitis, herpetic encephalitis, cold sores and genital infections (caused by herpes simplex), chicken pox and shingles (caused by varicella zoster) and CMV-pneumonia and retinitis, particularly in immunocompromised patients including renal and bone marrow transplant patients and patients with Acquired Immune Deficiency Syndrome (AIDS). Epstein-Barr virus can cause infectious mononucleosis, and is also

suggested as the causative agent of nasopharyngeal cancer, immunoblastic lymphoma and Burkitt's lymphoma. Additional specific examples of retroviral infections which may be suitably treated in accordance with the invention include human retroviral infections such as HIV-1, HIV-2, and Human T-cell Lymphotropic Virus (HTLV) e.g. HTLV-I or HTLV-II infections.

In another preferred embodiment, a pharmaceutical composition kit comprising i) angiogenin or a variant or fragment thereof and ii) directions (e.g. written) for use of the angiogenin or variant or fragment to treat against an RNA virus.

In another aspect, the invention provides use of angiogenin, or a fragment or variant thereof or a nucleic acid sequence encoding for the treatment or prevention (including prophylactic treatment) of a disease or condition as disclosed herein.

In further aspect, the invention provides a use of angiogenin or a variant or fragment thereof or a nucleic acid sequence encoding same for the preparation of a medicament for the treatment or prevention (including prophylactic treatment) of a disease or condition as disclosed herein.

Preferred methods of the invention including identifying and/or selecting a subject cells (e.g. a mammal or mammalian cells, particularly a human or human cells) that is susceptible to or suffering from a condition disclosed herein, and thereafter administering to the identified and selected subject or cells angiogenin, variants or fragments thereof or a nucleic acid sequence encoding same. Such identification and selection can be accomplished by any of a number of means, e.g. testing a subject or cells for the presence of HIV or other viral infection, or identifying a subject that is at high risk of developing such viral infections such as by intimate contact with previously infected individuals.

Other aspects of the invention are described *infra*.

DEFINITIONS

As used herein, the term "infectious agent" refers to an organism wherein growth/multiplication leads to pathogenic events in humans or animals. Examples of such agents are: bacteria, fungi, protozoa and viruses.

As used herein, "treatment" of a disease or other adverse medical condition, should be broadly interpreted based on the therapeutic effects described herein as variously including palliative, active, causal, conservative, medical, palliative, prophylactic, and/or symptomatic treatment, treatment designed to delay the onset or progression of the disease or other adverse medical condition, as well as treatment designed to arrest or reducing the severity of an ongoing disease or other adverse medical condition.

As used herein, a "pharmaceutically acceptable" component (such as a salt, carrier, excipient or diluent) of a formulation according to the present invention is a component which (1) is compatible with the other ingredients of the formulation in that it can be combined with the therapeutics of the invention without eliminating the biological activity of the therapeutics; and (2) is suitable for use in non-human animals or humans without undue adverse side effects (e.g., toxicity, irritation, and allergic response). Side effects are "undue" when their risk outweighs the benefit provided by the pharmaceutical composition.

As used herein, a "pharmaceutically acceptable" with reference to the degree of purity of a polypeptide (e.g., a chemokine or chemokine analog or chemokine fragment) or nucleic acid indicates that the polypeptide or nucleic acid (1) is free of contaminating materials that would eliminate the biological activity of the polypeptide or nucleic acid; and (2) is free of contaminating materials that would render the therapeutic (e.g., polypeptide or nucleic acid) unsuitable for administration to humans (for pharmaceutical use) or other animals (for veterinary use) by causing undue adverse side effects (e.g., toxicity, irritation, and allergic response). Side effects are "undue" when their risk outweighs the benefit provided by the therapeutic (e.g., polypeptide or nucleic acid).

The term "substantially pure" when used in reference to a polypeptide or nucleic acid is defined herein to mean a therapeutic (e.g., polypeptide or nucleic acid) that is substantially free from other contaminating proteins, nucleic acids, and other biologicals derived from an original source organism, recombinant DNA expression system, or from a synthetic procedure employed in the synthesis or purification of the therapeutic (e.g., chromatography reagents and polymers, such as acrylamide or agarose). Purity may be assayed by standard methods. Purity evaluation may be made on a mass or molar basis.

A "therapeutically effective" amount or dose is an amount or dose which prevents or delays the onset or progression of an indicated disease or other adverse medical condition. The term also includes an amount sufficient to arrest or reduce the severity of an ongoing disease or other adverse medical condition, and also includes an amount necessary to enhance normal physiological functioning.

"Diagnostic" or "diagnosed" means identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay, are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

The terms "patient" or "individual" are used interchangeably herein, and refers to a mammalian subject to be treated, with human patients being preferred. In some cases, the methods of the invention find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

"Sample" is used herein in its broadest sense. A sample comprising polynucleotides, polypeptides, peptides, antibodies and the like may comprise a bodily fluid; a soluble fraction of a cell preparation, or media in which cells were grown; a chromosome, an organelle, or membrane isolated or extracted from a cell; genomic DNA, RNA, or cDNA, polypeptides, or peptides in solution or bound to a substrate; a cell; a tissue; a tissue print; a fingerprint, skin or hair; and the like.

"Treatment" is an intervention performed with the intention of preventing the development or altering the pathology or symptoms of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. In antiviral (e.g., HIV) treatment, a therapeutic agent may directly decrease the pathology of HIV, or inhibit the replication of the HIV virus. As used herein, "ameliorated" or "treatment" refers to a symptom which is approaches a normalized value (for example a value obtained in a healthy patient or individual), e.g., is less than 50% different from a normalized value, preferably is less than about 25% different from a normalized value, more preferably, is less than 10% different from a normalized value, and still more preferably, is not significantly different from a normalized value as determined using routine statistical tests. For example, amelioration or treatment of a patient suffering from an infectious disease organism, such as for example, Human Immunodeficiency Virus (HIV), may be determined by a decrease of viral particles in a sample taken from a patient, as measured by, for example, a decrease in plaque forming units (p.f.u.) or by automated diagnostic methods such as ELISA, etc., which can be used to monitor efficacy of treatment.

As used herein, "an ameliorated symptom" or "treated symptom" refers to a symptom which is approaches a normalized value, e.g., is less than 50% different from a normalized value, preferably is less than about 25% different from a normalized value, more preferably, is less than 10% different from a normalized value, and still more

preferably, is not significantly different from a normalized value as determined using routine statistical tests.

"Cells of the immune system" or "immune cells" as used herein, is meant to include any cells of the immune system that may be assayed, including, but not limited to, B lymphocytes, also called B cells, T lymphocytes, also called T cells, natural killer (NK) cells, natural killer T (NK) cells, lymphokine-activated killer (LAK) cells, monocytes, macrophages, neutrophils, granulocytes, mast cells, platelets, Langerhans cells, stem cells, dendritic cells, peripheral blood mononuclear cells, tumor-infiltrating (TIL) cells, gene modified immune cells including hybridomas, drug modified immune cells, and derivatives, precursors or progenitors of the above cell types.

"T cells" or "T lymphocytes" are a subset of lymphocytes originating in the thymus and having heterodimeric receptors associated with proteins of the CD3 complex (e.g., a rearranged T cell receptor, the heterodimeric protein on the T cell surfaces responsible for antigen/MHC specificity of the cells). T cell responses may be detected by assays for their effects on other cells (e.g., target cell killing, activation of other immune cells, such as B-cells) or for the cytokines they produce.

"CD4" is a cell surface protein important for recognition by the T cell receptor of antigenic peptides bound to MHC class II molecules on the surface of an APC. Upon activation, naïve CD4 T cells differentiate into one of at least two cell types, Th1 cells and Th2 cells, each type being characterized by the cytokines it produces. "Th1 cells" are primarily involved in activating macrophages with respect to cellular immunity and the inflammatory response, whereas "Th2 cells" or "helper T cells" are primarily involved in stimulating B cells to produce antibodies (humoral immunity). CD4 is the receptor for the human immunodeficiency virus (HIV). Effector molecules for Th1 cells include, but are not limited to, IFN-γ, GM-CSF, TNF-α, CD40 ligand, Fas ligand, IL-3, TNF-β, and IL-2. Effector molecules for Th2 cells include, but are not limited to, IL-4, IL-5, CD40 ligand, IL-3, GS-CSF, IL-10, TGF-β, and eotaxin. Activation of the Th1 type cytokine response can suppress the Th2 type cytokine response.

A "chemokine" is a small cytokine involved in the migration and activation of cells, including phagocytes and lymphocytes, and plays a role in inflammatory responses.

A "cytokine" is a protein made by a cell that affect the behavior of other cells through a "cytokine receptor" on the surface of the cells the cytokine effects. Cytokines manufactured by lymphocytes are sometimes termed "lymphokines." Cytokines are also characterized as Type I (e.g. IL-2 and IFNγ) and Type II (e.g. IL-4 and IL-10).

The term "activated T cell," as used herein, refers to a T cell that expresses antigens indicative of T-cell activation (that is, T cell activation markers). Examples of T cell activation markers include, but are not limited to, CD25, CD26, CD30, CD38, CD69, CD70, CD71, ICOS, OX-40 and 4-1BB. The expression of activation markers can be measured by techniques known to those of skill in the art, including, for example, western blot analysis, northern blot analysis, RT-PCR, immunofluorescence assays, and fluorescence activated cell sorter (FACS) analysis.

The term "resting T cell," as used herein, refers to a T cell that does not express T-cell activation markers. Resting T cells include, but are not limited to, T cells which are $CD25^-$, $CD69^-$, $ICOS^-$, $SLAM^-$, and $4\text{-}1BB^-$. The expression of these markers can be measured by techniques known to those of skill in the art, including, for example, western blot analysis, northern blot analysis, RT-PCR, immunofluorescence assays, and fluorescence activated cell sorter (FACS) analysis.

As used herein, a cell has been "transformed", "transduced", by exogenous or heterologous nucleic acids and/or amino acids, proteins and the like, when such nucleic and/or amino acids, proteins and the like, have been introduced inside the cell.

A "vector" is a composition which can transduce, transfect, transform or infect a cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. A cell is "transduced" by a nucleic

acid when the nucleic acid is translocated into the cell from the extracellular environment. Any method of transferring a nucleic acid into the cell may be used; the term, unless otherwise indicated, does not imply any particular method of delivering a nucleic acid into a cell. A cell is "transformed" by a nucleic acid when the nucleic acid is transduced into the cell and stably replicated. A vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. A "cell transduction vector" is a vector which encodes a nucleic acid capable of stable replication and expression in a cell once the nucleic acid is transduced into the cell.

As used herein, a "target cell" or "recipient cell" refers to an individual cell or cell which is desired to be, or has been, a recipient of exogenous nucleic acid molecules, polynucleotides and/or proteins. The term is also intended to include progeny of a single cell.

The terms "nucleic acid molecule" or "polynucleotide" will be used interchangeably throughout the specification, unless otherwise specified. As used herein, "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogues thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA--DNA, DNA-RNA and RNA--RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence

homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

As used herein, the term "downstream" when used in reference to a direction along a nucleotide sequence means in the direction from the 5' to the 3' end. Similarly, the term "upstream" means in the direction from the 3' to the 5' end.

As used herein, the term "gene" means the gene and all currently known variants thereof and any further variants which may be elucidated.

As used herein, the terms "gene product" or "expression product" refers to the polypeptide encoded by the gene of interest. Expression of the polypeptide can be detected by a number of methods to one of ordinary skill in the art, such as RIA, ELISA, FACS, T-cell proliferation assays, cytotoxic T cell assays and the like.

The term "variant," when used in the context of a polynucleotide sequence, may encompass a polynucleotide sequence related to a wild type gene. This definition may also include, for example, "allelic", "splice," "species," or "polymorphic" variants. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or an absence of domains. Species variants are polynucleotide sequences that vary from one species to another. Of particular utility in the invention are variants of wild type target genes. Variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene may have none, one, or many allelic forms. Common mutational changes that give rise to variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

As used herein, "variant" of polypeptides refers to an amino acid sequence that is altered by one or more amino acid residues. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine). More rarely, a variant may have "nonconservative" changes (e.g., replacement of glycine with tryptophan). Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological activity may be found using computer programs well known in the art, for example, LASERGENE software (DNASTAR).

The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species. Polymorphic variants also may encompass "single nucleotide polymorphisms" (SNPs,) or single base mutations in which the polynucleotide sequence varies by one base. The presence of SNPs may be indicative of, for example, a certain population with a propensity for a disease state, that is susceptibility versus resistance.

The term, "complementary" means that two sequences are complementary when the sequence of one can bind to the sequence of the other in an anti-parallel sense wherein the 3'-end of each sequence binds to the 5'-end of the other sequence and each A, T(U), G, and C of one sequence is then aligned with a T(U), A, C, and G, respectively, of the other sequence. Normally, the complementary sequence of the oligonucleotide has at least 80% or 90%, preferably at least about 95%, most preferably 100%, complementarity to a defined sequence. Preferably, alleles or variants thereof can be identified. A BLAST program also can be employed to assess such sequence identity.

The term "complementary sequence" as it refers to a polynucleotide sequence, relates to the base sequence in another nucleic acid molecule by the base-pairing rules. More particularly, the term or like term refers to the hybridization or base pairing between nucleotides or nucleic acids, such as, for instance, between the two strands of a

double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid to be sequenced or amplified. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 95% of the nucleotides of the other strand, usually at least about 98%, and more preferably from about 99 % to about 100%. Complementary polynucleotide sequences can be identified by a variety of approaches including use of well-known computer algorithms and software, for example the BLAST program.

A "heterologous" component refers to a component that is introduced into or produced within a different entity from that in which it is naturally located. For example, a polynucleotide derived from one organism and introduced by genetic engineering techniques into a different organism is a heterologous polynucleotide which, if expressed, can encode a heterologous polypeptide. Similarly, a promoter or enhancer that is removed from its native coding sequence and operably linked to a different coding sequence is a heterologous promoter or enhancer. As used in relation to a human immunodeficiency virus "heterologous" may be used to refer to a non- human immunodeficiency viral sequence, or a sequence not of the specific herpes virus in question. Possible alternative terminology includes "foreign" or "exogenous". A heterologous nucleotide sequence may encode a sequence of amino acids, i.e. a peptide or a polypeptide.

As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise.

A "promoter," as used herein, refers to a polynucleotide sequence that controls transcription of a gene or coding sequence to which it is operably linked. A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources, are well known in the art and are available as or within cloned

polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources).

An "enhancer," as used herein, refers to a polynucleotide sequence that enhances transcription of a gene or coding sequence to which it is operably linked. A large number of enhancers, from a variety of different sources are well known in the art and available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides comprising promoter sequences (such as the commonly-used CMV promoter) also comprise enhancer sequences.

"Operably linked" refers to a juxtaposition, wherein the components so described are in a relationship permitting them to function in their intended manner. A promoter is operably linked to a coding sequence if the promoter controls transcription of the coding sequence. Although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. An enhancer is operably linked to a coding sequence if the enhancer increases transcription of the coding sequence. Operably linked enhancers can be located upstream, within or downstream of coding sequences. A polyadenylation sequence is operably linked to a coding sequence if it is located at the downstream end of the coding sequence such that transcription proceeds through the coding sequence into the polyadenylation sequence.

"Gene delivery," "gene transfer," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgenes") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically

requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

"*In vivo*" gene delivery, gene transfer, gene therapy and the like as used herein, are terms referring to the introduction of a vector comprising an exogenous polynucleotide directly into the body of an organism, such as a human or non-human mammal, whereby the exogenous polynucleotide is introduced to a cell of such organism in vivo.

A cell is "transduced" by a nucleic acid when the nucleic acid is translocated into the cell from the extracellular environment. Any method of transferring a nucleic acid into the cell may be used; the term, unless otherwise indicated, does not imply any particular method of delivering a nucleic acid into a cell. A cell is "transformed" by a nucleic acid when the nucleic acid is transduced into the cell and stably replicated. A vector includes a nucleic acid (ordinarily RNA or DNA) to be expressed by the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. A "cell transduction vector" is a vector which encodes a nucleic acid capable of stable replication and expression in a cell once the nucleic acid is transduced into the cell.

As used herein, a "target cell" or "recipient cell" refers to an individual cell or cell which is desired to be, or has been, a recipient of exogenous nucleic acid molecules, polynucleotides and/or proteins. The term is also intended to include progeny of a single cell.

As used herein, the term "fragment or segment", as applied to a nucleic acid sequence, gene or polypeptide, will ordinarily be at least about 5 contiguous nucleic acid bases (for nucleic acid sequence or gene) or amino acids (for polypeptides), typically at

least about 10 contiguous nucleic acid bases or amino acids, more typically at least about 20 contiguous nucleic acid bases or amino acids, usually at least about 30 contiguous nucleic acid bases or amino acids, preferably at least about 40 contiguous nucleic acid bases or amino acids, more preferably at least about 50 contiguous nucleic acid bases or amino acids, and even more preferably at least about 60 to 80 or more contiguous nucleic acid bases or amino acids in length. "Overlapping fragments" as used herein, refer to contiguous nucleic acid or peptide fragments which begin at the amino terminal end of a nucleic acid or protein and end at the carboxy terminal end of the nucleic acid or protein. Each nucleic acid or peptide fragment has at least about one contiguous nucleic acid or amino acid position in common with the next nucleic acid or peptide fragment, more preferably at least about three contiguous nucleic acid bases or amino acid positions in common, most preferably at least about ten contiguous nucleic acid bases amino acid positions in common.

A significant "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least 20 nucleotides, more generally at least 23 nucleotides, ordinarily at least 26 nucleotides, more ordinarily at least 29 nucleotides, often at least 32 nucleotides, more often at least 35 nucleotides, typically at least 38 nucleotides, more typically at least 41 nucleotides, usually at least 44 nucleotides, more usually at least 47 nucleotides, preferably at least 50 nucleotides, more preferably at least 53 nucleotides, and in particularly preferred embodiments will be at least 56 or more nucleotides.

Homologous nucleic acid sequences, when compared, exhibit significant sequence identity or similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. The hybridization conditions are described in greater detail below.

As used herein, "substantial homology" in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared,

are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 50% of the nucleotides, generally at least 56%, more generally at least 59%, ordinarily at least 62%, more ordinarily at least 65%, often at least 68%, more often at least 71%, typically at least 74%, more typically at least 77%, usually at least 80%, more usually at least about 85%, preferably at least about 90%, more preferably at least about 95 to 98% or more, and in particular embodiments, as high at about 99% or more of the nucleotides. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See Kanehisa (1984) *Nuc. Acids Res.* 12:203-213. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides. The endpoints of the segments may be at many different pair combinations.

"Amplification products", "amplified products" "PCR products" or "amplicons" comprise copies of the target sequence and are generated by hybridization and extension of an amplification primer. This term refers to both single stranded and double stranded amplification primer extension products which contain a copy of the original target sequence, including intermediates of the amplification reaction.

"Target" or "target sequence" refers to nucleic acid sequences to be amplified. These include the original nucleic acid sequence to be amplified, its complementary second strand and either strand of a copy of the original sequence which is produced in the amplification reaction. The target sequence may also be referred to as the template for extension of hybridized amplification primers.

"Nucleotide" as used herein, is a term of art that refers to a base-sugar-phosphate combination. Nucleotides are the monomeric units of nucleic acid polymers, i.e. of DNA

and RNA. The term includes ribonucleoside triphosphates, such as rATP, rCTP, rGTP, or rUTP, and deoxyribonucleotide triphosphates, such as dATP, dCTP, dUTP, dGTP, or dTTP. A "nucleoside" is a base-sugar combination, i.e. a nucleotide lacking phosphate. It is recognized in the art that there is a certain interchangability in usage of the terms nucleoside and nucleotide. For example, the nucleotide deoxyuridine triphosphate, dUTP, is a deoxyribonucleoside triphosphate. After incorporation into DNA, it serves as a DNA monomer, formally being deoxyuridylate, i.e. dUMP or deoxyuridine monophosphate. One may say that one incorporates dUTP into DNA even though there is no dUTP moiety in the resultant DNA. Similarly, one may say that one incorporates deoxyuridine into DNA even though that is only a part of the substrate molecule.

The term "nucleic acid" is defined to include DNA and RNA, and their analogs, and is preferably DNA. Further, the methods of the present invention are not limited to the detection of mRNAs. Other RNAs that may be of interest include tRNAs, rRNAs, and snRNAs.

"Incorporating" as used herein, means becoming part of a nucleic acid polymer.

"Terminating" as used herein, means causing a treatment to stop. The term includes means for both permanent and conditional stoppages. For example, if the treatment is enzymatic, a permanent stoppage would be heat denaturation; a conditional stoppage would be, for example, use of a temperature outside the enzyme's active range. Preferred methods of termination include the use of abasic regions. It is also expedient to use deoxyribonucleoside triphosphates as chain termination molecules which are modified at the 3' position of the deoxyribose in such a way that they have no free -OH group but are nevertheless accepted as a substrate by the polymerase. Examples of such chain termination molecules are 3' fluoro, 3'-O-alkyl and 3'H-modified deoxyribonucleosides. 3'-H-modified deoxyribonucleotides are preferably used as chain termination molecules i.e. dideoxyribonucleoside triphosphates (ddNTP). It is preferable to use unlabeled chain termination molecules in the method according to the invention but it is also possible to use labeled chain termination molecules as known to a person

skilled in the art. Any type of termination procedures are intended to fall within the scope of this term.

"Oligonucleotide" as used herein refers collectively and interchangeably to two terms of art, "oligonucleotide" and "polynucleotide". Note that although oligonucleotide and polynucleotide are distinct terms of art, there is no exact dividing line between them and they are used interchangeably herein. An oligonucleotide is said to be either an adapter, adapter/linker or installation oligonucleotide (the terms are synonymous) if it is capable of installing a desired sequence onto a predetermined oligonucleotide. An oligonucleotide may serve as a primer unless it is "blocked". An oligonucleotide is said to be "blocked," if its 3' terminus is incapable of serving as a primer.

The term "probe" refers to a strand of nucleic acids having a base sequence substantially complementary to a target base sequence. Typically, the probe is associated with a label to identify a target base sequence to which the probe binds, or the probe is associated with a support to bind to and capture a target base sequence.

"Oligonucleotide-dependent amplification" as used herein refers to amplification using an oligonucleotide or polynucleotide or probe to amplify a nucleic acid sequence. An oligonucleotide-dependent amplification is any amplification that requires the presence of one or more oligonucleotides or polynucleotides or probes that are two or more mononucleotide subunits in length and that end up as part of the newly-formed, amplified nucleic acid molecule.

"Primer" as used herein refers to a single-stranded oligonucleotide or a single-stranded polynucleotide that is extended by covalent addition of nucleotide monomers during amplification. Nucleic acid amplification often is based on nucleic acid synthesis by a nucleic acid polymerase. Many such polymerases require the presence of a primer that can be extended to initiate such nucleic acid synthesis. Here through the selection of primers, modified or otherwise, which determine the average molecular weight of the DNA segments (or size), the result can be achieved that the variations of size or

molecular weights for the DNA segments formed by the various primer pairs only prevents superimposition or overlap.

The term "label" refers to a molecular moiety capable of detection including, by way of example, without limitation, radioactive isotopes, enzymes, luminescent agents, dyes, and detectable intercalating agents. Any suitable means of detection may be employed, thus, the label maybe an enzyme label, a fluorescent label, a radioisotopic label, a chemiluminescent label, etc. Examples of suitable enzyme labels include alkaline phosphatase, acetylcholine esterase, α-glycerol phosphate dehydrogenase, alkaline phosphatase, asparaginase, β-galactosidase, catalase, δ-5-steroid isomerase, glucose oxidase, glucose-6-phosphate dehydrogenase, luciferase, malate dehydrogenase, peroxidase, ribonuclease, staphylococcal nuclease, triose phosphate isomerase, urease, and yeast alcohol dehydrogenase. Examples of suitable fluorescent labels include fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an o-phthaldehyde label, a fluorescamine label, 5,6-carboxymethyl fluorescein, Texas red, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), coumarin, dansyl chloride, and rhodamine. Preferred fluorescent labels are fluorescein (5-carboxyfluorescein-N-hydroxysuccinimide ester) and rhodamine (5,6-tetramethyl rhodamine), etc. Examples of suitable chemiluminescent labels include luminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate label, a luciferin label an aequorin label. Alternatively, the sample may be labeled with non-radioactive label such as biotin. The biotin labeled probe is detected via avidin or streptavidin through a variety of signal generating systems known in the art. Labeled nucleotides are preferred form of detection label since they can be directly incorporated into the products of PCR during synthesis. Examples of detection labels that can be incorporated into amplified DNA include nucleotide analogs such as BrdUrd (Hoy and Schimke, *Mutation Research*, 290:217-230 (1993)), BrUTP (Wansick et al., *J. Cell Biology*, 122:283-293 (1993)) and nucleotides modified with biotin (Langer et al., *Proc. Natl. Acad. Sci.* USA, 78:6633 (1981)) or with suitable haptens such as digoxygenin (Kerkhof, *Anal. Biochem.*, 205:359-364 (1992)). Suitable fluorescence-labeled nucleotides are Fluorescein-isothiocyanate-dUTP, Cyanine-3-dUTP and Cyanine-5-dUTP

(Yu et al., Nucleic Acids Res., 22:3226-3232 (1994)). A preferred nucleotide analog detection label for DNA is Cyanine-5-dUTP or BrdUrd (BUDR triphosphate, Sigma), and a preferred nucleotide analog detection label is Biotin-16-uridine-5'-triphosphate (Biotin-16-dUTP, Boehringher Mannheim).

The term "agent" is used in a broad sense, in reference to labels, and includes any molecular moiety which participates in reactions which lead to a detectable response.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A and 1B are graphs showing the inhibitory effect of rRNases on HIV-1 replication. Figure 1A shows the mean percentages of viral inhibition by four different rRNases (±SD). Figure 1B shows the dose response effect of rRNases on HIV-1 replication. RNases were added to HIV-1 infected PHA blasts two hours after infection.

Figure 2 is a graph showing the inhibitory effect of RNases on HIV-1 replication blocked by RNase-specific polyclonal antibodies. Mean percentages of viral inhibition (from two or three independent experiments (±SD)). RNases were added to HIV-1-infected PHA blasts two hours after infection.

Figure 3A and 3B are graphs showing the inhibitory effect of RNases on HIV-1 replication prior to infection. Figure 3A shows the results obtained when HIV-1 was incubated with the RNases one hour prior to infection of the PHA blasts. Figure 3B shows the results obtained when PHA blasts were incubated with RNases one hour prior to infection. Mean percentages of viral inhibition by four different rRNases in three independent experiments (±SD).

DETAILED DESCRIPTION OF THE INVENTION

We now provide methods and compositions for treatment of highly mutating viruses and for viruses which establish chronic or latent infections in an individual.

The invention thus provides methods of treatment against virus infections, including retroviral infections such as HIV infections, and treatment of other diseases caused by or otherwise associated with a virus such as influenza including influenza A and B; diseases associated with viruses of the herpes family, e.g., herpes simplex viruses (HSV) including herpes simplex 1 and 2 viruses (HSV 1, HSV 2), varicella zoster virus (VZV; shingles), human herpes virus 6, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and other herpes virus infections such as feline herpes virus infections; diseases associated with hepatitis viruses including hepatitis B viruses (HBV); and the like.

In one aspect, methods of the invention include use (particularly administration) of angiogenin, or a fragment or variant thereof, to treat against viral infections, particularly virally infected mammalian cells, especially virally infected human cells, such as HIV-infected cells. Thus, methods of the invention are useful to treat of HIV infected individuals.

According to one embodiment, administration of angiogenin, or a fragment or variant thereof, or a nucleic acid sequence that encodes angiogenin, or a fragment or variant thereof, to treat against viral infections, is done, for example, according to procedures disclosed in Example 1 herein. According to certain preferred embodiments, the administration of angiogenin, for example, according to the procedures disclosed in the examples herein, may reduce the viral load of a cell from between about 18 % to about 100%.

As discussed above, we have found that angiogenin is a potent inhibitor of viral replication, such as HIV-1. Preferred methods of the invention can inhibit HIV-1 replication in primary activated T lymphocyte cultures as well as chronically infected cell lines. Inhibition of HIV-1 replication in primary activated T lymphocytes would decrease the risk of HIV spreading to other susceptible cells.

Preferably, the virus load in an infected individual is decreased by at least about 50% during a course of treatment with a therapeutically effective dose of angiogenin,

variants or fragments thereof, more preferably the virus load is decreased by at least about 60%, 70%, 80% during a course of treatment with a therapeutically effective dose of angiogenin variants or fragments thereof. Viral load decreases can be determined by a decrease in the production of p24 viral antigen in a sample culture supernatant using an HIV-1 p24 antigen capture ELISA assay (Coulter Immunology, Hialeah, Fla.). A typical course of treatment will include administration of angiogenin, variant or fragment thereof, to an individual or cells for 1, 2, 3, 4, 5, or more days.

In a preferred embodiment, a patient suffering from an infectious disease organism is treated with a pancreatic-like ribonuclease, such as for example, recombinant angiogenin, in doses which are not toxic to the patient but are effective in inhibiting replication of an RNA virus, such as HIV, in primary activated T lymphocytes. A preferred dose for treating a patient depends on the body weight of the patients, seriousness of the disease, and doctor's opinion. However, it is generally advisable to administer 0.01 to 10 mg/kg body weight a day, preferably 0.02 to 2 mg in case of injection. Other than injection, it is advisable to take it into consideration the concentration of the protein circulating in the blood.

In accordance with the invention, angiogenin can be used to treat chronic or latent infections. Many other important infectious pathogens can exist in a latent state where they are dormant or replicate very slowly. Examples of these pathogens include retroviruses, e.g., human immunodeficiency virus type 2 (HIV-2), human T lymphotropic virus type 2 (HTLV-2); herpesviruses, e.g., Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex type 1 (HSV-1), herpes simplex type 2 (HSV-2), herpes zoster virus (HZV), herpes virus type 6 (HHV-6), herpes virus type 7 (HHV-7); hepatitis viruses, e.g., hepatitis B (HBV), hepatitis C (HCV), the delta agent, and hepatitis E.

In another preferred embodiment, the angiogenin molecule inhibits any of the mechanisms involved in HIV replication, such as inhibition of reverse transcriptase, integrase, and protease activities in primary activated T lymphocytes. Preferably the

angiogenin molecule targets more than one activity of a virus, such as for example, reverse transcriptase, and/or protease, and/or integrase.

Human immunodeficiency virus type 1 (HIV-1), like all retroviruses, depends upon the integration of a DNA copy of its viral genome into host cell chromosomes as part of its infection cycle. Multiple steps in this integration process are catalyzed by HIV-1 integrase. The integration of HIV-1 DNA into the host chromosome is achieved by the integrase protein performing a series of DNA "cutting" and "joining" reactions (A-C):

(A) The first step in the integration process is 3' processing. This step requires linear double-stranded viral DNA with sequence specific 3' ends, synthesized by reverse transcription from the viral RNA genome. The integrase protein removes two nucleotides from each 3' end of this viral DNA, leaving recessed hydroxyls (-OH's) at the 3' ends.

(B) In a second step, termed strand transfer, the integrase protein joins the previously processed 3' ends to the 5' ends of strands of target DNA at the site of integration. The 5' ends are produced by integrase-catalyzed staggered cuts, 5 base pairs (bp) apart. A Y-shaped, gapped, recombination intermediate results, with the 5' ends of the viral DNA strands and the 3' ends of target DNA strands remaining unjoined, flanking a gap of 5 bp.

(C) Integrase may catalyze the excision of viral DNA, termed disintegration. Alternatively, integration may ensue. This involves host DNA repair synthesis in which the 5 bp gaps between the unjoined strands are filled in and then ligated. Since this process occurs at both cuts flanking the HIV genome a 5 bp duplication of host DNA is produced at the ends of HIV-1 integration.

Treatment of a patient with Angiogenin

In a preferred embodiment, a single agent, preferably angiogenin, is administered to a patient in need of such therapy. Preferably, administration of angiogenin, is used to treat an individual infected with an RNA virus, such as HIV. In particular, the

angiogenin targets more than one replication mechanism of a highly mutating virus, such as HIV, and the therapeutic activity of angiogenin is not affected by the high mutagenesis rate of HIV. While administration of a single agent is preferred, angiogenin or other agents can be administered with one or more additional, distinct therapeutic agents, such as for example, AZT (zidovudine), ddI, ddC, d4T, 3TC, FTC, DAPD, 1592U89 or CS92; TAT antagonists such as Ro 3-3335 and Ro 24-7429; protease inhibitors such as saquinavir, ritonavir, indinavir or AG1343 (Viracept); and other agents such as 9-(2-hydroxyethoxymethyl)guanine (acyclovir), ganciclovir or penciclovir, interferon, e.g., alpha-interon or interleukin II.

Studies of HIV-1 isolates have revealed a heterogeneity in their ability to infect different human cell types (reviewed by Miedema et al., 1994, *Immunol. Rev.* 140:35-72). The majority of extensively passaged laboratory strains of HIV-1 readily infect cultured T cell lines and primary T lymphocytes, but not primary monocytes or macrophages. These strains are termed T-tropic. T-tropic HIV-1 strains are more likely to be found in HIV-1 infected individuals during the late stages of aids (Weiss et al., 1996, *Science* 272:1885-1886). The majority of primary HIV-1 isolates (i.e., viruses not extensively passaged in culture) replicate efficiently in primary lymphocytes, monocytes and macrophages, but grow poorly in established T cell lines. These isolates have been termed M-tropic. The viral determinant of T- and M-tropism maps to alterations in the third variable region of gp120 (the V3 loop) (Choe et al., 1996, *Cell* 85:1135-1148; Cheng-Mayer et al., 1991, *J. Virol.* 65:6931-6941; Hwang et al., 1991, *Science* 253:71-74; Kim et al., 1995, *J. Virol.*, 69:1755-1761; and O'Brien et al., 1990. *Nature* 348:69-73). The characterization of HIV isolates with distinct tropisms taken together with the observation that binding to the CD4 cell surface protein alone is insufficient to lead to infection, suggest a requirement for cell-type specific cofactors, in addition to CD4, for HIV-1 entry into the host cell.

In one embodiment, a patient suffering from an infection by an RNA virus is treated with angiogenin peptides, variants or fragments thereof. Angiogenin may be administered by a variety of suitable routes including oral, topical (including transdermal,

buccal or sublingual), nasal and parenteral (including intraperitoneal, subcutaneous, intravenous, intradermal or intramuscular injection. See generally Reminington's Pharmaceutical Sciences, Mack Pub. Co., Easton, Pa., 1980. Nasal or oral routes leading significant contact believe one or more of the angiogenin and with airway epithelia, lung tissue being generally preferred.

Angiogenin of the present invention can be administered as a sole active agent, in combination with one or more other RNases as provided herein or in combination with other medicaments, for example, a protease inhibitor such as indinavir or ritonavir, or other agents as discussed above, e.g. AZT, ddI, ddC, d4T, 3TC, FTC, DAPD, 1592U89 or CS92; TAT antagonists such as Ro 3-3335 and Ro 24-7429; protease inhibitors such as saquinavir, ritonavir, indinavir or AG1343 (Viracept); and other agents such as 9-(2-hydroxyethoxymethyl)guanine (acyclovir), ganciclovir or penciclovir, interferon, e.g., alpha-interon or interleukin II. Administration of two or more medicaments, including the angiogenin of the invention is illustrative of a "cocktail" or "cocktail" therapy.

While angiogenin may be administered alone, it can also be present as part of a pharmaceutical composition in mixture with conventional excipient, preferably a pharmaceutically acceptable organic or inorganic carrier substances that is generally suitable for oral or nasal delivery as mentioned previously. However, in some cases, other modes of administration may be indicated in which case the angiogenin can be combined with a vehicle suitable for parenteral, oral or other desired administration and which do not deleteriously react with the angiogenin and are not deleterious to the recipient thereof. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings,

flavorings and/or aromatic substances and the like which do not deleteriously react with the angiogenin.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin *Remington's Pharm. Sci.*, 15th Ed. (Mack Publ. Co., Easton (1975)). Pharmaceutically acceptable carriers are sterile, and pyrogen free.

For parenteral application, particularly suitable are solutions, preferably oily or aqueous solutions as well as, suspensions, emulsions, or implants, including suppositories. Ampules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees or capsules having talc and/or carbohydrate carrier binder or the like, the carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle, is employed. Sustained release compositions can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

Therapeutic angiogenin of the invention also may be incorporated into liposomes. The incorporation can be carried out according to known liposome preparation procedures, e.g. sonication and extrusion.

It will be appreciated that the actual preferred amounts of angiogenin used in a given therapy will vary according to the activity of the angiogenin being utilized, the particular anti-pathogen system formulated, the mode of application, the particular site of administration, etc. Optimal administration rates for a given protocol of administration

can be readily ascertained by those skilled in the art using conventional dosage determination tests conducted with regard to the foregoing guidelines.

Treatment of a Patient with Vectors Encoding Angiogenin or Variants Thereof

Cells (e.g. virally infected mammalian cells) or a subject (e.g. an individual such as a mammal, particularly a primate such as a human) may be treated in accordance with the invention by administering to the cells or subject a nucleic acid molecule (typically a vector is administered comprising the nucleic acid sequence) that encodes for the angiogenin peptide, variants or fragments thereof. As discussed above, a preferred use of angiogenin is for the inhibition of replication of an RNA virus. Angiogenin nucleic acid molecules can be expressed by a vector containing a DNA segment encoding the wild-type, alleles, variants, mutations or fragments of the genes. Mutations and alleles of the nucleic acid molecules are also preferably used in the construction of a vector for use in treatment. The vector comprising the desired nucleic acid sequence for inhibiting replication of, for example, HIV-1, preferably has at least one such nucleic acid sequence. Alternatively, the vector may be comprised of more than one such nucleic acid sequence, or combinations of variants. The vector can also be comprised of cassettes of mutant angiogenin with higher inhibitory activity and/ or wild type nucleic acid molecules.

According to the present invention, the coding sequence on the plasmid that encodes the nucleic acid molecules is provided with a coding sequence that encodes an amino acid sequence whose presence on the protein results in a specific intracellular localization of the expressed protein. The nucleotide sequences that encode amino acid sequences which direct intracellular protein trafficking and which are included in the coding sequences of immunogenic proteins that are included in plasmid constructs used as DNA therapeutic compositions direct localization to specific areas in the cells which result in enhancement or activation of the immune response.

Introducing the genes, fragments or alleles thereof, into an individual can include use of vectors, liposomes, naked DNA, adjuvant-assisted DNA, gene gun, catheters, etc.

Vectors include chemical conjugates such as described in WO 93/04701, which has a targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage etc. The vectors can be chromosomal, non-chromosomal or synthetic.

It is a preferred embodiment of this invention that the choice of cells for delivery of the nucleic acid molecules include CD4+ T cells, monocytes, macrophages, dendritic cells and the like.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. Viral vectors can be chosen to introduce the genes to cells of choice. Such vectors include pox vectors such as orthopox or avipox vectors, herpesvirus vectors such as herpes simplex I virus (HSV) vector (Geller et al., 1995, *J. Neurochem.* 64: 487; Lim et al., 1995, *in* DNA Cloning: Mammalian Systems, D. Glover, ed., Oxford Univ. Press, Oxford, England; Geller et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 1149), adenovirus vectors (LeGal LaSalle et al., 1993, *Science* 259: 988; Davidson et al., 1993, *Nat. Genet.* 3: 219; Yang et al., 1995, *J. Virol.* 69: 2004) and adeno-associated virus vectors (Kaplitt et al., 1994, *Nat. Genet.* 8: 148).

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The vectors can be introduced by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include for example, naked DNA calcium phosphate precipitation, DEAE

dextran, electroporation, protoplast fusion, lipofection, cell microinjection and viral vectors.

The vector can be employed to target essentially any desired target cell. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location. Other methods that can be used include catheters, intravenous, parenteral, intraperitoneal, and subcutaneous injection, and oral or other known routes of administration.

Another preferred method is DNA immunization. DNA immunization employs the subcutaneous injection of a plasmid DNA (pDNA) vector encoding a tumor marker. The pDNA sequence is taken up by antigen presenting cells (APC). Once inside the cell, the DNA encoding protein is transcribed and translated and presented to lymphocytes.

Genetic constructs comprise a nucleotide sequence that encodes the nucleic acid sequence of choice and preferably includes an intracellular trafficking sequence operably linked to regulatory elements needed for gene expression.

When taken up by a cell, the genetic construct(s) may remain present in the cell as a functioning extrachromosomal molecule and/or integrate into the cell's chromosomal DNA. DNA may be introduced into cells where it remains as separate genetic material in the form of a plasmid or plasmids. Alternatively, linear DNA which can integrate into the chromosome may be introduced into the cell. When introducing DNA into the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication. Gene constructs may remain part of the genetic material in attenuated live microorganisms or recombinant microbial vectors which live in cells. Gene constructs may be part of genomes of recombinant viral vaccines where

the genetic material either integrates into the chromosome of the cell or remains extrachromosomal.

Genetic constructs include regulatory elements necessary for gene expression of a nucleic acid molecule. The elements include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers may be required for gene expression of angiogenin, or fragments thereof. It is necessary that these elements be operably linked to the sequence that encodes the desired proteins and that the regulatory elements are operable in the individual to whom they are administered.

Initiation codons and stop codons are generally considered to be part of a nucleotide sequence that encodes the immunogenic target protein. However, it is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and termination codons must be in frame with the coding sequence.

Promoters and polyadenylation signals used must be functional within the cells of the individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metallothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the

SV40 polyadenylation signal which is in pCEP4 plasmid (Invitrogen, San Diego Calif.), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. For example, plasmids pCEP4 and pREP4 from Invitrogen (San Diego, Calif.) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the cells the construct is administered into. Moreover, codons may be selected which are most efficiently transcribed in the cell. One having ordinary skill in the art can produce DNA constructs which are functional in the cells.

The method of the present invention comprises the steps of administering nucleic acid molecules to tissue of the individual. In some preferred embodiments, the nucleic acid molecules are administered intramuscularly, intranasally, intraperatoneally, subcutaneously, intradermally, or topically or by lavage to mucosal tissue selected from the group consisting of vaginal, rectal, urethral, buccal and sublingual.

In some embodiments, the nucleic acid molecule is delivered to the cells in conjunction with administration of a facilitating agent. Facilitating agents are also referred to as polynucleotide function enhancers or genetic vaccine facilitator agents.

Facilitating agents are described in *e.g.* International Application No. PCT/US94/00899 filed Jan. 26, 1994 and International Application No. PCT/US95/04071 filed Mar. 30, 1995, both incorporated herein by reference. Facilitating agents which are administered in conjunction with nucleic acid molecules may be administered as a mixture with the nucleic acid molecule or administered separately simultaneously, before or after administration of nucleic acid molecules.

In some preferred embodiments, the genetic constructs of the invention are formulated with or administered in conjunction with a facilitator selected from the group consisting of, for example, benzoic acid esters, anilides, amidines, urethans and the hydrochloride salts thereof such as those of the family of local anesthetics. The facilitating agent is administered prior to, simultaneously with or subsequent to the genetic construct. The facilitating agent and the genetic construct may be formulated in the same composition.

In some embodiments of the invention, the individual is first subject to injection of the facilitator prior to administration of the genetic construct. That is, for example, up to a about a week to ten days prior to administration of the genetic construct, the individual is first injected with the facilitator. In some embodiments, the individual is injected with the facilitator about 1 to 5 days; in some embodiments 24 hours, before or after administration of the genetic construct. Alternatively, if used at all, the facilitator is administered simultaneously, minutes before or after administration of the genetic construct. Accordingly, the facilitator and the genetic construct may be combined to form a single pharmaceutical composition.

In some embodiments, the genetic constructs are administered free of facilitating agents, that is in formulations free from facilitating agents using administration protocols in which the genetic constructions are not administered in conjunction with the administration of facilitating agents.

Nucleic acid molecules which are delivered to cells according to the invention may serve as genetic templates for proteins that function as prophylactic and/or therapeutic immunizing agents. In preferred embodiments, the nucleic acid molecules comprise the necessary regulatory sequences for transcription and translation of the coding region in the cells of the animal.

Production of Angiogenin Fusion Proteins

Angiogenin, fragments and variants thereof may be suitably purchased commercially or can be generated by PCR based strategies. A variety of amplification approaches can be utilized, e.g. a standard polymerase chain reaction, a ligase chain reaction, reverse transcriptase polymerase chain reaction, Rolling Circle polymerase chain reaction, multiplex polymerase chain reaction, LCR, RT-PCR, RCA and the like. "Amplification", as used herein, refers to any *in vitro* process for increasing the number of copies of a nucleotide sequence or sequences, i.e., creating an amplification product which may include, by way of example additional target molecules, or target-like molecules or molecules complementary to the target molecule, which molecules are created by virtue of the presence of the target molecule in the sample. In a situation where the target is a nucleic acid, an amplification product can be made enzymatically with DNA or RNA polymerases or transcriptases. Nucleic acid amplification results in the incorporation of nucleotides into DNA or RNA. As used herein, one amplification reaction may consist of many rounds of DNA replication. PCR is an example of a suitable method for DNA amplification. For example, one PCR reaction may consist of 30-100 "cycles" of denaturation and replication.

Each of these methods makes use of one or more oligonucleotide primers or splice templates able to hybridize to or near a given nucleotide sequence of interest. After hybridization of the primer, the target-complementary nucleic acid strand is enzymatically synthesized, either by extension of the 3' end of the primer or by transcription, using a promoter-primer or a splice template. In some amplification methods, such as PCR, rounds of primer extension by a nucleic acid polymerizing enzyme is alternated with thermal denaturation of complementary nucleic acid strands.

Other methods, such as those of WO91/02818, Kacian and Fultz, U.S. Pat. No. 5,480,783; McDonough, et al., WO 94/03472; and Kacian, et al., WO 93/22461, are isothermal transcription-based amplification methods. Primers for angiogenin can be purchased commercially.

In another preferred embodiment, angiogenin oligonucleotides, proteins, peptides, or variants thereof are generated using nucleic acid sequences of angiogenin chosen by the user. For applications in which the nucleic acid segments of the present invention are incorporated into vectors, such as plasmids, cosmids or viruses, these segments may be combined with other DNA sequences, such as promoters, polyadenylation signals, restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

The term "DNA construct" and "vector" are used herein to mean a purified or isolated polynucleotide that has been artificially designed and which comprises at least two nucleotide sequences that are not found as contiguous nucleotide sequences in their natural environment.

Once the coding sequence of angiogenin, variants or fragments thereof, are selected, the gene can be inserted into an appropriate expression system. The gene can be expressed in any number of different recombinant DNA expression systems.

Examples of expression systems known to the skilled practitioner in the art include bacteria such as *E. Coli*, yeast such as *Saccharomyces cerevisia* and *Pichia pastoris*, baculovirus, and mammalian expression systems such as in Cos or CHO cells. In one embodiment, polypeptides are expressed in *E. coli* and in baculovirus expression systems. A complete gene can be expressed or, alternatively, fragments of the gene encoding portions of polypeptide can be produced.

A most preferred vector is the pTAT-HA expression vector (Nagahara et al., 1998).

As used herein, the term "administering a molecule to a cell" (e.g., an expression vector, nucleic acid, amino acid, fusion proteins, a delivery vehicle, agent, and the like) refers to transducing, transfecting, microinjecting, electroporating, or shooting, the cell with the molecule. In some aspects, molecules are introduced into a target cell by contacting the target cell with a delivery cell (e.g., by cell fusion or by lysing the delivery cell when it is in proximity to the target cell).

Deletion of sequences from the angiogenin gene used for expression can be achieved by standard techniques. For example, fortuitously-placed restriction enzyme sites can be used to excise the desired gene fragment, or PCR-type amplification can be used to amplify only the desired part of the gene. The skilled practitioner will realize that such changes must be designed to not change the translational reading frame for downstream portions of the protein-encoding sequence. Minigenes or gene fusions encoding the desired polypeptide can be constructed and inserted into expression vectors by standard methods, for example, using PCR methodology.

The gene or gene fragment encoding a angiogenin polypeptide can be inserted into an expression vector by standard subcloning techniques. In one embodiment, an *E. coli* expression vector is used that produces the recombinant polypeptide as a fusion protein, allowing rapid affinity purification of the protein. Examples of such fusion protein expression systems are the glutathione S-transferase system (Pharmacia, Piscataway, N.J.), the maltose binding protein system (NEB, Beverley, Mass.), the FLAG system (IBI, New Haven, Conn.), and the 6x-His system (Qiagen, Chatsworth, Calif.).

In a most preferred embodiment, the fusion protein is comprised of an N terminal 6x-His purification tag, a TAT protein transduction domain of about 11 amino acid residues and an HA-epitope tag.

Some of these systems produce recombinant polypeptides bearing only a small number of additional amino acids, which are unlikely to affect the physical and chemical properties of the recombinant polypeptide. For example, both the FLAG system and the 6x-His system add only short sequences, both of that are known to be poorly antigenic and which do not adversely affect folding of the polypeptide to its native conformation. Other fusion systems produce polypeptide where it is desirable to excise the fusion partner from the desired polypeptide. In one embodiment, the fusion partner is linked to the recombinant polypeptide by a peptide sequence containing a specific recognition sequence for a protease.

In another embodiment, the expression system used is one driven by the baculovirus polyhedron promoter. The gene encoding the polypeptide can be manipulated by standard techniques in order to facilitate cloning into the baculovirus vector. One baculovirus vector is the pBlueBac vector (Invitrogen, Sorrento, Calif.). The vector carrying the gene for the polypeptide is transfected into Spodopterafrugiperda (Sf9) cells by standard protocols, and the cells are cultured and processed to produce the recombinant antigen. See Summers et al., A MANUAL OF METHODS FOR BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES, Texas Agricultural Experimental Station.

As an alternative to recombinant polypeptides, synthetic angiogenin peptides can be prepared. Such peptides are at least six amino acid residues long, and may contain up to approximately 35 residues, which is the approximate upper length limit of automated peptide synthesis machines, such as those available from Applied Biosystems (Foster City, Calif.).

Purification and Characterization of Angiogenin Fusion Proteins

Further aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree

relative to its naturally-obtainable state, *i.e.*, in this case, relative to its purity within a cell expressing angiogenin. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number". The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

In another preferred embodiment, a patient suffering from infection with an RNA virus such as HIV is treated with an angiogenin fusion protein, the angiogenin fusion protein comprising the angiogenin peptide, variants or fragments thereof and a transducing domain. The transducing domain of the fusion protein is preferably comprised of TAT protein or fragments thereof.

In one aspect of the invention, the angiogenin fusion protein further comprises 6x-His and HA-epitope tags useful for intracellular detection of the fusion protein.

In general, the transduction domain of the fusion molecule can be nearly any synthetic or naturally-occurring amino acid sequence that can transduce or assist in the transduction of the fusion molecule. For example, transduction can be achieved in accord with the invention by use of a protein sequence and particularly an HIV TAT protein or fragment thereof that is covalently linked to the fusion molecule. Alternatively, the transducing protein can be the Antennapedia homeodomain or the HSV VP22 sequence, or suitable transducing fragments thereof such as those known in the field.

The type and size of the transducing amino acid sequence will be guided by several parameters including the extent of transduction desired. Preferred sequences will be capable of transducing at least about 20%, 25%, 50%, 75%, 80% or 90% of the cells of interest, more preferably at least about 95%, 98%% and up to about 100% of the cells. Transduction efficiency, typically expressed as the percentage of transduced cells, can be determined by several conventional methods such as those specific microscopical methods discussed below (e.g., flow cytometric analysis).

Additionally preferred transducing sequences will manifest cell entry and exit rates (sometimes referred to as k_1 and k_2, respectively) that favor at least picomolar amounts of the fusion molecule in the cell. The entry and exit rates of the amino acid sequence can be readily determined or at least approximated by standard kinetic analysis using detectably-labeled fusion molecules. Typically, the ratio of the entry rate to the exit rate will be in the range of from between about 5 to about 100 up to about 1000.

Particularly preferred, are transducing amino acid sequences that include at least a peptide featuring substantial alpha-helicity. It has been discovered that transduction is optimized when the transducing amino acid sequence exhibits significant alpha-helicity. Also preferred are those sequences having basic amino acid residues that are substantially aligned alone at least one face of the peptide. Typically such preferred transduction sequences are synthetic protein or peptide sequences.

Additional transducing sequences in accord with this invention include a TAT fragment that comprises a fragment of TAT up to about the full-length TAT sequence. A preferred TAT fragment includes one or more amino acid changes sufficient to increase the alpha-helicity of that fragment. In most instances, the amino acid changes introduced will involve adding a recognized alpha-helix enhancing amino acid. Alternatively, the amino acid changes will involve removing one or more amino acids from the TAT fragment that impede alpha helix formation or stability. In more specific embodiments, the TAT fragment will include at least one amino acid substitution with an alpha-helix enhancing amino acid. Preferably the TAT fragment is made, for example, by standard peptide synthesis techniques although recombinant DNA approaches may be preferred in some cases.

Additional transduction proteins of this invention include the TAT fragment in which the sequence has been modified so that at least two basic amino acids in the sequence are substantially aligned along at least one face of the TAT fragment. In one embodiment, that alignment is achieved by making at least one specified amino acid addition or substitution to the TAT sequence. Additional transduction proteins in accord with this invention include the TAT fragment in which the sequence includes at least one substitution with an alpha-helix enhancing amino acid. In one embodiment, the substitution is selected so that at least two basic amino acid residues in the TAT fragment are substantially aligned along at least one face of that TAT fragment. In a more specific embodiment, the substitution is chosen so that at least two basic amino acid residues in the TAT sequence are substantially aligned along at least one face of that sequence.

Additionally provided are chimeric transducing proteins that include parts of at least two different transducing proteins. For example, chimeric transducing proteins can be formed by fusing two different TAT fragments, e.g., one from HIV-1 and the other from HIV-2. Alternatively, other transducing proteins can be formed by fusing a desired transducing protein to heterologous amino acid sequences such as 6xHis, (sometimes referred to as "HIS"), EE, HA or Myc.

The fusion proteins of the present invention can be separated and purified by appropriate combinations of known techniques. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

Methods for purification of the fusion proteins utilize several chemical and physical properties of the fusion proteins. These methods include, for example, methods utilizing solubility such as salt precipitation and solvent precipitation, methods utilizing the difference in molecular weight such as dialysis, ultra-filtration, gel-filtration, and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electrical charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatograph, methods utilizing a difference in hydrophobicity such as reverse-phase high performance liquid chromatograph and methods utilizing a difference in isoelectric point, such as isoelectric focusing electrorhoresis, metal affinity columns such as Ni-NTA. See generally Sambrook *et al.* and Ausubel *et al.* for disclosure relating to these methods.

It is preferred that the fusion proteins of the present invention be substantially pure. That is, the fusion proteins have been isolated from cell substituents that naturally accompany it so that the fusion proteins are present preferably in at least 80% or 90% to 95% homogeneity (w/w). Fusion proteins having at least 98 to 99% homogeneity (w/w) are most preferred for many pharmaceutical, clinical and research applications. Once substantially purified the fusion protein should be substantially free of contaminants for therapeutic applications. Once purified partially or to substantial purity, the soluble fusion proteins can be used therapeutically, or in performing *in vitro* or *in vivo* assays.

Substantial purity can be determined by a variety of standard techniques such as chromatography and gel electrophoresis.

Nucleic acid encoding a desired fusion protein can be introduced into a host cell by standard techniques for transfecting cells. The term "transfecting" or "transfection" is intended to encompass all conventional techniques for introducing nucleic acid into host cells, including calcium phosphate co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, viral transduction and/or integration. Suitable methods for transfecting host cells can be found in Sambrook *et al.*, and other laboratory textbooks.

Cells transduced by the fusion molecules of the present invention can be assayed for viability by standard methods. In one approach, cell viability can be readily assayed by measuring DNA replication following or during transduction. For example, a preferred assay involves cell uptake of one or more detectably-labeled nucleosides such as radiolabelled thymidine. The uptake can be conveniently measured by several conventional approaches including trichloroacetic acid (TCA) precipitation followed by scintillation counting. Other cell viability methods include well know trypan blue exclusion techniques.

As noted, fusion molecules of the present invention are efficiently transduced into target cells or groups of such cells. Transduction efficiency can be monitored and quantified if desired by one or a combination of different strategies.

For example, one approach involves an *in vitro* assay that measures uptake of the fusion protein by the cell. The assay includes detectably-labeling the fusion protein with, *e.g.*, a radioactive atom, fluorescent, phosphorescent, or luminescent tag (e.g., fluorescein, rhodamine or FITC) and then measuring uptake of the labeled fusion protein. Alternatively, the fusion protein can be labeled with an enzyme capable of forming a detectable label such as horseradish peroxidase, β-galactosidase, chloramphenicol acetyl transferase or luciferase. In a preferred approach, it is possible to genetically fuse a

desired fusion protein to the well-known green fluorescent protein (GFP) and then assaying the fusion protein. Uptake can be measured by several conventional methods such as by quantifying labeled cells in a standard cell sorter (e.g., FACS), by fluorescence microscopy or by autoradiography. See generally Sambrook *et al.* and Ausubel *et al.* *infra* for disclosure relating to the assays.

Biologically, GFP acts to shift the color of bioluminescence from blue to green in luminous coelenterates (jellyfish, hydroids, sea pansies, and sea pens) and to increase the quantum yield of light emission. This fluorescence can be visualized directly on culture plates upon illumination with either blue- or long-wave ultraviolet (UV) light. Any of the vectors designed for protein expression can be used to make constructs to express GFP in different cells or organisms, either alone or as a fusion protein.

Preferred fusion proteins of the invention are capable of transducing at least about 20%, to 80%, and more preferably at least about 90%, 95%, 99% up to 100% of the total number of target cells as determined by any conventional methods for monitoring protein uptake by cells and particularly the FACS or related microscopical techniques. The total number of target cells can be estimated by standard techniques.

In vivo *and* in vitro *Administration of Angiogenin Fusion Proteins*

The fusion proteins of the invention can be administered to cells *in vivo* or *in vitro* by one or a combination of strategies.

For example, the fusion proteins can be administered to primary or immortalized cells growing in culture *in vitro* by conventional cell culture techniques that generally include contacting the cells with the fusion protein and allowing the fusion protein to transduce through the cells for a specified period of time. Typically, cell media will be removed from the cells prior to the contact to increase fusion protein concentration.

In addition, the fusion proteins can be administered to cells *in vivo*, for example, by using a specified delivery mechanism suitable for introduction of fusion proteins into

those cells. In general, the type of delivery mechanism selected will be guided by several considerations including the location of the cells, the degree of transduction needed to induce motility of the cells, and the general health of the cells.

Preferred methods for determining intracellular localization of the fusion proteins, include but are not limited to immunofluorescence, leptomycin B assays and other techniques well known to one of skill in the art.

Treatment of a Patient with Angiogenin Fusion Proteins and/or Angiogenin

In accordance with the invention, the fusion protein is transduced into mammalian cells. The transduced fusion protein inhibits the replication of RNA viruses such as HIV-1 , HIV-2 and the like. Inhibition of replication can be measured by several methods. For example HIV-1 replication is measured using the commercially available p24 ELISA capture assay. Preferred cells that are transduced with the angiogenin fusion protein include all cells that are infected by HIV such as $CD4^+$ T cells, macrophages.

In a preferred embodiment, cells infected with an RNA virus are treated with angiogenin. Preferably, the angiogenin molecule inhibits HIV replication in primary activated T lymphocyte cultures as well as chronically infected cell lines. Inhibition of HIV-1 replication in primary activated T lymphocytes is important as it would decrease the risk of HIV spreading to other T cells.

In another preferred embodiment, angiogenin inhibits the replication of T lymphocyte tropic and/or macrophage tropic strains of HIV. Preferably, the infected lymphocyte is an activated primary $CD4^+$ T lymphocyte. Preferably, the activated primary T lymphocyte is a lymphocyte. Also preferred is a chronically infected lymphocyte is a $CD4^+$ T lymphocyte. Preferably, angiogenin inhibits the replication of an RNA virus in a chronically infected cell by at least 18%, or by at least 40% at least about, 50%,60%, 75%, 80%, 99.9% or by at least about100%.

Replication of, for example, HIV can be measured using a p24 commercially available assay. For example, for each infection, a total of about $1x10^4$ cells in exponential growth phase are harvested and washed once with medium and pelleted. The cell pellet is then resuspended in about 1 ml of diluted HIV virus stock comprising about 10 $TCID_{50}$ units of virus. After adsorption at 37°C. for about 2 hours, about 10 ml of medium was added, and the cells were pelleted by centrifugation. They are then resuspended in about 15 ml of Iscove's and 10% FCS medium, and transferred into a 25 cm^2 flask. Duplicate infections per cell line were employed in each challenge assay, and the infected cultures are incubated at 37°C. Every other day beginning from day 2 post infection, about 0.5 ml of culture supernatant is removed from the flasks, and virus replication is monitored by measuring the production of p24 viral antigen in culture supernatant using an HIV-1 p24 antigen capture ELISA assay (Coulter Immunology, Hialeah, Fla.).

In a preferred embodiment, a method for inhibiting replication of an RNA virus comprises administering to an infected lymphocyte and/or monocyte an effective amount of a pancreatic-like ribonuclease, fragments or variants thereof. Preferably the ribonuclease is angiogenin or a variant or fragment thereof.

Cells, such as lymphocytes can be activated using for example, cytokines, mitogens such as phorbol myristate acetate (PHA) and the like, prior to infection with virus. Pancreatic ribonuclease, angiogenin can be added at various time points before and/or after cells have been activated and incubated with virus. Inhibition of replication of HIV by the administration of angiogenin is measured preferably by HIV p24 antigen capture ELISA assay.

If desirable, a second agent can be given in conjunction with angiogenin, particularly when it is desirable to administer a lower dose of the second agent. Examples of a second agent include, but not limited to commonly used anti-retroviral drugs, such as reverse transcriptase inhibitors, protease inhibitors, and inhibitors of viral entry. Reverse transcriptase inhibitors can be nucleoside analogues, e.g., AZT

(Zidovudine; Glaxo-Burroughs Wellcome Co., Research Triangle Park, NC), ddI (Didanosine; Bristol-Myers Squibb; Wallingford, Conn.), 3TC (Glaxo-Burroughs Wellcome), d4T (Stavudine; Bristol-Myers Squibb), or ddC (Zalcitabine; Hoffman-La Roche; Basel, Switzerland); or non-nucleoside drugs, e.g., Nevirapine (Viramune; Roxane Laboratories; Columbus, Ohio), Delaviridine (Rescriptor; Pharmacia & Upjohn; Kalamazoo, Mich.)., Abacavir or Pyridnone (Merck, Sharp & Dohme; Rahway, N.J.). Protease inhibitors which can be used include, e.g., Indinavir (Crixivan; Merck; West Point, Pa.), Ritonavir (Novir; Abbott Laboratories; Abbott Park, Ill.), Saquinavir (Invirase; Roche; Palo Alto, Calif.), Nelfinavir (Agouron Pharmaceuticals; La Jolla, Calif.), and Amprenavir.

Inhibition of Replication of an RNA Virus by Angiogenin in Cells

In another preferred embodiment, angiogenin inhibits replication of an RNA virus in immune cells. Immune cells express a variety of cell surface molecules which can be detected with either monoclonal antibodies or polyclonal antisera. Immune cells that have undergone differentiation or activation can also be enumerated by staining for the presence of characteristic cell surface proteins by direct immunofluorescence in fixed smears of cultured cells. For example, T lymphocytes, at whichever stage of maturity and cell differentiation can be identified by measuring cell phenotypes. The phenotypes of immune cells and any phenotypic changes can be evaluated by flow cytometry after immunofluorescent staining using monoclonal antibodies that will bind membrane proteins characteristic of various immune cell types.

T cells at different stages of maturation or differentiation express surface molecules indicative of that stage or differentiation. For example, memory T cells express $CD45RO^+$. Memory T cells can be expanded (proliferated) without the need of specific antigenic stimulation to maintain the clonal size. Naïve T cell repertoires express $CD45RA^+$. For example, to evaluate the frequency of resting T cells with memory phenotype that could be stimulated by cytokines to grow, limiting dilution experiments can be performed. $CD45RO^+$ $CD4^+$ resting T cells can be cultured with IL-2 alone or in

combination with TNF-α and IL-6, in the presence of autologous irradiated macrophages and anti-DR antibodies to prevent autoreactive responses.

Systemic memory T cells are characterized according to the cell surface expression of certain known antigens. Typically, these cells are positive for CD4, and lack expression of CD45RA, and integrin α4β7. They are further characterized by expression of CCR4. A subset of cells of interest are common leukocyte antigen positive (CLA^+). Verification of the identity of the cells of interest may be performed by any convenient method, including antibody staining and analysis by fluorescence detection, ELISA, etc., reverse transcriptase PCR, transcriptional amplification and hybridization to nucleic acid microarrays, etc. Some memory T cells associated with the skin are known to express CLA. Thus, any type of cell can be identified when necessary.

A second means of assessing cell differentiation is by measuring cell function. This may be done biochemically, by measuring the expression of enzymes, mRNA's, genes, proteins, or other metabolites within the cell, or secreted from the cell.

In another preferred embodiment, a pharmaceutical composition kit comprising i) angiogenin or a variant or fragment thereof and ii) directions for use of the angiogenin or variant or fragment to treat against an RNA virus.

Optionally, the kit can further comprise instructions for suitable operational parameters in the form of a label or a separate insert. For example, the kit may have standard instructions informing a consumer how to dilute the angiogenin prior to administration, the final concentration of the diluted angiogenin, doses, the amount of time between treatments; contraindications and the like. Preferably, the angiogenin, variant or fragments thereof are supplied in an effective dose and in separate ampoules to provide at least about a weeks course of treatment. The course of treatment provided in a kit preferably decreases the viral load by at least about 50%. In some embodiments, the kit may further comprise instructions for suitable operation parameters in the form of a label or a separate insert.

All documents mentioned herein are incorporated herein by reference in their entirety.

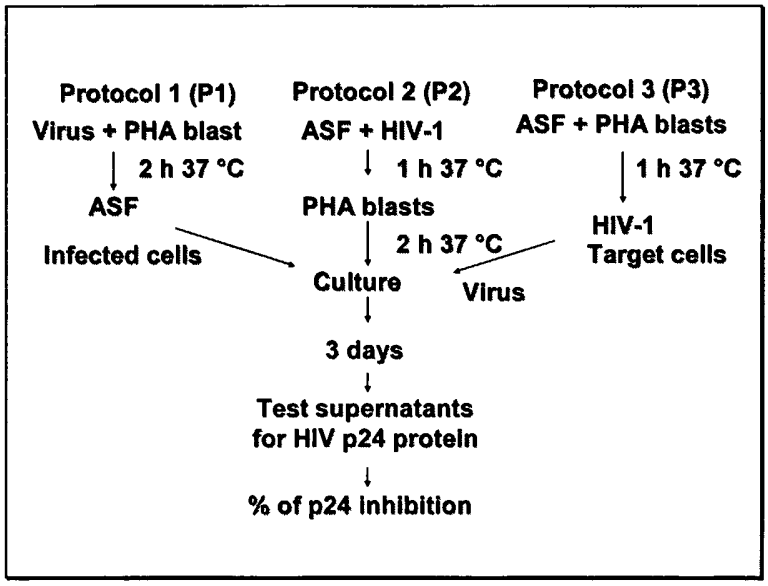
The following non-limiting examples are illustrative of the invention.

EXAMPLES

Materials and Methods

Three types of protocols were used in measuring the percent of p24 inhibition. This is schematically shown in Table 1:

Table 1



In the first protocol (P1) virus is cultured with the CD4+ cells for 2 hours before the ASF (allostimulated factor or EDN containing ASF) is added. P2 and P3 were developed for the purpose of blocking the anti-HIV effects of RNases with ribonuclease

inhibitors (RI), the RIs are not as effective in the presence of serum or plasma, as compared to serum-free medium.

In protocol 1, peripheral blood mononuclear cells (PBMC) were cultured for two days in the presence of PHA (PHA blasts) and were infected with HIV-1 for 2 hours. The cells were then washed in culture media, and ASF and negative control culture supernatants was added for three days. The supernatants from these infections were assayed for amount of the HIV-1 p 24 protein by ELISA.

Protocol 2 (P2) differs from P1 in that the ASF and HIV-1 are first incubated together for one hour, after which the PHA blasts are added for infection for two hours, and then excess virus is washed out. The infection cultures are cultured for three days to allow for any p24 production and quantitation by ELISA

Protocol 3 (P3) sets up the remaining situation, in which ASF and the PHA blasts are first incubated together for one hour. These cells are then washed, and HIV-1 is added for infection. After two hours of incubation, excess virus is removed by washing and the culture are incubated for three days and the supernatants are tested for p24 content by ELISA.

Leukocyte source and preparation

PBMC were isolated by Ficoll density centrifugation of heparinized whole blood from healthy HIV-seronegative blood donors (Pinto L.A. et al., *Blood*, 1998, 92:3346-3354).

Viral stock

HIV-1_{BZ167} and HIV-1_{GT} primary isolates were grown in human phytohemagglutinin (PHA)-stimulated PBMC (Pinto L.A. et al). Both primary virus isolates are dula tropic and use CCR5, CXCR4 and CCR3 co-receptors.

Generation and heat treatment of alloantigen-stimulated factors (ASF)

PBMC from healthy donors were cultured with HLA-unrelated (allogeneic) PBMC at 37°C in a 7% CO_2 atmosphere, and the supernatants were collected after 7 days of culture and frozen. Some culture supernatants were heated to 90°C for 3 min. Three different conditions were used to generate ASF: (i) PBMC were cultured with 5000 cGy-irradiated allogeneic stimulator PBMC from a pool of six unrelated donors in 10% fetal bovine serum (FBS)-supplemented RPMI 1640 medium; (ii) PBMC were cultured with the allogeneic pool for 4 days in FBS-containing RPMI 1640 medium, followed by an additional 3 days in serum-free AIM V medium (Gibco, Grand Island, New York, USA); or (iii) unirradiated PBMC from 2 HLA-A02-positive donors (two-way mixed lymphocyte reaction) were co-cultured in RPMI 1640 supplemented with human AB+ serum.

RNAse preparation and RNase-specific antibodies.

The recombinant ribonucleases (rRNases), eosinophil-derived neurotoxin (EDN), RNAse A and angiogenin were prepared as described by Newton D.L. and Rybak S.M. *J. Nat'l. Cancer Inst.*, 1998, 90:1787-1791. Polyclonal rabbit antibodies were purchased from Assay Research (College Park, Maryland, USA).

Acute infection of PHA-stimulated PBMC

HIV-1 infected target cells were prepared by stimulating PBMC from random blood bank donors for 2 days at 37 °C in 7% CO_2 with FBS-supplemented RPMI 1640. Viral infections were performed at 170-200 50% tissue culture infectious doses per 10^5 target cells and the infected targets were cultured in FBS-supplemented RPMI-1640 containing 10 U/ml recombinant rIL-2. The supernatants were later assayed in triplicate for HIV-1 p24 using a standard ELISA (Beckman-Coulter, Miami, Florida, USA).

Example 1: Inhibition of HIV Replication

The HIV-1-inhibitory activity of angiogenin is compared with three other RNases that are known to inhibit HIV replication. The inhibition of viral replication is expressed

in terms of percent inhibition (as shown in the figures 1-3). Table 2 gives the actual values of the HIV p24 protein, which is an indication of the amount of viral replication that is occurring in cell cultures, and also shows the percent (%) inhibition. The 162478 value at the top of the table is the denominator of the equation for % inhibition and represents no inhibition, or the amount of p24 that would be produced without an inhibitor. The values below that number are used as the numerator for calculating % inhibition of each row of data.

Table 2. Inhibition of HIV-1 p24 production by RNases

rRNase	Concentration (µg/well)	P24 (pg/mL)	% p24 Inhibition
None		162478	
EDN	1	154466	5
	10	107000	35
	50	NT	
-4EDN	1	177200	0
	10	80500	51
	50	64600	61
RNase A	1	159966	2
	10	77850	52
	50	24133	85
Angiogenin	1	133533	18
	10	37350	77
	50	16200	90

NT: not tested

As can be seen in Table 2, angiogenin is the most effective of the four ribonucleases that were compared for inhibiting HIV-1 replication. For ease of presentation, the three figures present the data in % inhibition. In Figures 1A, as well as 3A and 3B the cell cultures contain: target cells that are to be infected (PHA blasts); HIV-1 virus; and the RNase. There are different infection sequences in which these three components are put together: Figure 1A incubates PHA blasts and HIV together first for 2 hours adding the putative inhibiting RNase. This gives a "jump start" to the virus, and shows that these RNases are able to block viral replication, even after infection has been

initiated. Figure 3A shows the inhibitory effect using a protocol in which the Rnases are incubated with HIV-1 before infecting the PHA blasts. Figure 3B shows incubation of the RNase with the PHA targets before infecting with HIV-1. The results show that angiogenin inhibits HIV replication regardless of the order in which the different components are added. Even though the other ribonucleases inhibit HIV replication, none inhibited the replication of HIV as well as angiogenin.

All documents mentioned herein are incorporated herein by reference.

The invention has been described herein with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of the disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A method for inhibiting replication of an RNA virus comprising:

administering to an infected lymphocyte and/or monocyte an effective amount of a pancreatic-like ribonuclease, or a fragment or variant thereof.

2. The method of claim 1, wherein the ribonuclease is angiogenin or a variant or fragment thereof.

3. The method of claim 1, wherein the infected lymphocyte is an activated primary T lymphocyte.

4. The method of claim 3, wherein the activated primary T lymphocyte is a $CD4^+$ lymphocyte.

5. The method of claim 1, wherein the infected lymphocyte is a chronically infected lymphocyte.

6. The method of claim 5, wherein the chronically infected lymphocyte is a $CD4^+$ T lymphocyte.

7. The method of claim 1, wherein the RNA virus is a virus selected from the group consisting of Retroviridae, Paramyxoviridae, Orthomyxoviradae, Flaviviridae, Filoviridae, Rhabdoviridae, Coronaviridae, and Togaviridae.

8. The method of claim 7, wherein the RNA virus is human immunodeficiency virus.

9. The method of claim 8, wherein the human immunodeficiency virus is lymphocyte-tropic and/or macrophage-tropic.

10. A method for treating cells infected with RNA virus comprising:

administering to the cells an effective amount of a pancreatic-like ribonuclease, fragments or variants thereof.

11. The method of claim 10, wherein the ribonuclease is angiogenin or a variant or fragment thereof.

12. The method of claim 10 or 11, wherein the RNA virus is a virus selected from the group consisting of Retroviridae, Paramyxoviridae, Orthomyxoviradae, Flaviviridae, Filoviridae, Rhabdoviridae, Coronaviridae, and Togaviridae.

13. The method any one of claims 10 through 12, wherein the RNA virus is human immunodeficiency virus.

14. The method of claim 13, wherein the human immunodeficiency virus is lymphocyte-tropic and/or macrophage-tropic.

15. A method for treating an individual suffering from or susceptible to, infection by an RNA virus, comprising administering an effective amount of a pancreatic-like ribonuclease in a pharmaceutically acceptable carrier.

16. The method of claim 15, wherein the ribonuclease is angiogenin or a variant or fragment thereof.

17. The method of claim 15 or 16, wherein the RNA virus is a virus selected from the group consisting of Retroviridae, Paramyxoviridae, Orthomyxoviradae, Flaviviridae, Filoviridae, Rhabdoviridae, Coronaviridae, and Togaviridae.

18. The method of any one of claims 15 through 17, wherein the RNA virus is human immunodeficiency virus.

19. The method of claim 18, wherein the human immunodeficiency virus is lymphotropic and/or macrophage-tropic.

20. The method of any one of claims 16 through 19, wherein the angiogenin, variants or fragments thereof, inhibit the replication of the human immunodeficiency virus in activated primary T lymphocytes.

21. The method of any one claims 16 through 19, wherein the angiogenin, variants or fragments thereof, inhibit the replication of the human immunodeficiency virus in chronically infected T lymphocytes.

22. The method of any one of claims 16 through 21, wherein the virus load in a chronically infected individual is decreased by at least about 50% during a course of treatment with a therapeutically effective dose of angiogenin, variants or fragments thereof.

23. The method of any one of claims 1 through 22 wherein the cells or subject is identified being virally infected and the identified cells or individual are selected for treatment.

24. A pharmaceutical composition kit comprising i) angiogenin or a variant or fragment thereof or a nucleic acid sequence encoding angiogenin or a variant or fragment thereof; and ii) directions for use of the angiogenin or variant or fragment to treat against an RNA virus.

25. The pharmaceutical composition kit of claim 24, wherein angiogenin or a variant or a fragment thereof, inhibits the replication of the human immunodeficiency virus in activated primary T lymphocytes.

26. The pharmaceutical composition kit of claim 24, wherein the angiogenin, variants or fragments thereof, inhibit the replication of the human immunodeficiency virus in chronically infected T lymphocytes.

27. The pharmaceutical composition kit of claim 24, wherein the kit provides angiogenin, variants or fragments thereof sufficient to decrease the viral load in an infected individual by at least about 50%.

28. A method for inhibiting replication of an RNA virus comprising:

administering to an infected lymphocyte and/or monocyte with a viral load an effective amount of a pancreatic-like ribonuclease, or a fragment or variant thereof, wherein the viral load is decreased by between about 18% to about 100%.

29. A method for inhibiting replication of an RNA virus comprising:

administering to an infected lymphocyte and/or monocyte with a viral load an effective amount of a pancreatic-like ribonuclease, or a fragment or variant thereof, wherein the viral load is decreased by at least about 70%.

Figure 1A

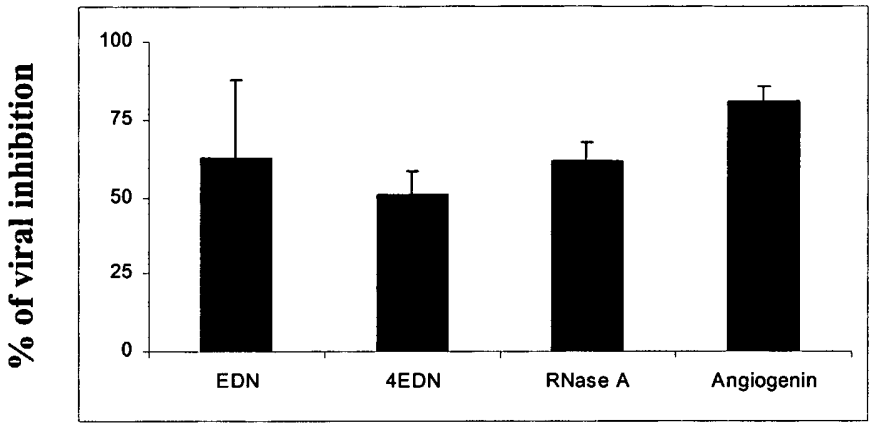

% of viral inhibition
100
75
50
25
0
EDN
4EDN
RNase A
Angiogenin

Figure 1B

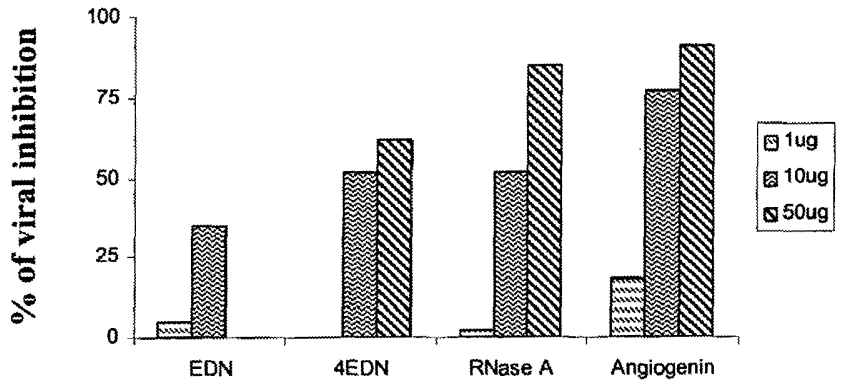

% of viral inhibition
100
75
50
25
0
EDN
4EDN
RNase A
Angiogenin
1ug
10ug
50ug

Figure 2

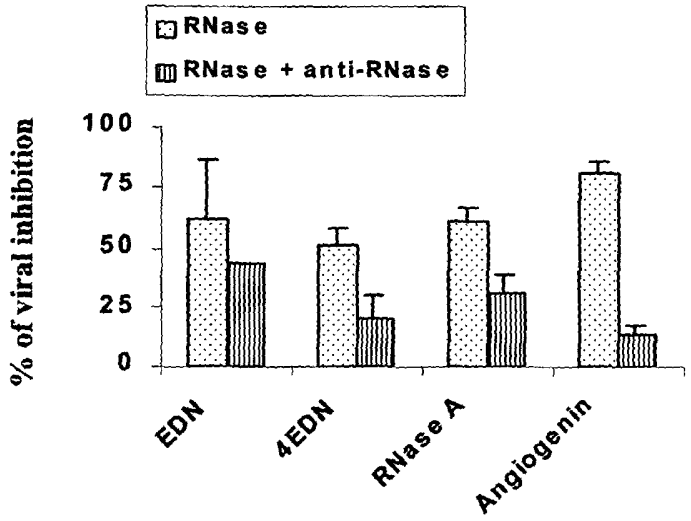

RNase
RNase + anti-RNase
% of viral inhibition
100
75
50
25
0
EDN
4EDN
RNase A
Angiogenin

Figure 3A

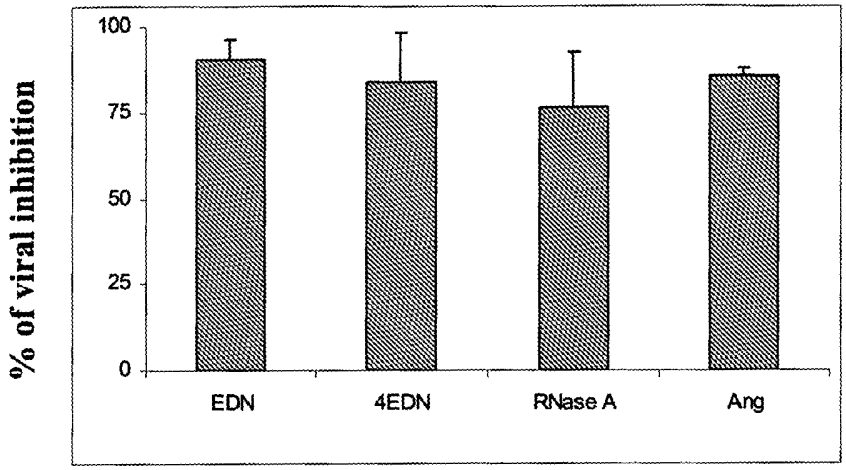


Figure 3B