COMPOSITIONS AND METHODS FOR TREATING HIV INFECTION WITH CUPREDOXIN AND CYTOCHROME C

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §§ 119 and 120 to co-filed U.S. Patent Application Serial No. _____, entitled "Compositions and Methods for Treating Malaria with Cupredoxins and Cytochrome", filed May 18, 2006, U.S. Provisional Patent Application Serial No. 60/780,868, filed March 10, 2006, U.S. Provisional Patent Application Serial No. 60/682,833, filed May 20, 2005, and U.S. Patent Application No. 11/244,105, filed October 6, 2005, which claims priority to U.S. Provisional Patent Application Serial No. 60/616,782, filed October 7, 2004, and U.S. Provisional Patent Application Serial No. 60/680,500, filed May 13, 2005, and is a continuation-in-part of U.S. Patent Application Serial Number 10/720,603, filed November 11, 2003, which claims priority to U.S. Provisional Patent Application Serial No. 60/414,550, filed August 15, 2003, and which is a continuation-in-part of U.S. Patent Application Serial Number 10/047,710, filed January 15, 2002, which claims priority to U.S. Provisional Patent Application Serial Number 60/269,133, filed February 15, 2001. The entire content of these prior applications is fully incorporated herein by reference.

STATEMENT OF GOVERNMENTAL INTEREST

The subject matter of this application has been supported by research grants from the National Institutes of Health (NIH), Bethesda, Maryland, U.S.A., (Grant Numbers AI 16790-21, ES 04050-16, AI 45541, CA09432 and N01-CM97567). The government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to cupredoxin, specifically *Pseudomonas aeruginosa* azurin, and/or *Pseudomonas aeruginosa* cytochrome c_{551} and their use in inhibiting of viral infection, and in particular infection of mammalian cells by the Human Immunodeficiency Virus (HIV). The invention also relates to variants and derivatives of cupredoxin and cytochrome c that retain the ability to inhibit viral infection, and in particular infection by the Human Immunodeficiency Virus (HIV). The invention also relates to research methods for studying viral and bacterial infection in mammalian cells.

BACKGROUND

While human immunodeficiency virus (HIV) infection, which results in AIDS, is a relatively new infection in the human population, it has quickly risen to the foremost health problem in the world. HIV/AIDS is now the leading cause of death in sub-Saharan Africa, and is the fourth biggest killer worldwide. At the end of 2001, it was estimated that 40 million people were living with HIV infection world wide. The Centers for Disease Control (CDC) estimates that nearly 800,000 people are living with AIDS in the US, and 40,000 new cases diagnosed each year. While better treatment methods are now known to prolong the life of patients with HIV infection, there is still no cure.

Modern anti-HIV drugs target several different stages of the HIV life cycle, and several of the enzymes that HIV requires to replicate and survive. Some of the commonly used anti-HIV drugs include nucleoside reverse transcriptase inhibitors such as AZT, ddI, ddC, d4T, 3TC, and abacavir; nucleotide reverse transcriptase inhibitors such as tenofovir; non-nucleoside reverse transcriptase inhibitors such as nevirapine, efavirenz and delavirdine; protease inhibitors such as saquinavir, ritonavir, indinavir, nelfinavir, amprinavir, lopinavir and atazanavir; and fusion inhibitors such as enfuvirtide. However, in many HIV infected patients, none of these antiviral drugs, alone or in combination, is effective to prevent the progression of chronic infection or treat acute AIDS. The high mutation rate of the HIV virus and associated emergence of HIV strains resistant to drugs is one large factor that results in the inability to effectively treat HIV infection. New and better treatments are required for HIV infection.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods of use of cupredoxins and /or cytochrome c_{551} and their use to inhibit viral infections in mammalian cells, and in particular HIV infection. Specifically, the present invention relates to compositions comprising the peptides *Pseudomonas aeruginosa* azurin and/or cytochrome c_{551}, and/or variants and/or derivatives thereof, and methods to use these compositions and peptides to inhibit the growth of HIV-1 infection in mammalian cells and patients. The invention also relates to variants and derivatives of cupredoxin and cytochrome c_{551} that retain the ability to inhibit the growth of viral infection, and in particular infection by the HIV.

One aspect of the invention is an isolated peptide that is a variant, derivative or structural equivalent of a cupredoxin or cytochrome c_{551} and that can inhibit the growth of HIV-1 infection in mammalian cells. In some embodiments, the cupredoxin is azurin, pseudoazurin, plastocyanin, rusticyanin, Laz or auracyanin, and specifically azurin or Laz. In other embodiments, the cupredoxin is from *Pseudomonas aeruginosa, Alcaligenes faecalis, Achromobacter xylosoxidan, Bordetella bronchiseptica, Methylomonas sp., Neisseria meningitidis, Neisseria gonorrhea, Pseudomonas fluorescens, Pseudomonas chlororaphis, Xylella fastidiosa* or *Vibrio parahaemolyticus*, and specifically *Pseudomonas aeruginosa* or *Neisseria gonorrhea.* In some embodiments, the peptide is part of one of SEQ ID NOS: 1-17. In some embodiments, the peptide has at least about 90% amino acid sequence identity to a sequence in SEQ ID NOS: 1-17.

In some embodiments, the isolated peptide may be a truncation of cupredoxin or cytochrome c_{551}. The peptide may be more than about 10 residues and not more than about 100 residues. In some embodiments, the isolated peptide comprises a region of azurin that is residues 36-128, residues 36-88 or residues 88-113. In other embodiments, the isolated peptide consists of a region of azurin that is residues 36-128, residues 36-88 or residues 88-113. In other embodiments, the peptide comprises equivalent residues of a cupredoxin as a region of azurin that is residues 36-128, residues 36-88 and residues 88-113.

Another aspect of the invention is a composition which comprises at least one cupredoxin, cytochrome c_{551}, or isolated peptide that is a variant, derivative or structural equivalent of a cupredoxin or cytochrome c_{551} and that can inhibit the growth of HIV-1 infection in mammalian cells, in a pharmaceutical composition. In some embodiments, the pharmaceutical composition is formulated for intravenous administration. In other embodiments, the cupredoxin is from *Pseudomonas aeruginosa, Alcaligenes faecalis, Achromobacter xylosoxidan, Bordetella bronchiseptica, Methylomonas sp., Neisseria* meningitidis, *Neisseria gonorrhea, Pseudomonas fluorescens, Pseudomonas chlororaphis, Xylella fastidiosa* or *Vibrio parahaemolyticus,* and specifically *Pseudomonas aeruginosa* or *Neisseria gonorrhea.* In some embodiments, the cupredoxin or cytochrome c_{551} is selected SEQ ID NOS: 1-17.

Another aspect of the invention is a method to treat a patient infected with a virus or bacteria, comprising administering to the patient a therapeutically effective amount of a composition which comprises at least one cupredoxin, cytochrome c551, or isolated peptide

that is a variant, derivative or structural equivalent of a cupredoxin or cytochrome c551 and that can inhibit the growth of HIV-1 infection in mammalian cells, in a pharmaceutical composition. The patient may be infected with HIV-1, Herpes simplex virus (HSV), Ebola virus, cytomeglovirus (CMV), parainfluenza viruses types A, B and C, hepatitis virus A, B, C, and G, the delta hepatitis virus (HDV), mumps virus, measles virus, respiratory syncytial virus, bunyvirus, arena virus, Dhori virus, poliovirus, rubella virus, dengue virus; SIV or Mycobacterium tuberculosis, and specifically HIV-1. In some embodiments, the patient is human. In some embodiments, the composition is administered by intravenous injection, intramuscular injection, subcutaneous injection, inhalation, topical administration, transdermal patch, suppository or oral, and specifically by intravenous injection. The composition may be administered within 0 minutes to 1 week of the administration of another anti-HIV drug., specifically at about the same time as another anti viral, anti-bacterial and/or anti-HIV drug.

Another aspect of the invention is a composition comprising at least two of an isolated cupredoxin, a cytochrome c551, and variant, derivative or structural equivalent of a cupredoxin or a cytochrome c551. In some embodiments, the composition is in a pharmaceutical composition.

Another aspect of the invention is a kit comprising the composition comprising at least one cupredoxin, cytochrome c551, or isolated peptide that is a variant, derivative or structural equivalent of a cupredoxin or cytochrome c551 and that can inhibit the growth of HIV-1 infection in mammalian cells in a pharmaceutical composition in a vial. In some embodiments, the kit is designed for intravenous administration.

Another aspect of the invention is a method to study viral and bacterial infection in mammalian cells, comprising the steps of contacting the cells with a cupredoxin or cytochrome c551, or variant, derivative or structural equivalent thereof; contacting the cells with a bacteria or virus; and measuring the growth of infection of the virus or bacteria. In some embodiments, the step of contacting the cells with a cupredoxin or cytochrome c551, or variant, derivative or structural equivalent thereof occurs before the step of contacting the cells with a bacteria or virus. In other embodiments, the step of contacting the cells with a cupredoxin or cytochrome c551, or variant, derivative or structural equivalent thereof occurs after the step of contacting the cells with a bacteria or virus. In some embodiments, the cells are human cells. In other embodiments, the cells are lymphoma cells or peripheral blood

mononuclear cells. In other embodiments, the virus or bacteria is HIV-1, Herpes simplex virus (HSV), Ebola virus, cytomeglovirus (CMV), parainfluenza viruses types A, B and C, hepatitis virus A, B, C, and G, the delta hepatitis virus (HDV), mumps virus, measles virus, respiratory syncytial virus, bunyvirus, arena virus, Dhori virus, poliovirus, rubella virus, dengue virus, SIV and Mycobacterium tuberculosis, specifically HIV-1.

Another aspect of the invention is an expression vector, which encodes peptide that is a variant, derivative or structural equivalent of a cupredoxin or cytochrome c551 and that can inhibit the growth of HIV-1 infection in mammalian cells.

These and other aspects, advantages, and features of the invention will become apparent from the following figures and detailed description of the specific embodiments.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1. Amino acid sequence of azurin from Pseudomonas aeruginosa.

SEQ ID NO: 2. Amino acid sequence of cytochrome c551 from Pseudomonas aeruginosa.

SEQ ID NO: 3. Amino acid sequence of plastocyanin from Phormidium laminosum.

SEQ ID NO: 4. Amino acid sequence of rusticyanin from Thiobacillus ferrooxidans.

SEQ ID NO: 5. Amino acid sequence of pseudoazurin from Achromobacter cycloclastes.

SEQ ID NO: 6. Amino acid sequence of azurin from Alcaligenes faecalis.

SEQ ID NO: 7. Amino acid sequence of azurin from Achromobacter xylosoxidans ssp.denitrificans I.

SEQ ID NO: 8. Amino acid sequence of azurin from Bordetella bronchiseptica.

SEQ ID NO: 9. Amino acid sequence of azurin from Methylomonas sp. J.

SEQ ID NO: 10. Amino acid sequence of azurin from Neisseria meningitidis Z2491.

SEQ ID NO: 11. Amino acid sequence of azurin from Pseudomonas fluorescen.

SEQ ID NO: 12. Amino acid sequence of azurin from Pseudomonas chlororaphis.

SEQ ID NO: 13. Amino acid sequence of azurin from Xylella fastidiosa 9a5c.

SEQ ID NO: 14. Amino acid sequence of stellacyanin from Cucumis sativus

SEQ ID NO: 15. Amino acid sequence of auracyanin A from Chloroflexus aurantiacus

SEQ ID NO: 16. Amino acid sequence of auracyanin B from Chloroflexus aurantiacus

SEQ ID NO: 17. Amino acid sequence of cucumber basic protein from Cucumis sativus.

SEQ ID NO: 18 is the amino acid sequence of the H.8 region from Laz from Neisseria gonorrhoeae F62.

SEQ ID NO: 19 is the amino acid sequence of Laz from Neisseria gonorrhoeae F62.

SEQ ID NO: 20 is the forward primer to PCR amplify the Laz-encoding gene (laz) of Neisseria gonorrhoeae.

SEQ ID NO: 21 is the reverse primer to PCR amplify the Laz-encoding gene (laz) of Neisseria gonorrhoeae.

SEQ ID NO: 22 is the forward primer to PCR amplify a 3.1 kb fragment of pUC18-laz .

SEQ ID NO: 23 is the reverse primer to PCR amplify a 3.1 kb fragment of puc18-laz.

SEQ ID NO: 24 is the forward primer to PCR amplify a 0.4 kb fragment of pUC19-paz.

SEQ ID NO: 25 is the reverse primer to PCR amplify a 0.4 kb fragment of pUC19-paz.

SEQ ID NO: 26 is the forward primer for pGST-azu 36-128.

SEQ ID NO: 27 is the reverse primer for pGST-azu 36-128.

SEQ ID NO: 28 is the forward primer for pGST-azu 36-89.

SEQ ID NO: 29 is the reverse primer for pGST-azu 36-89.

SEQ ID NO: 30 is the forward primer for pGST-azu 88-113.

SEQ ID NO: 31 is the reverse primer for pGST-azu 88-113.

SEQ ID NO: 32 is an oligonucleotide for site directed mutagenesis for the preparation of pGST-azu 88-113.

SEQ ID NO: 33 is an oligonucleotide for site directed mutagenesis for the preparation of pGST-azu 88-113.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Figure 1 depicts the inhibition of HIV-1 viral growth by azurin, H.8-azurin (H.8-Az) and Laz. These three proteins were incubated at different concentrations with PBMC followed by addition of the three subtypes of HIV-1. After 2 h incubation, the virus was removed but the proteins added back as described in Example 3. Suppression of virus growth was determined by ELISA assays of p24.

Figure 2. Figure 2 depicts surface plasmon resonance binding curves depicting the binding patterns of cupredoxins with CD4 and HIV-1 gp120. (A) SPR titration curves showing novel and specific binding of azurin, and GST-Azu 36-128 (shown as an inset) with immobilized CD4 on carboxymethyldextran coated gold sensor chips (CD4-CM5). HIV-1 gp120, HIV-1 gag, and HIV-1 nef served as the positive and negative controls respectively. Relative binding affinities were determined via fitting the data to $R_{eq} = R_{max}/(1+(K_d/C))$ with the curve fits connecting the data points above. The CD4 binding K_d values are: 36.9 ± 2.0 nM (azurin), 0.34 ± 0.04 nM (GST-Azu 36-128), and 48.1 ± 3.1 nM (HIV-1 gp120). (B) The binding titrations when immobilized azurin (Az-CM5) is in contact with HIV proteins. Due to large nonspecific binding to the bare Au-CM5 chip, CM5 was added as an eluent to the running buffer (1 mg/ml CM5 to HBS-EP buffer). Curve fits gave Kd's of 25.1 ± 3.1 nM (CD4), and 8.9 ± 0.8 nM (HIV-1 gp120). (C) SPR curves for the binding of ICAMs (ICAM-1, ICAM-2, ICAM-3 and NCAM, inset) with immobilized azurin were determined under similar conditions as for experiments in part (B). The selective recognition of azurin with ICAM-3, but not with ICAM-1 or ICAM-2, is notable and the binding strength was 19.5 ± 5.4 nM. The Kd for NCAM binding with azurin, as shown in the inset, was 20 ± 5.0 nM. (D) SPR binding competition studies with CD4 immobilized on CM5 sensor chips. Azurin + HIV-1 gp120 solutions were added at different azurin concentrations (0-4500 nM, [HIV-1 gp120] is 242 nM) to the sensor surface and the data were plotted as a ratio of resonances, % total response [R_{eq} (azurin+HIV-1 gp120)/(R_{eq}/(HIV-1 gp120))]. GST-Azu 36-128 was titrated with HIV-1 gp120 to immobilized CD4 and analyzed in a similar manner. Competition data suggests 1:1 stoichiometry of binding between azurin and GST-Azu 36-128 with immobilized CD4.

Figure 3. Figure 3 depicts surface plasmon resonance binding titrations depicting the interactions of azurin, and GST-Azurin fusions with DC-SIGN. (A) Concentration dependent binding of azurin, ICAM-3, and GST-Azu 36-89 with DC-SIGN were determined via

injection of various concentrations of the proteins (0 - 100 nM) over a DC-SIGN modified CM5 sensor surface and the extent of binding was evaluated as a function of the equilibrium resonance response value measured in resonance units (RU). (B) The binding titration curve of GST-Azu 88-113 with DC-SIGN using the same sensor chip and protocol as described for azurin in part A. The positive interaction of GST-Azu 88-113 with DC-SIGN suggests its potential role as the recognition sequence for azurin. The binding affinities (Kd) for azurin, ICAM-3 and GST-Azu 88-113 were determined by fitting the data to Req = Rmax/(1+(Kd/C)) and the curve fits connect the data points in these plots. The extrapolated Kd values are 0.83 ± 0.05 nM (azurin), 0.93 ± 0.39 nM (ICAM-3), and 5.98 ± 1.13 nM (GST-Azu 88-113).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein, the term "cell" includes both the singular or the plural of the term, unless specifically described as a "single cell."

As used herein, the terms "polypeptide," "peptide," and "protein" are used interchangeably to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid. The terms also apply to naturally occurring amino acid polymers. The terms "polypeptide," "peptide," and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. It will be appreciated that polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination and they may be circular (with or without branching), generally as a result of post-translation events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods as well.

As used herein, the term "pathological condition" includes anatomic and physiological deviations from the normal that constitute an impairment of the normal state of the living animal or one of its parts, that interrupts or modifies the performance of the bodily functions, and is a response to various factors (as malnutrition, industrial hazards, or climate),

to specific infective agents (as worms, parasitic protozoa, bacteria, or viruses), to inherent defects of the organism (as genetic anomalies), or to combinations of these factors.

As used herein, the term "condition" includes anatomic and physiological deviations from the normal that constitute an impairment of the normal state of the living animal or one of its parts, that interrupts or modifies the performance of the bodily functions.

As used herein, the term "suffering from" includes presently exhibiting the symptoms of a pathological condition, having a pathological condition even without observable symptoms, in recovery from a pathological condition, or recovered from a pathological condition.

A used herein, the term "treatment" includes preventing, lowering, stopping, or reversing the progression or severity of the condition or symptoms associated with a condition being treated. As such, the term "treatment" includes medical, therapeutic, and/or prophylactic administration, as appropriate.

As used herein, the term " inhibit the growth of HIV infection" means any means by which HIV infection is decreased, or prevented from increasing in the human body. These means can include, but are not limited to, inhibition of replication of the HIV genome, inhibition of synthesis and/or assembly of the HIV coat proteins, and inhibition of HIV entry into uninfected cells. This definition includes any the method of action of any of the currently known HIV therapies. One method to determine if the growth of HIV infection is inhibited in found in Example 3.

A "therapeutically effective amount" is an amount effective to prevent or slow the development of, or to partially or totally alleviate the existing symptoms in a particular condition for which the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

The term "substantially pure", when used to modify the term a polypeptide or other compound, as used herein, refers to a polypeptide or compound, for example, a polypeptide isolated from the growth medium, in a form substantially free of, or unadulterated by, active inhibitory agents. The term "substantially pure" refers to a compound in an amount of at least about 75%, by dry weight, of isolated fraction, or "75% substantially pure." More specifically, the term "substantially pure" refers to a compound of at least about 85%, by dry weight, active compound, or "85% substantially pure." Most specifically, the term "substantially pure" refers to a compound of at least about 95%, by dry weight, active

compound, or "95% substantially pure." The substantially pure cupredoxin or cytochrome c551 or a variant or derivative thereof can be used in combination with one or more other substantially pure compounds, or another isolated cupredoxin or cytochrome c551.

The phrases "isolated," "purified" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany the material as it is found in its native state. Thus, isolated peptides in accordance with the invention preferably do not contain materials normally associated with the peptides in their in situ environment. An "isolated" region refers to a region that does not include the whole sequence of the polypeptide from which the region was derived. An "isolated" nucleic acid, protein, or respective fragment thereof has been substantially removed from its in vivo environment so that it may be manipulated by the skilled artisan, such as but not limited to nucleotide sequencing, restriction digestion, site-directed mutagenesis, and subcloning into expression vectors for a nucleic acid fragment as well as obtaining the protein or protein fragment in substantially pure quantities.

The term "variant" as used herein with respect to a peptide, refers to amino acid sequence variants which may have amino acids replaced, deleted, or inserted as compared to the wild-type polypeptide. Variants may be truncations of the wild-type peptide. Thus, a variant peptide may be made by manipulation of genes encoding the polypeptide. A variant may be made by altering the basic composition or characteristics of the polypeptide, but not at least some of its fundamental activities. For example, a "variant" of azurin can be a mutated azurin that retains its ability to inhibit the growth of HIV infection in mammalian cells. In some cases, a variant peptide is synthesized with non-natural amino acids, such as ε-(3,5-dinitrobenzoyl)-Lys residues. (Ghadiri & Fernholz, J. Am. Chem. Soc., 112:9633-9635 (1990)) In some embodiments, the variant has not more than 20 amino acids replaced, deleted or inserted compared to wild-type peptide. In some embodiments, the variant has not more than 15 amino acids replaced, deleted or inserted compared to wild-type peptide. In some embodiments, the variant has not more than 10 amino acids replaced, deleted or inserted compared to wild-type peptide. In some embodiments, the variant has not more than 6 amino acids replaced, deleted or inserted compared to wild-type peptide. In some embodiments, the variant has not more than 5 amino acids replaced, deleted or inserted compared to wild-type peptide. In some embodiments, the variant has not more than 3 amino acids replaced, deleted or inserted compared to wild-type peptide.

The term "amino acid," as used herein, means an amino acid moiety that comprises any naturally-occurring or non-naturally occurring or synthetic amino acid residue, i.e., any moiety comprising at least one carboxyl and at least one amino residue directly linked by one, two three or more carbon atoms, typically one (α) carbon atom.

The term "derivative" as used herein with respect to a peptide refers to a peptide that is derived from the subject peptide. A derivation includes chemical modifications of the peptide such that the peptide still retains some of its fundamental activities. For example, a "derivative" of azurin can, for example, be a chemically modified azurin that retains its ability to inhibit the growth of HIV infection in mammalian cells. Chemical modifications of interest include, but are not limited to, amidation, acetylation, sulfation, polyethylene glycol (PEG) modification, phosphorylation or glycosylation of the peptide. In addition, a derivative peptide maybe a fusion of a polypeptide or fragment thereof to a chemical compound, such as but not limited to, another peptide, drug molecule or other therapeutic or pharmaceutical agent or a detectable probe.

The term "percent (%) amino acid sequence identity" is defined as the percentage of amino acid residues in a polypeptide that are identical with amino acid residues in a candidate sequence when the two sequences are aligned. To determine % amino acid identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum % sequence identity; conservative substitutions are not considered as part of the sequence identity. Amino acid sequence alignment procedures to determine percent identity are well known to those of skill in the art. Often publicly available computer software such as BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR) software is used to align peptide sequences. In a specific embodiment, Blastp (available from the National Center for Biotechnology Information, Bethesda MD) is used using the default parameters of long complexity filter, expect 10, word size 3, existence 11 and extension 1.

When amino acid sequences are aligned, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) can be calculated as:

% amino acid sequence identity = X/Y*100

where

X is the number of amino acid residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B and

Y is the total number of amino acid residues in B.

If the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. When comparing longer sequences to shorter sequences, the shorter sequence will be the "B" sequence. For example, when comparing truncated peptides to the corresponding wild-type polypeptide, the truncated peptide will be the "B" sequence.

General

The present invention provides compositions comprising cupredoxin and/or cytochrome c_{551} and methods to inhibit viral and bacterial infection in mammalian cells and in mammalian patients. The present invention specifically relates to compositions comprising cupredoxin and/or cytochrome c_{551}, and their use in inhibiting the growth of Human Immunodeficiency Virus (HIV) infection. The invention also relates to variants, derivatives and structural equivalents of cupredoxin and cytochrome c_{551} that retain the ability to inhibit the growth of viral infection, and in particular infection by the HIV, and compositions comprising the same. Most particularly, the invention provides compositions comprising *Pseudomonas aeruginosa* azurin and cytochrome c_{551}, variants, derivatives and structural equivalents of azurin and cytochrome c_{551}, and their use to treat patients with viral or bacterial infection, and specifically HIV-1 infection, or prevent infection in those at risk thereof. Finally, the invention provides methods to study infection of mammalian cells by viruses or bacteria by contacting the cells with cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof before or after the cells are infected, and measuring the growth of infection.

Previously, it was know that two redox proteins elaborated by *Pseudomonas aerugisnosa*, the cupredoxin azurin and cytochrome c_{551} (cyt c_{551}), both selectively enter J774 cells and show significant cytotoxic activity towards these cancerous cells but not normal cells. (Zaborina et al., Microbiology 146: 2521-2530 (2000).) Azurin can also selectively enter human melanoma UISO-Mel-2 or human breast cancer MCF-7 cells. (Yamada et al., PNAS 99:14098-14103 (2002); Punj et al., Oncogene 23:2367-2378 (2004).) Azurin from P. aeruginosa preferentially enters J774 murine reticulum cell sarcoma cells, forms a complex

with and stabilizes the tumor suppressor protein p53, enhances the intracellular concentration of p53, and induces apoptosis. (Yamada et al., Infection and Immunity 70:7054-7062 (2002).)

Surprisingly, it is now known that azurin can induce about a 90% suppression of growth of HIV-1 in peripheral blood mononuclear cell (PBMC) cultures. See, Example 3. Azurin is now known to inhibit the growth of three strains of HIV-1, Bal (the most predominant clade B circulating in the US and Western Europe), a clade B African isolate RW/92/008/RE1, and a clade C Indian isolate IN/2167 D15. See, Example 3. Additionally, a cupredoxin-like protein from Neisseria, Laz, is nowalso known to inhibit the growth of these three HIV-1 strains, as well as a fusion of the H.8 region of the Laz protein with P. aeruginosa azurin. See, Example 3. Finally, it is now known that M44KM64E mutant of azurin and cytochrome c551 from P. aeruginosa can inhibit HIV infection in HIV-infected human blood lymphocytes. See, Example 1.

It is now known that azurin from Pseudomonas aeruginosa has a structural similarly with ICAM-1 (found in HIV-1 particles and known to enhance HIV-1 infectivity), ICAM-2 , and CD4 (the primary cell surface receptor for HIV-1). See, Example 2. Surface plasmon resonance experiments have now revealed that in vitro azurin shows significant binding to the cell surface receptor CD4, and surprisingly a higher affinity for CD4 than the HIV-1 surface ligand gp120. See, Example 4. Further, GST-Azu 36-128, a glutathione S- transferase (GST) fusion to azurin amino acids 36-128, showed stronger binding to CD4 than azurin itself, while GST-Azu 88-113 showed no binding to CD4, suggesting that only a part of the azurin protein is responsible for binding to CD4. See, Example 4. Gp120 showed a somewhat stronger binding to azurin than CD4, indicating that azurin binds both gp120 and CD4. Further, ICAM-3 and NCAM showed strong binding the azurin in vitro in surface plasmon resonance assays. See, Example 5. Finally, azurin is now known to bind another receptor of HIV-1, dendritic cell-specific adhesion receptor (DC-SIGN). See, Example 7. Other proteins implicated in HIV-1 entry into host cells, such as gp41, did not show any binding to azurin. See, Example 4.

Through competition experiments, it is now known that azurin can interfere with gp120 binding with its cognate receptor CD4 in vitro. Specifically, GST-Azu 36-128 showed significant ability to compete with gp120 for CD4 binding, and GST-Azu 88-113 showed little ability to compete. See, Example 6. Laz, an azurin like protein from Neisseria, is now

know to inhibit the growth of HIV-1 infection in PBMC culture by 73% if added before HIV-1 infection, and very little if added after HIV-1 infection. See, Example 8. While not limiting the operation of the invention to any one mechanism, it now appears that azurin may inhibit the growth of HIV-1 infection in peripheral blood mononuclear cells by interfering with the interaction between the HIV and cell surface receptors such as ICAMs, CD4 and DC-SIGN, to prevent HIV from entering the cell and infecting it.

Accordingly, due to the high degree of structural similarity between cupredoxins, that other cupredoxins will as well inhibit the growth of HIV-1 infection in mammalian blood cells. It follows that in addition to HIV infection, cupredoxins also will suppress other infections of viruses that bind to cell surface receptors similar in structure to the ICAMs, DC-SIGN and CD4. For example, DC-SIGN is also a binding receptor of other viral and bacterial pathogens, including hepatitis C virus (HCV), Ebola virus, cytomeglovirus (CMV) *and Mycobacterium tuberculosis*. (Wang *et al.*, Chin. Med. J. 117:1395-1400 (2004))

Compositions of the Invention

The invention provides for peptides that are variants, derivatives or structural equivalents of cupredoxin or *Pseudomonas aeruginosa* cytochrome c_{551}. In some embodiments, the peptide is substantially pure. In other embodiments, the peptide is in a composition that comprises or consists essentially of the peptide. In other embodiments, the peptide is isolated. In some embodiments, the peptide is less that a full length cupredoxin or cytochrome c_{551}, and retains some of the functional characteristics of the cupredoxin or cytochrome c_{551}. In some embodiments, the peptide retains the ability to inhibit the growth of viral or bacterial infection, and more specifically HIV-1 infection in peripheral blood mononuclear cells. In a specific embodiment, the cytochrome c_{551} is SEQ ID NO: 2. In another specific embodiment, the peptide does not raise an immune response in a mammal, and more specifically a human. The invention also provides compositions comprising at least one peptide that is a cupredoxin, *Pseudomonas aeruginosa* cytochrome c_{551}, or variant, derivative or structural equivalent of a cupredoxin or cytochrome c_{551}. The invention also provides compositions comprising at least one peptide that is a cupredoxin, *Pseudomonas aeruginosa* cytochrome c_{551}, or variant, derivative or structural equivalent of a cupredoxin or cytochrome c_{551} in a pharmaceutical composition.

Because of the high structural homology between the cupredoxins, it is contemplated that other cupredoxins will have the same anti-HIV activity as *Pseudomonas aeruginosa* azurin with regards to inhibition of growth of HIV-1 infection. In some embodiments, the cupredoxin is, but is not limited to, azurin, pseudoazurin, plastocyanin, rusticyanin or auracyanin. In particularly specific embodiments, the azurin is derived from *Pseudomonas aeruginosa, Alcaligenes faecalis, Achromobacter xylosoxidans ssp.denitrificans I, Bordetella bronchiseptica, Methylomonas sp., Neisseria meningitidis Z2491, Neisseria gonorrhea, Pseudomonas fluorescens, Pseudomonas chlororaphis, Xylella fastidiosa 9a5* or *Vibrio parahaemolyticus.* In a very specific embodiment, the azurin is from *Pseudomonas aeruginosa.* In other specific embodiments, the cupredoxin comprises an amino acid sequence that is SEQ ID NO: 1, 3-17. In other specific embodiments, the cupredoxin is the Laz protein from *Neisseria meningitidis* or *Neisseria gonorrhea.*

The invention provides for amino acid sequence variants of a cupredoxin or cytochrome c_{551} which have amino acids replaced, deleted, or inserted as compared to the wild-type polypeptide. Variants of the invention may be truncations of the wild-type polypeptide. In some embodiments, the composition comprises a peptide that consists of a region of a cupredoxin or cytochrome c_{551} that is less that the full length wild-type polypeptide. In some embodiments, the composition comprises a peptide that consists of more than about 10 residues, more than about 15 residues or more than about 20 residues of a truncated cupredoxin or cytochrome c_{551}. In some embodiments, the composition comprises a peptide that consists of not more than about 100 residues, not more than about 50 residues, not more than about 40 residues or not more than about 30 residues of a truncated cupredoxin or cytochrome c_{551}. In some embodiments, composition comprises a peptide to which a cupredoxin or cytochrome c_{551}, and more specifically to SEQ ID NOS: 1-17 has at least about 90% amino acid sequence identity, at least about 95% amino acid sequence identity or at least about 99% amino acid sequence identity.

In specific embodiments, the variant of cupredoxin comprises *P. aeruginosa* azurin residues 36-128, azurin residues 36-88, or azurin residues 88-113. In other embodiments, the variant of cupredoxin consists of *P. aeruginosa* azurin residues 36-128, azurin residues 36-88, or azurin residues 88-113. In other specific embodiments, the variant consists of the equivalent residues of a cupredoxin other that azurin. It is also contemplated that other cupredoxin variants can be designed that have a similar activity to azurin residues 36-128,

azurin residues 36-88, or azurin residues 88-113. To do this, the subject cupredoxin amino acid sequence will be aligned to the *Pseudomonas aeruginosa* azurin sequence using BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR), the relevant residues located on the *P. aeruginosa* azurin amino acid sequence, and the equivalent residues found on the subject cupredoxin sequence, and the equivalent truncated peptide thus designed.

The variants also include peptides made with synthetic amino acids not naturally occurring. For example, non-naturally occurring amino acids may be integrated into the variant peptide to extend or optimize the half-life of the composition in the bloodstream. Such variants include, but are not limited to, D,L-peptides (diastereomer), (Futaki *et al.*, J. Biol. Chem. 276(8):5836-40 (2001); Papo *et al.*, Cancer Res. 64(16):5779-86 (2004); Miller *et al,* Biochem. Pharmacol. 36(1):169-76, (1987)); peptides containing unusual amino acids (Lee *et al.*, J. Pept. Res. 63(2):69-84 (2004)), and olefin-containing non-natural amino acid followed by hydrocarbon stapling (Schafmeister *et al.*, J. Am. Chem. Soc. 122:5891-5892 (2000); Walenski *et al.*, Science 305:1466-1470 (2004)) and peptides conprising ε-(3,5-dinitrobenzoyl)-Lys residues.

In other embodiments, the peptide of the invention is a derivative of a cupredoxin or cytochrome c_{551}. The derivatives of cupredoxin/or cytochrome c_{551} are chemical modifications of the peptide such that the peptide still retains some of its fundamental activities. For example, a "derivative" of azurin can be a chemically modified azurin that retains its ability to inhibit the growth of HIV infection in mammalian cells. Chemical modifications of interest include, but are not limited to, amidation, acetylation, sulfation, polyethylene glycol (PEG) modification, phosphorylation and glycosylation of the peptide. In addition, a derivative peptide maybe a fusion of a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof to a chemical compound, such as but not limited to, another peptide, drug molecule or other therapeutic or pharmaceutical agent or a detectable probe. Derivatives of interest include chemical modifications by which the half-life in the bloodstream of the peptides and compositions of the invention can be extended or optimized, such as by several methods well known to those in the art, including but not limited to, circularized peptides (Monk *et al.*, BioDrugs 19(4):261-78, (2005); DeFreest *et al.*, J. Pept. Res. 63(5):409-19 (2004)), N- and C- terminal modifications (Labrie *et al.*, Clin. Invest. Med. 13(5):275-8, (1990)), and olefin-containing non-natural amino acid followed by

hydrocarbon stapling (Schafmeister *et al.*, J. Am. Chem. Soc. 122:5891-5892 (2000); Walenski *et al.*, Science 305:1466-1470 (2004)).

It is contemplated that the peptides of the composition of invention may be a variant, derivative and/or structural equivalent of a cupredoxin or cytochrome c551. For example, the peptides may be a truncation of azurin that has been PEGylated, thus making it both a variant and a derivative. In one embodiment, the peptides of the invention are synthesized with α,α-disubstituted non-natural amino acids containing olefin-bearing tethers, followed by an all-hydrocarbon "staple" by ruthenium catalyzed olefin metathesis. (Scharmeister *et al.*, J. Am. Chem. Soc. 122:5891-5892 (2000); Walensky *et al.*, Science 305:1466-1470 (2004)) Additionally, peptides that are structural equivalents of azurin may be fused to other peptides, thus making a peptide that is both a structural equivalent and a derivative. These examples are merely to illustrate and not to limit the invention. Variants, derivatives or structural equivalents of cupredoxin or cytochrome c_{551} may or may not bind copper.

In another embodiment, the peptide is a structural equivalent of a cupredoxin or cytochrome c_{551}. Examples of studies that determine significant structural homology between cupredoxins and other proteins include Toth *et al.* (*Developmental Cell* 1:82-92 (2001)). Specifically, significant structural homology between a cupredoxin and the structural equivalent is determined by using the VAST algorithm (Gibrat *et al.,* Curr Opin Struct Biol 6:377-385 (1996); .Madej *et al.,* Proteins 23:356-3690 (1995)). In specific embodiments, the VAST p value from a structural comparison of a cupredoxin to the structural equivalent is less than about 10^{-3}, less than about 10^{-5}, or less than about 10^{-7}. In other embodiments, significant structural homology between a cupredoxin and the structural equivalent is determined by using the DALI algorithm (Holm & Sander, J. Mol. Biol. 233:123-138 (1993)). In specific embodiments, the DALI Z score for a pairwise structural comparison is at least about 3.5, at least about 7.0, or at least about 10.0.

In some embodiments, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof has some of the functional characteristics of the *P. aeruginosa* azurin or cytochrome c_{551}. In a specific embodiment, the cupredoxin or cytochrome c_{551} inhibits the growth of viral or bacterial infection, and specifically HIV infection in mammalian cells, more specifically in peripheral blood mononuclear cells infected with HIV. The invention also provides for the variants, derivatives and structural equivalents of cupredoxin and cytochrome c_{551} that retain the ability to inhibit the growth of viral or

bacterial infection, and specifically HIV infection in mammalian cells. The growth of HIV-1 infection in the cells may be determined by measuring the change in the production of HIV-1 p24 antigen in the cell culture supernatant by a commercial p24 enzyme immunoassay (PerkinElmer Life Sciences, Inc., Wellseley, Mass.). Inhibition of a growth of infection is any decrease or lessening of the rate of increase of that infection that is statistically signification as compared to control treatments.

Because it is now known that cupredoxins and cytochrome c_{551} can limit the growth of viral or bacterial infection, and specifically infection in HIV-infected mammalian cells, it is now possible to design variants and derivatives of cupredoxins that retain this anti-viral or anti-bacterial, and specifically anti-HIV activity. Such variants and derivatives can be made by, for example, creating a "library" of various variants and derivatives of cupredoxins and cytochrome c_{551}, and then testing each for anti-viral or anti-bacterial, and specifically anti-HIV activity using one of many methods known in the art, such the exemplary method in Example 3. It is contemplated that the resulting variants and derivatives of cupredoxins with anti-viral or anti-bacterial, and specifically anti-HIV activity can be used in the methods of the invention, in place of or in addition to cupredoxins or cytochrome c_{551}.

In some specific embodiments, the cupredoxin or cytochrome, or variant, derivative or structural equivalent thereof binds to CD4 with a binding constant that is statistically different a non-binding control protein. A peptide can be tested for this activity by using surface plasmon resonance analysis as described in Example 4. Other methods to determine whether one protein binds to another are well known in the art and may be used as well.

In some specific embodiments, the cupredoxin or cytochrome, or variant, derivative or structural equivalent thereof binds to gp120 with a binding constant that is statistically different a non-binding control protein. A peptide can be tested for this activity by using surface plasmon resonance analysis as described in Example 4. Other methods to determine whether one protein binds to another are well known in the art and may be used as well.

In some specific embodiments, the cupredoxin or cytochrome, or variant, derivative or structural equivalent thereof binds to ICAM3 with a binding constant that is statistically different a non-binding control protein. A peptide can be tested for this activity by using surface plasmon resonance analysis as described in Examples 4 and 5. Other methods to determine whether one protein binds to another are well known in the art and may be used as well.

In some specific embodiments, the cupredoxin or cytochrome, or variant, derivative or structural equivalent thereof binds to DC-SIGN with a binding constant that is statistically different a non-binding control protein. A peptide can be tested for this activity by using surface plasmon resonance analysis as described in Examples 4 and 5. Other methods to determine whether one protein binds to another are well known in the art and may be used as well.

In some specific embodiments, the peptide of the invention induces apoptosis in a mammalian cancer cell, more specifically a J774 cell. The ability of a cupredoxin or other polypeptide to induce apoptosis may be observed by mitosensor ApoAlert confocal microscopy using a MITOSENSOR™ APOLERT™ Mitochondrial Membrane Sensor kit (Clontech Laboratories, Inc., Palo Alto, California, U.S.A.), by measuring caspase-8, caspase-9 and caspase-3 activity using the method described in Zou *et al.* (*J. Biol. Chem.* 274: 11549-11556 (1999)), and by detecting apoptosis-induced nuclear DNA fragmentation using, for example, the APOLERT™ DNA fragmentation kit (Clontech Laboratories, Inc., Palo Alto, California, U.S.A.).

In another specific embodiment, the peptide of the invention induces cellular growth arrest in a mammalian cancer cell, more specifically a J774 cell. Cellular growth arrest can be determined by measuring the extent of inhibition of cell cycle progression, such as by the method found in Yamada *et al.* (*PNAS* 101:4770-4775 (2004)). In another specific embodiment, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof inhibits cell cycle progression in a mammalian cancer cell, more specifically a J774 cell.

Cupredoxins

These small blue copper proteins (cupredoxins) are electron transfer proteins (10-20 kDa) that participate in bacterial electron transfer chains or are of unknown function. The copper ion is solely bound by the protein matrix. A special distorted trigonal planar arrangement to two histidine and one cysteine ligands around the copper gives rise to very peculiar electronic properties of the metal site and an intense blue color. A number of cupredoxins have been crystallographically characterized at medium to high resolution.

The cupredoxins in general have a low sequence homology but high structural homology. (Gough & Clothia, *Structure* 12:917-925 (2004); De Rienzo *et al., Protein Science* 9:1439-1454 (2000)). For example, the amino acid sequence of azurin is 31%

identical to that of auracyanin B, 16.3% to that of rusticyanin, 20.3 % to that of plastocyanin, and 17.3% to that of pseudoazurin. *See*, Table 1. However, the structural similarity of these proteins is more pronounced. The VAST p value for the comparison of the structure of azurin to auracyanin B is $10^{-7.4}$, azurin to rusticyanin is 10^{-5}, azurin to plastocyanin is $10^{-5.6}$, and azurin to psuedoazurin is $10^{-4.1}$.

All of the cupredoxins possess an eight-stranded Greek key beta-barrel or beta-sandwich fold and have a highly conserved site architecture. (De Rienzo *et al., Protein Science* 9:1439-1454 (2000)) A prominent hydrophobic patch, due to the presence of many long chain aliphatic residues such as methionines and leucines, is present around the copper site in azurins, amicyanins, cyanobacterial plastocyanins, cucumber basic protein and to a lesser extent, pseudoazurin and eukaryotic plastocyanins. *Id.* Hydrophobic patches are also found to a lesser extent in stellacyanin and rusticyanin copper sites, but have different features. *Id.*

Table 1. Sequence and structure alignment of azurin (1JZG) from *P. aeruginosa* to other proteins using VAST algorithm.

PDB	Alignment length[1]	% aa identity	P-value[2]	Score[3]	RMSD[4]	Description
1AOZ A 2	82	18.3	10 e-7	12.2	1.9	Ascorbate oxidase
1QHQ_A	113	31	10e-7.4	12.1	1.9	AuracyaninB
1V54 B 1	79	20.3	10e-6.0	11.2	2.1	Cytocrome c oxidase
1GY2 A	92	16.3	10e-5.0	11.1	1.8	Rusticyanin
3MSP A	74	8.1	10e-6.7	10.9	2.5	Motile Major Sperm Protein[5]
1IUZ	74	20.3	10e-5.6	10.3	2.3	Plastocyanin
1KGY E	90	5.6	10e-4.6	10.1	3.4	Ephrinb2
1PMY	75	17.3	10e-4.1	9.8	2.3	Pseudoazurin

[1]Aligned Length: The number of equivalent pairs of C-alpha atoms superimposed between the two structures, i.e. how many residues have been used to calculate the 3D superposition.

[2]P-VAL: The VAST p value is a measure of the significance of the comparison, expressed as a probability. For example, if the p value is 0.001, then the odds are 1000 to 1 against seeing a match of this quality by pure chance. The p value from VAST is adjusted for the effects of multiple comparisons using the assumption that there are 500 independent and

unrelated types of domains in the MMDB database. The p value shown thus corresponds to the p value for the pairwise comparison of each domain pair, divided by 500.

[3]Score: The VAST structure-similarity score. This number is related to the number of secondary structure elements superimposed and the quality of that superposition. Higher VAST scores correlate with higher similarity.

[4]RMSD: The root mean square superposition residual in Angstroms. This number is calculated after optimal superposition of two structures, as the square root of the mean square distances between equivalent C-alpha atoms. Note that the RMSD value scales with the extent of the structural alignments and that this size must be taken into consideration when using RMSD as a descriptor of overall structural similarity.

[5] *C. elegans* major sperm protein proved to be an ephrin antagonist in oocyte maturation (Kuwabara, 2003 "The multifaceted C. elegans major sperm protein: an ephrin signalling antagonist in oocyte maturation" *Genes and Development,* 17:155-161.

Azurin

The azurins are copper containing proteins of 128 amino acid residues which belong to the family of cupredoxins involved in electron transfer in certain bacteria. The azurins include those from *P. aeruginosa* (PA) (SEQ ID NO: 1), *A. xylosoxidans, and A. denitrificans.* (Murphy *et al.*, J. Mol. Biol. 315:859-871 (2002)) The amino acid sequence identity between the azurins varies between 60-90%, these proteins showed a strong structural homology. All azurins have a characteristic β-sandwich with Greek key motif and the single copper atom is always placed at the same region of the protein. In addition, azurins possess an essentially neutral hydrophobic patch surrounding the copper site. *Id.*

Plastocyanins

The plastocyanins are soluble proteins of cyanobacteria, algae and plants that contain one molecule of copper per molecule and are blue in their oxidized form. They occur in the chloroplast, where they function as electron carriers. Since the determination of the structure of poplar plastocyanin in 1978, the structure of algal (*Scenedesmus, Enteromorpha, Chlamydomonas*) and plant (French bean) plastocyanins has been determined either by crystallographic or NMR methods, and the poplar structure has been refined to 1.33 Å resolution. SEQ ID NO: 3 shows the amino acid sequence of plastocyanin from *Phormidium laminosum*, a thermophilic cyanobacterium.

Despite the sequence divergence among plastocyanins of algae and vascular plants (e.g., 62% sequence identity between the Chlamydomonas and poplar proteins), the three-dimensional structures are conserved (e.g., 0.76 Å rms deviation in the C alpha positions between the *Chlamydomonas* and *Poplar* proteins). Structural features include a distorted tetrahedral copper binding site at one end of an eight-stranded antiparallel beta-barrel, a pronounced negative patch, and a flat hydrophobic surface. The copper site is optimized for its electron transfer function, and the negative and hydrophobic patches are proposed to be involved in recognition of physiological reaction partners. Chemical modification, cross-linking, and site-directed mutagenesis experiments have confirmed the importance of the negative and hydrophobic patches in binding interactions with cytochrome f, and validated the model of two functionally significant electron transfer paths involving plastocyanin. One putative electron transfer path is relatively short (approximately 4 Å) and involves the solvent-exposed copper ligand His-87 in the hydrophobic patch, while the other is more lengthy (approximately 12-15 Å) and involves the nearly conserved residue Tyr-83 in the negative patch, Redinbo *et al., J. Bioenerg. Biomembr.* 26:49-66 (1994).

Rusticyanins

Rusticyanins are blue-copper containing single-chain polypeptides obtained from a *Thiobacillus* (now called *Acidithiobacillus*). The X-ray crystal structure of the oxidized form of the extremely stable and highly oxidizing cupredoxin rusticyanin from *Thiobacillus* ferrooxidans (SEQ ID NO: 4) has been determined by multiwavelength anomalous diffraction and refined to 1.9Å resolution. The rusticyanins are composed of a core beta-sandwich fold composed of a six- and a seven-stranded b-sheet. Like other cupredoxins, the copper ion is coordinated by a cluster of four conserved residues (His 85, Cys138, His143, Met148) arranged in a distorted tetrahedron. Walter, R.L. *et al., J. Mol. Biol.* 263:730-51 (1996).

Pseudoazurins

The pseudoazurins are a family of blue-copper containing single-chain polypeptide. The amino acid sequence of pseudoazurin obtained from *Achromobacter cycloclastes* is shown in SEQ ID NO: 5. The X-ray structure analysis of pseudoazurin shows that it has a similar structure to the azurins although there is low sequence homology between these

proteins. Two main differences exist between the overall structure of the pseudoazurins and azurins. There is a carboxy terminus extension in the pseudoazurins, relative to the azurins, consisting of two alpha-helices. In the mid-peptide region azurins contain an extended loop, shortened in the pseudoazurins, which forms a flap containing a short α-helix. The only major differences at the copper atom site are the conformation of the MET side-chain and the Met-S copper bond length, which is significantly shorter in pseudoazurin than in azurin.

Phytocyanins

The proteins identifiable as phytocyanins include, but are not limited to, cucumber basic protein, stellacyanin, mavicyanin, umecyanin, a cucumber peeling cupredoxin, a putative blue copper protein in pea pods, and a blue copper protein from *Arabidopsis thaliana*. In all except cucumber basic protein and the pea-pod protein, the axial methionine ligand normally found at blue copper sites is replaced by glutamine.

Auracyanin

Three small blue copper proteins designated auracyanin A, auracyanin B-1, and auracyanin B-2 have been isolated from the thermophilic green gliding photosynthetic bacterium *Chloroflexus aurantiacus*. The two B forms are glycoproteins and have almost identical properties to each other, but are distinct from the A form. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrates apparent monomer molecular masses as 14 (A), 18 (B-2), and 22 (B-1) kDa.

The amino acid sequence of auracyanin A has been determined and showed auracyanin A to be a polypeptide of 139 residues. (Van Dreissche *et al;. Protein Science* 8:947-957 (1999).) His58, Cys123, His128, and Met132 are spaced in a way to be expected if they are the evolutionary conserved metal ligands as in the known small copper proteins plastocyanin and azurin. Secondary structure prediction also indicates that auracyanin has a general beta-barrel structure similar to that of azurin from *Pseudomonas aeruginosa* and plastocyanin from poplar leaves. However, auracyanin appears to have sequence characteristics of both small copper protein sequence classes. The overall similarity with a consensus sequence of azurin is roughly the same as that with a consensus sequence of plastocyanin, namely 30.5%. The N-terminal sequence region 1-18 of auracyanin is remarkably rich in glycine and hydroxy amino acids. *Id. See* exemplary amino acid

sequence SEQ ID NO: 15 for chain A of auracyanin from *Chloroflexus aurantiacus* (NCBI Protein Data Bank Accession No. AAM12874).

The auracyanin B molecule has a standard cupredoxin fold. The crystal structure of auracyanin B from *Chloroflexus aurantiacus* has been studied. (Bond *et al., J. Mol. Biol.* 306:47-67 (2001).) With the exception of an additional N-terminal strand, the molecule is very similar to that of the bacterial cupredoxin, azurin. As in other cupredoxins, one of the Cu ligands lies on strand 4 of the polypeptide, and the other three lie along a large loop between strands 7 and 8. The Cu site geometry is discussed with reference to the amino acid spacing between the latter three ligands. The crystallographically characterized Cu-binding domain of auracyanin B is probably tethered to the periplasmic side of the cytoplasmic membrane by an N-terminal tail that exhibits significant sequence identity with known tethers in several other membrane-associated electron-transfer proteins. The amino acid sequences of the B forms are presented in McManus et al. (*J Biol Chem.* 267:6531-6540 (1992).). *See* exemplary amino acid sequence SEQ ID NO: 16 for chain B of auracyanin from *Chloroflexus aurantiacus* (NCBI Protein Data Bank Accession No. 1QHQA).

Stellacyanin

Stellacyanins are a subclass of phytocyanins, a ubiquitous family of plant cupredoxins. An exemplary sequence of a stellacyanin is included herein as SEQ ID NO: 14. The crystal structure of umecyanin, a stellacyanin from horseradish root (Koch *et al., J. Am. Chem. Soc.* 127:158-166 (2005)) and cucumber stellacyanin (Hart *el al., Protein Science* 5:2175-2183 (1996)) is also known. The protein has an overall fold similar to the other phytocyanins. The ephrin B2 protein ectodomain tertiary structure bears a significant similarity to stellacyanin. (Toth *et al., Developmental Cell* 1:83-92 (2001).) An exemplary amino acid sequence of a stellacyanin is found in the National Center for Biotechnology Information Protein Data Bank as Accession No. 1JER, SEQ ID NO: 14.

Cucumber basic protein

An exemplary amino acid sequence from a cucumber basic protein is included herein as SEQ ID NO: 17. The crystal structure of the cucumber basic protein (CBP), a type 1 blue copper protein, has been refined at 1.8 Å resolution. The molecule resembles other blue copper proteins in having a Greek key beta-barrel structure, except that the barrel is open on

one side and is better described as a "beta-sandwich" or "beta-taco". (Guss *et al., J. Mol. Biol.* 262:686-705 (1996).) The ephrinB2 protein ectodomian tertiary structure bears a high similarity (rms deviation 1.5Å for the 50 α carbons) to the cucumber basic protein. (Toth *et al., Developmental Cell* 1:83-92 (2001).)

The Cu atom has the normal blue copper NNSS' co-ordination with bond lengths Cu-N(His39) = 1.93 A, Cu-S(Cys79) = 2.16 A, Cu-N(His84) = 1.95 A, Cu-S(Met89) = 2.61 A. A disulphide link, (Cys52)-S-S-(Cys85), appears to play an important role in stabilizing the molecular structure. The polypeptide fold is typical of a sub-family of blue copper proteins (phytocyanins) as well as a non-metalloprotein, ragweed allergen Ra3, with which CBP has a high degree of sequence identity. The proteins currently identifiable as phytocyanins are CBP, stellacyanin, mavicyanin, umecyanin, a cucumber peeling cupredoxin, a putative blue copper protein in pea pods, and a blue copper protein from *Arabidopsis thaliana.* In all except CBP and the pea-pod protein, the axial methionine ligand normally found at blue copper sites is replaced by glutamine. An exemplary sequence for cucumber basic protein is found in NCBI Protein Data Bank Accession No. 2CBP, SEQ ID NO: 17.

Methods of Use

The invention provides a method to treat a patient infected with viral or bacterial infection, and specifically HIV-1 infection, comprising administering to the patient at least one polypeptide that is a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof, as described above. It is also contemplated that the same method will be effective to treat patients with other viral or bacterial infections, such as, but not limited to, Herpes simplex virus (HSV), Ebola virus, cytomeglovirus (CMV), parainfluenza viruses types A, B and C, etc., hepatitis A, B, C, G, the delta hepatitis virus (HDV), mumps viruses, measles viruses, respiratory syncytial viruses, bunyviruses, arena viruses, the orthomyxo-like insect virus called Dhori, polioviruses, rubella virus, dengue virus; SIV and *Mycobacterium tuberculosis.*

It has also been learned that cupredoxins and cytochrome c are also effective against malarial infections, as disclosed in a co-filed application. Further, co-infections with HIV and malaria are very common in many areas of the world, and in particular sub-Saharan Africa. In some embodiments, the patient suffering from infection by HIV is also suffering from infection by a malaria parasite. In some embodiments, the method of treatment of the

invention also comprises administering anti-malarial drugs. In some embodiments, the anti-malarial drugs are co-administered.

The methods of the invention include contacting mammalian cells with a composition comprising cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof. In some embodiments, the mammalian cells are infected with a virus or bacteria, such as HIV-1. In other embodiments, the mammalian cells will be exposed to a virus or bacteria, such as HIV-1.

The composition comprising cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof can be administered to the patient by many routes and in many regimens that will be well known to those in the art. In specific embodiments, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof is administered intravenously, intramuscularly or subcutaneously.

In one embodiment, the methods may comprise co-administering to a patient one unit dose of a composition comprising a cupredoxin or cytochrome c_{551}, or a variant, derivative or structural equivalent of cupredoxin or cytochrome c_{551}, and one unit dose of a composition comprising another anti-viral or anti-bacterial, and specifically anti-HIV drug and/or an anti-malarial drug, in either order, administered at about the same time, or within about a given time following the administration of the other, for example, about one minute to about 6o minutes following the administration of the other drug, or about 1 hour to about 12 hours following the administration of the other drug.

In addition, the present invention includes methods useful for studying viral and bacterial infection. In some embodiments, the method comprises contacting mammalian cells infected with a virus or bacteria with a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof, and measuring the growth of infection. In other embodiments, the method comprises contacting mammalian cells with a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof, contacting the mammalian cells with a virus or bacteria, and measuring the growth of infection in the cells. In some embodiments, the bacteria or virus is HIV, Herpes simplex virus (HSV), Ebola virus, cytomeglovirus (CMV), parainfluenza viruses types A, B and C, etc., hepatitis A, B, C, G, the delta hepatitis virus (HDV), mumps viruses, measles viruses, respiratory syncytial viruses, bunyviruses, arena viruses, the orthomyxo-like insect virus called Dhori, polioviruses, rubella virus, dengue virus; SIV and *Mycobacterium tuberculosis*. In a specific embodiment, the

virus is HIV-1, and particularly strains Bal, 2167, or RW/92/008/RE1. In other embodiments, the mammalian cells are human. In other embodiments, the cells are blood lymphocytes or peripheral blood mononuclear cells. In various embodiments, the cells are contacted in the animal (*in vivo*), or outside the animal (*in vitro*). In some embodiments, the cells are contacted *in vitro* and them introduced into an animal.

Pharmaceutical Compositions Comprising Cupredoxin Or Cytochrome C_{551}, Or Variant, Derivative Or Structural Equivalent Thereof

Pharmaceutical compositions comprising cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalents thereof, can be manufactured in any conventional manner, *e.g.* by conventional mixing, dissolving, granulating, dragee-making, emulsifying, encapsulating, entrapping, or lyophilizing processes. The substantially pure cupredoxin and/or cytochrome c_{551}, and variants, derivatives and structural equivalents thereof can be readily combined with a pharmaceutically acceptable carrier well-known in the art. Such carriers enable the preparation to be formulated as a tablet, pill, dragee, capsule, liquid, gel, syrup, slurry, suspension, and the like. Suitable carriers or excipients can also include, for example, fillers and cellulose preparations. Other excipients can include, for example, flavoring agents, coloring agents, detackifiers, thickeners, and other acceptable additives, adjuvants, or binders. In some embodiments, the pharmaceutical preparation is substantially free of preservatives. In other embodiments, the pharmaceutical preparation may contain at least one preservative. General methodology on pharmaceutical dosage forms is found in Ansel *et al., Pharmaceutical Dosage Forms and Drug Delivery Systems* (Lippencott Williams & Wilkins, Baltimore MD (1999)).

The composition comprising a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof used in the invention may be administered in a variety of ways, including by injection (*e.g.*, intradermal, subcutaneous, intramuscular, intraperitoneal and the like), by inhalation, by topical administration, by suppository, by using a transdermal patch or by mouth. General information on drug delivery systems can be found in Ansel *et al., Id.*. In some embodiments, the composition comprising a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof can be formulated and used directly as injectibles, for subcutaneous and intravenous injection, among others. The injectable formulation, in particular, can advantageously be used to treat patients that are at risk of,

likely to have or have a viral or bacterial infection, and specifically an HIV-infection. The composition comprising a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof can also be taken orally after mixing with protective agents such as polypropylene glycols or similar coating agents.

When administration is by injection, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof may be formulated in aqueous solutions, specifically in physiologically compatible buffers such as Hanks solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use. In some embodiments, the pharmaceutical composition does not comprise an adjuvant or any other substance added to enhance the immune response stimulated by the peptide. In some embodiments, the pharmaceutical composition comprises a substance that inhibits an immune response to the peptide.

When administration is by intravenous fluids, the intravenous fluids for use administering the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof may be composed of crystalloids or colloids. Crystalloids as used herein are aqueous solutions of mineral salts or other water-soluble molecules. Colloids as used herein contain larger insoluble molecules, such as gelatin. Intravenous fluids may be sterile.

Crystalloid fluids that may be used for intravenous administration include but are not limited to, normal saline (a solution of sodium chloride at 0.9% concentration), Ringer's lactate or Ringer's solution, and a solution of 5% dextrose in water sometimes called D5W, as described in Table 2.

Table 2. Composition of Common Crystalloid Solutions

Solution	Other Name	$[Na^+]$	$[Cl^-]$	[Glucose]
D5W	5% Dextrose	0	0	252
2/3 & 1/3	3.3% Dextrose / 0.3% saline	51	51	168
Half-normal saline	0.45% NaCl	77	77	0

Normal saline	0.9% NaCl	154	154	0
Ringer's lactate*	Ringer's solution	130	109	0

*Ringer's lactate also has 28 mmol/L lactate, 4 mmol/L K^+ and 3 mmol/L Ca^{2+}.

When administration is by inhalation, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof may be delivered in the form of an aerosol spray from pressurized packs or a nebulizer with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the proteins and a suitable powder base such as lactose or starch.

When administration is by topical administration, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof may be formulated as solutions, gels, ointments, creams, jellies, suspensions, and the like, as are well known in the art. In some embodiments, administration is by means of a transdermal patch. When administration is by suppository (*e.g.*, rectal or vaginal), cupredoxin and/or cytochrome c and variants and derivatives thereof compositions may also be formulated in compositions containing conventional suppository bases.

When administration is oral, a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof can be readily formulated by combining the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof with pharmaceutically acceptable carriers well known in the art. A solid carrier, such as mannitol, lactose, magnesium stearate, and the like may be employed; such carriers enable the cupredoxin and/or cytochrome c and variants and derivatives thereof to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. For oral solid formulations such as, for example, powders, capsules and tablets, suitable excipients include fillers such as sugars, cellulose preparation, granulating agents, and binding agents.

Other convenient carriers, as well-known in the art, also include multivalent carriers, such as bacterial capsular polysaccharide, a dextran or a genetically engineered vector. In

addition, sustained-release formulations that include a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof allow for the release of cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof over extended periods of time, such that without the sustained release formulation, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof would be cleared from a subject's system, and/or degraded by, for example, proteases and simple hydrolysis before eliciting or enhancing a therapeutic effect.

The half-life in the bloodstream of the compositions of the invention can be extended or optimized by several methods well known to those in the art, including but not limited to, circularized peptides (Monk *et al.*, BioDrugs 19(4):261-78, (2005); DeFreest et al., J. Pept. Res. 63(5):409-19 (2004)), D,L-peptides (diastereomer), (Futaki *et al.*, J. Biol. Chem. Feb 23;276(8):5836-40 (2001); Papo *et al.*, Cancer Res. 64(16):5779-86 (2004); Miller *et al.*, Biochem. Pharmacol. 36(1):169-76, (1987)); peptides containing unusual amino acids (Lee *et al.*, J. Pept. Res. 63(2):69-84 (2004)), N- and C- terminal modifications (Labrie *et al.*, Clin. Invest. Med. 13(5):275-8, (1990)), and hydrocarbon stapling (Schafmeister *et al.*, J. Am. Chem. Soc. 122:5891-5892 (2000); Walenski *et al.*, Science 305:1466-1470 (2004)). Of particular interest are d-isomerization (substitution) and modification of peptide stability via D-substitution or L- amino acid substitution.

In various embodiments, the pharmaceutical composition includes carriers and excipients (including but not limited to buffers, carbohydrates, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents, suspending agents, thickening agents and/or preservatives), water, oils, saline solutions, aqueous dextrose and glycerol solutions, other pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like. It will be recognized that, while any suitable carrier known to those of ordinary skill in the art may be employed to administer the compositions of this invention, the type of carrier will vary depending on the mode of administration. Compounds may also be encapsulated within liposomes using well-known technology. Biodegradable microspheres may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344 and 5,942,252.

The pharmaceutical compositions may be sterilized by conventional, well-known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration.

Administration Of Cupredoxin Or Cytochrome C_{551}, Or Variant, Derivative Or Structural Equivalent Thereof

The cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof can be administered formulated as pharmaceutical compositions and administered by any suitable route, for example, by oral, buccal, inhalation, sublingual, rectal, vaginal, transurethral, nasal, topical, percutaneous, i.e., transdermal or parenteral (including intravenous, intramuscular, subcutaneous and intracoronary) administration. The pharmaceutical formulations thereof can be administered in any amount effective to achieve its intended purpose. More specifically, the composition is administered in a therapeutically effective amount. In specific embodiments, the therapeutically effective amount is generally from about 0.01-20 mg/day/kg of body weight.

The compounds comprising cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof are useful for the treatment and/or prophylaxis of HIV infection or other viral or bacterial infections, alone or in combination with other active agents. The appropriate dosage will, of course, vary depending upon, for example, the compound of cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof employed, the host, the mode of administration and the nature and severity of the conditions being treated. However, in general, satisfactory results in humans are indicated to be obtained at daily dosages from about 0.01-20 mg/kg of body weight. An indicated daily dosage in humans is in the range from about 0.7 mg to about 1400 mg of a compound of cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof conveniently administered, for example, in daily doses, weekly doses, monthly doses, and/or continuous dosing. Daily doses can be in discrete dosages from 1 to 12 times per day. Alternatively, doses can be administered every other day, every third day, every fourth day, every fifth day, every sixth day, every week, and similarly in day increments up to 31 days. Alternatively, dosing can be continuous using patches, i.v. administration and the like.

The method of introducing cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof to patients is, in some embodiments, the same as currently used to introduce anti-HIV drugs, such as the protease-inhibitor-containing cocktails. Such methods are well-known in the art. In a specific embodiment, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof are part of an cocktail or co-dosing containing or with other anti-HIV therapeutics. Other anti-HIV drugs include, but are not limited to, reverse transcriptase inhibitors: AZT (zidovudine [Retrovir]), ddC (zalcitabine [Hivid], dideoxyinosine), d4T (stavudine [Zerit]), and 3TC (lamivudine [Epivir]), nonnucleoside reverse transcriptase inhibitors (NNRTIS): delavirdine (Rescriptor) and nevirapine (Viramune), protease inhibitors: ritonavir (Norvir), a lopinavir and ritonavir combination (Kaletra), saquinavir (Invirase), indinavir sulphate (Crixivan), amprenavir (Agenerase), and nelfinavir (Viracept). Presently, a combination of several drugs called highly active antiretroviral therapy (HAART) is used to treat people with HIV.

The method of introducing cupredoxin or cytochrome, or variant, derivative or structural equivalent thereof to patients is, in some embodiments, through the co-administration of cupredoxin or cytochrome, or variant, derivative or structural equivalent thereof with drugs used for malaria therapy. Such methods are well-known in the art. In a specific embodiment, the cupredoxin and/or cytochrome c are part of an cocktail or co-dosing containing or with malaria therapeutics. Malaria therapeutics of interest include, but are not limited to, proguanil, chlorproguanil, trimethoprim, chloroquine, mefloquine, lumefantrine, atovaquone, pyrimethamine-sulfadoxine, pyrimethamine-dapsone, halofantrine, quinine, quinidine, amodiaquine, amopyroquine, sulphonamides, artemisinin, arteflene, artemether, artesunate, primaquine, pyronaridine, proguanil, chloroquine, mefloquine, pyrimethamine-sulfadoxine, pyrimethamine-dapsone, halofantrine, quinine, proguanil, chloroquine, mefloquine, 1,16-hexadecamethylenebis(N-methylpyrrolidinium)dibromide, and combinations thereof.

The exact formulation, route of administration, and dosage is determined by the attending physician in view of the patient's condition. Dosage amount and interval can be adjusted individually to provide plasma levels of the active cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof which are sufficient to maintain therapeutic effect. Generally, the desired cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof is administered in an admixture with a pharmaceutical carrier

selected with regard to the intended route of administration and standard pharmaceutical practice.

In one aspect, the cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof is delivered as DNA such that the polypeptide is generated *in situ*. In one embodiment, the DNA is "naked," as described, for example, in Ulmer *et al.*, (Science 259:1745-1749 (1993)) and reviewed by Cohen (Science 259:1691-1692 (1993)). The uptake of naked DNA may be increased by coating the DNA onto a carrier, *e.g.*, biodegradable beads, which are then efficiently transported into the cells. In such methods, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacterial and viral expression systems. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. See, *e.g.*, WO90/11092, WO93/24640, WO 93/17706, and U.S. Pat. No. 5,736,524.

Vectors, used to shuttle genetic material from organism to organism, can be divided into two general classes: Cloning vectors are replicating plasmid or phage with regions that are essential for propagation in an appropriate host cell and into which foreign DNA can be inserted; the foreign DNA is replicated and propagated as if it were a component of the vector. An expression vector (such as a plasmid, yeast, or animal virus genome) is used to introduce foreign genetic material into a host cell or tissue in order to transcribe and translate the foreign DNA, such as the DNA of a cupredoxin. In expression vectors, the introduced DNA is operably-linked to elements such as promoters that signal to the host cell to highly transcribe the inserted DNA. Some promoters are exceptionally useful, such as inducible promoters that control gene transcription in response to specific factors. Operably-linking a cupredoxin and/or cytochrome c and variants and derivatives thereof polynucleotide to an inducible promoter can control the expression of the cupredoxin and/or cytochrome c and variants and derivatives thereof in response to specific factors. Examples of classic inducible promoters include those that are responsive to α-interferon, heat shock, heavy metal ions, and steroids such as glucocorticoids (Kaufman, Methods Enzymol. 185:487-511 (1990)) and tetracycline. Other desirable inducible promoters include those that are not endogenous to the cells in which the construct is being introduced, but, are responsive in those cells when the induction agent is exogenously supplied. In general, useful expression vectors are often

plasmids. However, other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses) are contemplated.

Vector choice is dictated by the organism or cells being used and the desired fate of the vector. In general, vectors comprise signal sequences, origins of replication, marker genes, polylinker sites, enhancer elements, promoters, and transcription termination sequences.

Kits Comprising Cupredoxin Or Cytochrome C_{551}, Or Variant, Derivative Or Structural Equivalent Thereof

In one aspect, the invention provides kits containing one or more of the following in a package or container: (1) a biologically active composition comprising at least one cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof; (2) an anti-viral or anti-bacterial drug, specifically an anti-HIV drug, including, but not limited to, reverse transcriptase inhibitors: AZT (zidovudine [Retrovir]), ddC (zalcitabine [Hivid], dideoxyinosine), d4T (stavudine [Zerit]), and 3TC (lamivudine [Epivir]), nonnucleoside reverse transcriptase inhibitors (NNRTIS): delavirdine (Rescriptor) and nevirapine (Viramune), protease inhibitors: ritonavir (Norvir), a lopinavir and ritonavir combination (Kaletra), saquinavir (Invirase), indinavir sulphate (Crixivan), amprenavir (Agenerase), and nelfinavir (Viracept); (3) a pharmaceutically acceptable excipient; (4) a vehicle for administration, such as a syringe; (5) instructions for administration. Embodiments in which two or more of components (1) - (5) are found in the same packaging or container are also contemplated.

In some embodiments, the kit also comprises an anti-malarial therapeutic. Anti-malarial therapeutics of interest include, but are not limited to, proguanil, chlorproguanil, trimethoprim, chloroquine, mefloquine, lumefantrine, atovaquone, pyrimethamine-sulfadoxine, pyrimethamine-dapsone, halofantrine, quinine, quinidine, amodiaquine, amopyroquine, sulphonamides, artemisinin, arteflene, artemether, artesunate, primaquine, pyronaridine, proguanil, chloroquine, mefloquine, pyrimethamine-sulfadoxine, pyrimethamine-dapsone, halofantrine, quinine, proguanil, chloroquine, mefloquine, 1,16-hexadecamethylenebis(N-methylpyrrolidinium)dibromide.

When a kit is supplied, the different components of the composition may be packaged in separate containers and admixed immediately before use. Such packaging of the

components separately may permit long-term storage without losing the active components' functions.

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain lyophilized cupredoxin and/or cytochrome c and variants and derivatives thereof, or buffers that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, etc., ceramic, metal or any other material typically employed to hold similar reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes, that may comprise foil-lined interiors, such as aluminum or an alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to be mixed. Removable membranes may be glass, plastic, rubber, etc.

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, audiotape, flash memory device etc. Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

Modification Of Cupredoxin And/Or Cytochrome C And Variants And Derivatives Thereof

Cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalents thereof may be chemically modified or genetically altered to produce variants and derivatives as explained above. Such variants and derivatives may be synthesized by standard techniques.

In addition to naturally-occurring allelic variants of cupredoxin and cytochrome c_{551}, changes can be introduced by mutation into cupredoxin or cytochrome c_{551} coding sequence that incur alterations in the amino acid sequences of the encoded cupredoxin or cytochrome c_{551} that do not significantly alter the ability of cupredoxin or cytochrome c to inhibit the growth of a viral or bacterial infection, and specifically an HIV infection in mammalian cells.

A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequences of the cupredoxin without altering biological activity, whereas an "essential" amino acid residue is required for such biological activity. For example, amino acid residues that are conserved among the cupredoxins are predicted to be particularly non-amenable to alteration, and thus "essential."

Amino acids for which conservative substitutions that do not change the activity of the polypeptide can be made are well known in the art. Useful conservative substitutions are shown in Table 3, "Preferred substitutions." Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the invention so long as the substitution does not materially alter the biological activity of the compound.

Table 3. Preferred substitutions

Original residue	Exemplary substitutions	Preferred substitutions
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln, His, Lys, Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro, Ala	Ala
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala, Tyr	Leu
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr, Phe	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Leu, Met, Phe, Ala, Norleucine	Leu

Non-conservative substitutions that affect (1) the structure of the polypeptide backbone, such as a β-sheet or α-helical conformation, (2) the charge, (3) hydrophobicity, or (4) the bulk of the side chain of the target site can modify the cytotoxic factor function. Residues are divided into groups based on common side-chain properties as denoted in Table 4. Non-conservative substitutions entail exchanging a member of one of these classes for another class. Substitutions may be introduced into conservative substitution sites or more specifically into non-conserved sites.

Table 4. Amino acid classes

Class	Amino acids
hydrophobic	Norleucine, Met, Ala, Val, Leu, Ile
neutral hydrophilic	Cys, Ser, Thr
acidic	Asp, Glu
basic	Asn, Gln, His, Lys, Arg
disrupt chain conformation	Gly, Pro
aromatic	Trp, Tyr, Phe

The variant polypeptides can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter, Biochem *J.* 237:1-7 (1986); Zoller and Smith, Methods Enzymol. 154:329-350 (1987)), cassette mutagenesis, restriction selection mutagenesis (Wells *et al.*, Gene 34:315-323 (1985)) or other known techniques can be performed on the cloned DNA to produce the cupredoxin or cytochrome c_{551} variant DNA.

Known mutations of cupredoxins and cytochrome c_{551} can also be used to create variant cupredoxin and cytochrome c_{551} to be used in the methods of the invention. For example, the C112D and M44KM64E mutants of azurin are known to have cytotoxic and growth arresting activity that is different from the native azurin, and such altered activity can be useful in the treatment methods of the present invention. One embodiment of the methods of the invention utilize cupredoxin and/or cytochrome c_{551} and variants and derivatives thereof retaining the ability inhibit the growth of an viral or bacterial infection, and specifically HIV infection in mammalian cells. In another embodiment, the methods of the

present invention utilize cupredoxin variants such as the M44KM64E mutant, having the ability to cause cellular growth arrest.

A more complete understanding of the present invention can be obtained by reference to the following specific Examples. The Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations. Modifications and variations of the invention as hereinbefore set forth can be made without departing from the spirit and scope thereof, and, therefore, only such limitations should be imposed as are indicated by the appended embodiments.

EXAMPLES

Example 1: *In Vivo* Inhibition Of HIV Infection Of Lymphocytes By Azurin Mutant And Cytochrome C_{551}.

The M44KM64E mutant of azurin was mixed with cytochrome c_{551} on a 1:1 basis (1 μM azurin : 1 μM cytochrome c_{551}). HIV-infected human blood lymphocytes were incubated with the mixed azurin/cytochrome c_{551} proteins at concentrations of 0, 500 to 1000 μg/ml protein for 7 days. The HIV p24 levels were then measured in the infected lymphocytes. p24 levels are known to be colinear with HIV virus levels in infected blood. Measuring the change in p24 concentrations in blood will indicate the change of HIV virus titer in the blood. Controls with non-infected human blood lymphocytes were also run in a parallel manner. After the 7 day incubation, the HIV p24 levels in the infected lymphocytes were reduced by 25% to 90% as compared to the control infected lymphocytes with 0 μg/ml azurin and cytochrome c_{551}. In the non-infected control cells, after 7 days of incubation with the protein mixture, neither cell death nor cytotoxicity was found.

Example 2. Azurin exhibits structural similarity with ICAM-1 from HIV-1

Previous studies (Gough & Chothia, Structure 12:917-925 (2004); Stevens *et al.*, J. Mol. Recognit. 18:150-157 (2005)) have shown that cupredoxins show structural similarity to the variable domains of the immunoglobulin superfamily members. The DALI algorithm (Holm & Park, Bioinformatics 16:566-567 (2000)) was used to search the 3D databases for

structural homologs of azurin (1JZG) from *P. aeruginosa*. *See*, Table 5. Azurin exhibits structural similarity to not only the fab fragment of the monoclonal antibody in complexation with PfMSP1-19, but also to ICAM-1 (Table 5) involved in cerebral malaria (Smith *et al.*, Proc. Natl. Acad. Sci. USA 97:1766-1771 (2000); Franke-Fayard *et al.*, Proc. Natl. Acad. Sci. USA 102:11468-11473 (2005))

ICAM-1 is not only implicated as a receptor on the endothelial cells in the microvasculture of the brain and other tissues for sequestering *P. falciparum* – infected erythrocytes, but is also found in HIV-1 particles during their passage through the host cells and is known to enhance HIV-1 infectivity by enhancing cytosolic delivery of the viral materials. (Fortin *et al.*, J. Virol. 71:3588-3596 (1997); Tardif & Tremblay, J. Virol. 77:12299-12309 (2003)) ICAM-1 is known also to be subverted as receptors for major groups of rhinoviruses and coxsackie viruses. (Bella & Rossmann, J. Struct. Biol. 128:69-74 (1999))

Azurin demonstrates structural similarity to CD4 (Table 5), the primary host cell surface receptor for HIV-1. (Maddon et al., Cell 47:333-348 (1986)) This example shows that cupredoxins including azurin demonstrate structural similarities in having two anti-parallel β sheets packed face to face and linked by a disulfide bridge to the variable domains of the immunoglobulin superfamily members as well as extracellular domains of the intercellular adhesion molecules (ICAM) and their ligands.

Table 5. Structural similarity of *P. aeruginosa* azurin with various pathogenesis-related proteins

PDB	Annotation	Reference	DALI z score[1]	Azurin (1jzg) RMSD to Azurin[2]	Alignment length
1VCA B1	Human Vascular Cell Adhesion	17	3.5	3.2	80
1CDH	CD4 (D1D2 Fragment) Type I Crystal Form	18	3.4	2.8	73
1ZXQ1	Crystal Structure of ICAM-2	19	3.3	2.9	80
1IAM1	Structure of the Two Amino-Terminal Adhesion Molecule-1, ICAM-1	20	3.3	3.1	74
1OB1 A1	Crystal Structure of a Fab Complex with *Plasmodium falciparum* MSP1-19	21	2.9	3.7	84

Structural alignment of azurin were made using DALI (Holm & Park, Bioinformatics 16:566-567 (2000)). Structure pairs with DALI z scores <2 are considered dissimilar.

RMSD-Root-mean-square deviation of backbone residues in angstroms between the aligned parts of the pair of structure.

Example 3. Effect of Azurin, H.8-Azurin and Laz on HIV-1 Entry and Viral Growth.

The effect of various concentrations of azurin, H.8-azurin and Laz on the growth of three subtypes of HIV-1 in peripheral blood mononuclear cells (PBMCs), Bal, RW/92/008/RE1 clade A and IN/2157 D15 clade C

Cloning and Expression of the *paz* and *laz* Genes. The *laz* gene from *Neisseria gonorrhoeae* was cloned based on its known sequence (SEQ ID NO: 19). The *P. aeruginosa* azurin gene (SEQ ID NO: 1), termed *paz* , and the sequence of the H.8 epitope of *laz* from *N. gonnerrhoeae* (SEQ ID NO: 18), were used to clone in frame the H.8 epitope gene in the 5'-end of *paz* to produce H.8-*paz* or in the 3'-end of *paz* to generate *paz*-H.8. *See,* Table 6 for bacterial strains and genetic constructs used in this and related Examples.

Table 6. Bacterial strains and genetic constructs

Cells/strains/ plasmids	Relevant characteristics*	Reference
P. aeruginosa PAO1	Prototroph, FP- (sex factor minus)	Holloway, *et al.*, Microbiol. Rev. 43:73-102 (1979)
E. coli JM109	Cloning and azurin expression strain	Yanisch-Perron, *et al.*, Gene 33:103-119 (1985)
E. coli BL21 (DE3)	GST expression strain	Novagen
N. gonorrhoeae F62	Prototroph used for DNA isolation	American Type Culture Collection
pUC18	General cloning vector, Ap^r	Yanisch-Perron, *et al., id.*
pUC19	General cloning vector, Ap^r	Yanisch-Perron, *et al., id.*
pUC18-*laz*	A 1 kb PCR fragment from genomic DNA of *N. gonorrhoeae* F62 cloned into pUC18	Herein
pUC19-*paz*	A 0.55 kb PCR fragment from *P. aeruginosa* PAO1 cloned into *HindIII* and *PstI* digested pUC19, Ap^r	Yamada, *et al.*, Proc. Natl. Acad. Sci. USA 99:14098-14103 (2002); Yamada, *et al.*, Proc. Natl. Acad. Sci. USA 101:4770-4775 (2004)
pUC18-H.8-*paz*	Fusion plasmid encoding H.8 from N. gonorrhoeae and azurin from *P. aeruginosa* PAO1, Ap^r	Herein
pGEX-5X-3	GST gene fusion vectors, Ap^r	Amersham
pET29a	*E. coli* expression vector, Km^r	Novagen
pET29a-gst	pET29a derivative containing the gst gene, Km^r	Herein
pGEX-5X-3-H.8	pGEX-5X-3 derivative containing H.8-encoding region, Ap^r	Herein
pET29a-*gst*-H.8	pET29a derivative containing gst-H.8 gene, Km^r	Herein

*Ap, ampicillin; Km, kanamycin; GST, Glutathione S-transferase.

The cloning and hyperexpression of the azurin gene has been described. (Yamada, *et al.*, Proc. Natl. Acad. Sci. USA 99:14098-14103 (2002); Punj, *et al.*, Oncogene 23:2367-2378 (2004)) The Laz-encoding gene (*laz*) of *Neisseria gonorrhoeae* was amplified by PCR with genomic DNA of *N. gonorrhoeae* strain F62 as template DNA. The forward and reverse primers used were 5'-CCGGAATTCCGGCAGGGATGTTGTAAATATCCG-3' (SEQ ID NO: 20) and 5'-GGGGTACCGCCGTGGCAGGCATACAGCATTTCAATCGG-3' (SEQ ID NO: 21) where the additionally introduced restriction sites of *EcoRI* and *KpnI* sites are underlined respectively. The amplified DNA fragment of 1.0 kb, digested with *EcoRI* and *KpnI*, was inserted into the corresponding sites of pUC18 vector (Yanisch-Perron, *et al.*, Gene 33:103-119 (1985)) so that the *laz* gene was placed downstream of the lac promoter to yield an expression plasmid pUC18-*laz* (Table 6).

The plasmids expressing fusion H.8 of *N. gonorrhoeae* Laz and azurin of *P. aeruginosa* (Paz) were constructed by PCR with pUC19-*paz* and pUC18-*laz* as templates. For H.8-Paz fusion, a 3.1 kb fragment was amplified with pUC18-*laz* as a template and primers, 5'-(phosphorylated)GGCAGCAGGGGCTTCGGCAGCATCTGC-3' (SEQ ID NO: 22) and 5'-CTGCAGGTCGACTCTAGAGGATCCCG-3' (SEQ ID NO: 23) where a *SalI* site is underlined. A PCR amplified a 0.4 kb fragment was obtained from pUC19-*paz* as a template and primers, 5'-(phosphorylated)GCCGAGTGCTCGGTGGACATCCAGG-3' (SEQ ID NO: 24) and 5'-TACTCGAGTCACTTCAGGGTCAGGGTG-3' (SEQ ID NO: 25) where a *XhoI* site is underlined. A *SalI* digested PCR fragment from pUC18-*laz* and *XhoI* digested PCR fragment from pUC19-*paz* were cloned to yield an expression plasmid pUC18-H.8-*paz* (Table 6).

E. coli JM109 was used as a host strain for expression of azurin and its derivative genes. Recombinant *E. coli* strains were cultivated in 2 X YT medium containing 100 μg/ml ampicillin, 0.1 mM IPTG and 0.5 mM $CuSO_4$ for 16 h at 37°C to produce the azurin proteins.

When *E. coli* strains harboring these plasmids were grown in presence of IPTG, cells lysed and the proteins purified as described for azurin (Yamada, *et al.*, Proc. Natl. Acad. Sci. USA 99:14098-14103 (2002); Punj, *et al.*, Oncogene 23:2367-2378 (2004);Yamada, *et al.*, Cell. Microbiol. 7:1418-1431 (2005)), the various azurin derivatives migrated on SDS-PAGE as single components, although the H.8 containing proteins (about 17 kDa) showed anomalous migrations, as noted before. (Cannon, Clin. Microbiol. Rev. 2:S1-S4 (1989); Fisette, *et al.*, J. Biol. Chem. 278:46252-46260 (2003))

HIV-1 Suppression Assay. Azurin, H.8-azurin and Laz were filter sterilized through a 0.45 μM filter. Peripheral blood mononuclear cells (PBMC) were treated with polybrene (5 μg/ml) for 1 h and seeded at 250,000 cells/well in a microtiter plate. The plate was spun at 800 rpm for 5 min to collect the cells. The supernatant was taken off and media with protein (at concentrations of 0.3, 0.6, 1.2, 6.0 and 30 μM) was added (100 μl). The cells were then incubated for 1 h. AZT (25 μM) was used as a control. The proteins were left on cells and 100 μl of virus (Bal, 2167, or RW/92/008/RE1) was added and incubated for 2 h. The plate was spun again at 800 rpm for 5 minutes and protein and virus was removed. Protein and media were added back for a total volume of 100 μl and incubated for 5 days. At the end of the 5 day period, the culture supernatant was tested for HIV/p24 by ELISA.

The results in Figure 1 show that azurin at a concentration of 6.0 μM shows about 90% suppression of the growth of HIV-1 Bal, the most predominant clade B circulating in the US and Western Europe, a clade B African isolate RW/92/008/RE1 and a clade C Indian isolate IN/2167 D15. However, H.8-azurin (azurin with the H.8 epitope in the N-terminal) had high inhibitory activity against all the three subtypes at concentrations as low as 0.3 μM, particularly for the African and the Indian subtypes (Fig.1).

The *Neisserial* protein Laz, which also harbors the H.8 epitope in the N-terminal part of the Neisserial azurin homolog (Gotschlich & Seiff *FEMS Microbiol. Lett.* 43:253-255 (1987); Kawula *et al.*, Mol. Microbiol. 1:179-185 (1987)), had similar inhibitory activity for the three subtypes, particularly for the African and the Indian subtypes (Fig. 1), demonstrating a role of the H.8 epitope in promoting enhanced anti-HIV-1 activity by azurin. No effect on host cell (PBMC) death by MTT assay (Yamada *et al.*, Proc. Natl. Acad. Sci. USA 99:14098-14103 (2002); Punj *et al.*, Oncogene 23:2367-2378 (2004)) was discernible for all concentrations of these three proteins, suggesting that inhibition of HIV-1 growth was not due to death of the host cells.

Example 4. Azurin Binding with gp120 and CD4 as Studied by Surface Plasmon Resonance.

Surface Plasmon Resonance experiments were conducted to determine the extent of azurin binding not only to CD4 but also to HIV-1 surface proteins such as gp120 or gp41 known to be involved in HIV-1 entry and other proteins such as Nef or Gag that are involved in intracellular virus multiplication.

Plasmid Construction for Fusion GST Proteins. Plasmids expressing fusion glutathione S-transferase (GST)-truncated wt-azurin (azu) derivatives were constructed by a polymerase chain reaction using proofreading DNA polymerase. For pGST-azu 36-128, an amplified PCR fragment was introduced into the BamHl and EcoRl sites of the commercial GST expression vector pGEX-5X (Amersham Biosciences, Piscataway, NJ). The fragment was amplified with pUC19-azu as a template and primers, 5'-CGGGATCC CCG GCA ACC TGC CGA AGA ACG TCA TGG GC-3'(SEQ ID NO: 26) and 5'-CGGAATTC GCA TCA CTT CAG GGT CAG GG-3' (SEQ ID NO: 27), where the additionally introduced *BamHl* and *EcoRI* sites are underlined respectively. Carboxyl-terminus truncation of *azu* gene was cumulatively performed by introducing a stop codon using QuickChange site-direct mutagenesis kit (Stratagene, La Jolla, CA).

For pGST-azu 36-89, a stop codon were introduced into Gly90. The plasmid carrying pGST-azu 36-128 was used as template DNA. Three sets of oligonuclotides for site-direct mutagenesis are shown as follows. For pGST-azu 36-89: 5'-CCA AGC TGA TCG GCT CGT GAG AGAAGG ACT CGG TGA CC-3' (SEQ ID NO: 28), and 5'-GGT CAC CGA GTC CTT CTC TCA CGA GCC GAT CAG CTT GG-3 (SEQ ID NO: 29).

For pGST-azu 88-113, carboxyl terminus truncation of *azu* gene was cumulatively performed by introducing stop codon using QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA). For pGST-azu 88-113, a stop codon was introduced into Phe114. The plasmid carrying pGST-azu 88-128 was used as the template. For pGST-azu 88-128 an amplified PCR fragment was introduced into the *BamHl* and *EcoRl* sites of the commercial GST expression vector pGEX-5X (Amersham Biosciences). The fragment was amplified with pUC19-azu as the template and primers, 5'-CGGGGATCC CCG GCT CGG GCG AGA AGG AC-3' (SEQ ID NO: 30) and 5'-CGGGAATTC TCC ACT TCA GGG TCA GGG TG-3' (SEQ ID NO: 31) where the additionally introduced *BamHl* and *EcoRl* sites are underlined respectively.

One set of oligonucleotides for site directed mutagenesis are shown as follows for the preparation of pGST-azu 88-113: 5'-GTT CTT CTG CAC CTA GCC GGG CCA CTC CG-3' (SEQ ID NO: 32) and 5'-CGG AGT GGC CCG GCT AGG TGC AGA AGA AC-3' (SEQ ID NO: 33). pGST-azu 88-113 was used to transform *E. coli* XL-1 Blue strains. Plasmid extraction was performed using a commercial kit (Qiagen, Venlo, The Netherlands) and PCR sequencing were performed to assess plasmid insertion and transfection.

E. coli BL21 (DE3) was used as a host strain for expression of the *gst* and its fusions derivatives. *E. coli* strain XL1-Blue transformed with pGST-azu plasmids was grown in LB media with ampicillin for three hours at 37°C upon which IPTG induction (0.4 mM) was performed an subsequent incubation for 2-4 h at 37°C to maximize the expression levels. Cells were isolated by centrifugation, resuspended in 25 mL of 1X PBS buffer. Subsequent cell lysis involved two sequential treatments of the cell suspension via sonication (20 min on ice) and heat-cold shock in acetone-dry ice bath (using the appropriate protease inhibitors). Supernatants of the cell lysis mixture were isolated and passed through a freshly packed and PBS equilibrated 1 mL glutathione-sepharose 4B (Amersham Biosciences) column. After column washing and subsequent elution of GST-azu product using 10 mM glutathione in 20 mM Tris-HCl pH 8. GST-Azu 88-113 purity was tested via electrophoresis using a 10% SDS-PAGE Tris-Gly gel stained with Coomassie Brilliant Blue R reagent. Protein concentration was determined using the Bradford Method.

Surface Plasmon Resonance (SPR) Studies. *In vitro* protein-protein interactions were evaluated using a Biacore X spectrometer from Biacore AB International (Uppsala, Sweden). All experiments were conducted at 25°C in HBS-EP running buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20)) using Au-CM5 sensor chips purchased from Biacore. Protein stock solutions were prepared in PBS after desalting on G-75 column and lyophilizaiton in order to preconcentrate and exchange the buffer.

Protein immobilizations on CM5 chips were conducted according to the amine coupling procedure. Due to differences in protein crosslinking efficiencies, proteins were immobilized under various conditions after NHS/EDC preactivation of the CM5 surface: 50 µl injections of azurin (510 µM), or 35 µl injections of CD4 (25 µM, 2x), or HIV-1 gp120 (10 µM). Subsequent treatment of CM5 surface with ethanolamine (1M, pH 8.8) removed uncrosslinked proteins prior to binding studies. Binding studies were performed by injecting protein eluents (50 µl) over the protein-CM5 surface at flow rates of 30 µl/min with a 120 sec time delay at the end of the injections. Protein eluents included CD4 (Protein Sciences Corp., Meriden, CT), HIV-1 gp120 (Immunodiagnostics Inc., Woburn, MA), HIV-1 gp41 (Bioclone Inc., San Diego, CA), HIV-1 gag and HIV-1-nef (Chemicon International, Temecula, CA) and GST-azurin fusion proteins (GST, GST-Azu 36-128, GST-Azu 36-89, and GST-Azu 88-113, expressed in inventor's laboratory). Sensor chip surfaces were regenerated between

protein injections using 100 mM NaOH (10 µl injection pulse). All binding studies were run against a negative flow channel containing bare Au-CM5 to correct for nonspecific binding effects. For the binding experiments wherein CD4 and HIV-1 gp120 served as the eluents (not immobilized), 1 mg/mL of carboxymethyldextran (CarboMer Inc., San Diego CA) was added to the running buffer in order to reduce nonspecific protein binding to the bare Au-CM5 flow channel surface.

To generate binding constant data, titration experiments were designed via injection of increasing concentrations of protein eluents (0.05-2000 nM) and the data collected. The SPR data could be fit to a Langmuir equilibrium binding model [Req = Rmax/(1 + Kd/C] form which binding constants (Kd) were determined. Similar to the binding constant studies described above, competition studies with CD4-CM5 were performed using similar protocols but with injections of HIV-1 gp120 + the competitor proteins (azurin, GST-Azu 36-128 and GST-Azu 88-113).

With CD4 immobilized in the sensor chip, both azurin and gp120 showed significant binding to CD4 (Fig. 2A). Azurin demonstrated a higher affinity for binding CD4 (Kd = 36.9 nM) than the HIV-1 ligand gp120 (Kd = 48.1 nM). While a GST-azurin fusion such as GST-Azu 88-113 showed no binding (Fig. 2A), another GST-azurin fusion protein, GST-Azu 36-128 showed even stronger binding than azurin itself with a Kd value of 0.34 nM (Fig. 4A inset), suggesting that parts of azurin might retain a stronger binding affinity than the full length protein. When azurin was immobilized on the sensor chip, gp120 showed somewhat stronger binding to azurin than CD4 (Fig. 2B), clearly demonstrating that azurin binds both to gp120 and CD4 with a high affinity. Interestingly, gp41, also involved in HIV-1 entry into the host cell, did not show any binding to azurin (Fig. 2B). Similar lack of binding was demonstrated for Gag and Nef.

Example 5. Azurin Binding with ICAMs and CD5 as Studied by Surface Plasmon Resonance.

There is a structural similarity between azurin and ICAMs (Table 5) that are known to be involved as receptors HIV-1 infections. (Liao *et al.*,. AIDS Res. Hum. Retroviruses 16:355-366 (2000); Hioe *et al.*, J. Virol. 75:1077-1082 (2001)) ICAM-3 has been implicated in stimulating HIV-1 transcription and viral production, thereby contributing additionally to intracellular viral growth. (Barat *et al.*, J. Virol. 78, 6692-6697 (2004))

Protein-protein interactions as measured by SPR between azurin and ICAMs such as ICAM-1, ICAM-2, ICAM-3 and NCAM were therefore studied. With immobilized azurin on the CM5 chip, ICAM-3 (Fig. 2C, $K_d = 19.5 \pm 5.4$ nM) and NCAM (Fig. 2C, inset), but not ICAM-1 and ICAM-2, showed strong binding. While not limiting the operation of the invention to any one mechanism, part of azurin suppression of HIV-1 growth might also be mediated through its interaction with ICAM-3 or NCAM.

Example 6. Azurin Compeition with gp120 for CD4 as Studied by Surface Plasmon Resonance.

Due to the higher affinity of binding of azurin to CD4, as compared to gp120 (Fig. 2A), a competition experiment was performed to see if azurin can interfere in gp120 binding with its cognate receptor CD4. As the concentration of the competitor protein (azurin, GST-Azu 36-128 or GST-Azu 88-113) was increased in presence of a fixed concentration of gp120 adsorbed to the immobilized CD4 chip, both azurin and GST-Azu 36-128 demonstrated significant decrease in the total protein binding of gp120 from the CD4-CM5 chip (Fig. 2D). Such apparent displacement of gp120 from the chip was not observed in case of GST-Azu 88-113 (Fig. 2D). GST-Azu 88-113 is known not to bind CD4 (Fig. 2A). While not limiting the operation of the invention to any one mechanism, this indicates that azurin or GST-Azu 36-128 fusion protein may successfully inhibit the complex formation between gp120 and CD4.

Example 7. Azurin and ICAM-3 Binding with DC-SIGN as Studied by Surface Plasmon Resonance.

The strong binding of azurin with gp120, CD4 and ICAM-3 (Fig. 2) mimics the binding of another very important HIV-1 binding protein present on the surface of dendritic cells (DC) known as DC-SIGN (DC-specific intercellular adhesion molecule 3-grabbing nonintegrin) and a related protein called DC-SIGN/R. DC-SIGN is expressed abundantly on DC while DC-SIGN/R is expressed primarily on sinusoidal and endothelial cells. DC-SIGN plays a major role in HIV-1 immunopathogenesis by allowing DC, which are professional antigen presenting cells, to capture and present pathogens including HIV-1 to resting T cells through their interactions with ICAM-3 on the T cell surface. (Geijtenbeek *et al.*, Cell 100, 575-585 (2000); Soilleux, Clin. Sci. 104, 437-446 (2003); Geijtenbeek *et al.*, Placenta 22,

S19-S23 (2001)). DC-SIGN has also been shown to bind avidly to HIV-1 envelope protein gp120, thereby capturing HIV-1 and transporting it to $CD4^+$ T cells, where HIV-1 can replicate freely. (Snyder *et al.*, J. Virol. 79:4589-4598 (2005))

In SPR experiments with immobilized DC-SIGN on the sensor chip, both azurin (K_d=0.83±0.05 nM) and ICAM-3 (K_d=0.93± 0.39 nM) bound strongly to DC-SIGN (Fig. 3A). While the GST-fusion derivative GST-Azu 36-89 showed very little binding (Fig. 3A), another GST-fusion derivative GST-Azu 88-113 exhibited relatively strong binding (K_d=5.98±1.13 nM), demonstrating the involvement of the C-terminal part of azurin in DC-SIGN binding (Fig. 3B). GST-Azu 88-113, however, does not bind with CD4 (Fig. 2A), suggesting that different parts of azurin have different binding specificities.

While not limiting the operation of the invention to any one mechanism, such binding with DC-SIGN demonstrates azurin's potential ability to interfere in the binding of HIV-1 with DCs. Thus DC-SIGN, a critical molecule on DC surface responsible for transmitting HIV-1 from the mucosal cells to the lymphoid T cells, may well find a strong competitor in azurin or Laz that can also avidly bind gp120, CD4 and ICAM-3.

Example 8. Azurin/Laz Acts in the Entry Stage of HIV-1 Infection.

To determine if azurin acts at the entry or post entry step of HIV-1 infection, the effect of Laz on the Indian isolate IN/2167 of HIV-1 was investigated. In one experiment, activated PBMC (25,000 cells/well) were incubated with 6.0 μM Laz and HIV-1 for 2 h. The mixture was centrifuged to remove Laz and HIV-1, fresh medium without Laz was added back, and the culture was grown for 5 days. HIV-1 growth was monitored by measurement of p24 in the culture supernatant. Under this condition, Laz (6.0 μM) suppressed the HIV-1 growth by 43%. With higher concentration of Laz (30 μM), the extent of suppression was 76%. In a parallel experiment, when the Laz (6 or 30 μM) was added to the PBMC after the HIV-1 infection and its removal, very little suppression of viral growth was observed. As a positive control, when Laz (6.0 μM) was present both during infection and after removal of the virus with fresh medium during 5 days of the culture, the extent of inhibition was about 93%. While not limiting the operation of the invention to any one mechanism. such data clearly indicate that azurin or Laz exerts its effect primarily at the entry stage of infection.

Example 9: Treatment of HIV-infected patients with azurin

Twenty four patients with AIDS presents with low to non-detectable HIV viral loads (RNA PCR) in the plasma as measured by PCR techniques, and increased CD4+ counts. Next, CD4+RO+ cells are enriched by magnetic separation and FACS sorting, and assayed to determine infectivity with respect to naive and uninfected cell co-culture experiments. This analysis of CD4+RO+ memory cells shows the presence of infective HIV.

Azurin is therefore administered to twenty of the patients at a dose of 4 mg/m^2 by intravenous infusion once every two weeks for a period of 3 months until CD4+ cells, including memory cells, are at low levels. Four patients receive placebo infusions. During administration of azurin and for a period of approximately 1-2 months thereafter, or until CD4+ cells recover, the patients are maintained with antibiotics and antifungal therapy. Stem cell or precursor cell replacement is provided through a bone marrow transplant and cytokine therapy, both of which are performed according to conventional techniques.

During and following therapy, the patients are followed at frequent intervals and monitored for CD34 cell level, reestablishment of CD4+ cells and quantitation of CD4+RO+ cells. Additionally, the patients' plasma is assayed for viral load by cell co-culture experiments. On reducing virus load in active and memory CD4+ T cells to low or non-detectable concentrations, the patients are weaned from azurin. After 3 months, the patients are weaned from antibiotic and antifungal therapy. Following this, the patients are followed at 6 month intervals and assayed for viral content. The results demonstrate the effectiveness of azurin therapy for patients with HIV infection.

What is claimed:

1. An isolated peptide that is a variant, derivative or structural equivalent of a cupredoxin or cytochrome c_{551}; and that can inhibit the growth of HIV-1 infection in mammalian cells.

2. The isolated peptide of claim 1, wherein the cupredoxin is selected from the group consisting of azurin, pseudoazurin, plastocyanin, rusticyanin, Laz and auracyanin.

3. The isolated peptide of claim 2, wherein the cupredoxin is azurin or Laz.

4. The isolated peptide of claim 1, wherein the cupredoxin is from an organism selected from the group consisting of *Pseudomonas aeruginosa, Alcaligenes faecalis, Achromobacter xylosoxidan, Bordetella bronchiseptica, Methylomonas sp., Neisseria meningitidis, Neisseria gonorrhea, Pseudomonas fluorescens, Pseudomonas chlororaphis, Xylella fastidiosa* and *Vibrio parahaemolyticus.*

5. The isolated peptide of claim 4, that is from *Pseudomonas aeruginosa* or *Neisseria gonorrhea.*

6. The isolated peptide of claim 1, which is part of a peptide selected from the group consisting of SEQ ID NOS: 1-17.

7. The isolated peptide of claim 1, to which a sequence selected from the group consisting of SEQ ID NOS: 1-17 has at least 90% amino acid sequence identity.

8. The isolated peptide of claim 1, which is a truncation of cupredoxin or cytochrome c_{551}.

9. The isolated peptide of claim 1, wherein the peptide is more than about 10 residues and not more than about 100 residues.

10. The isolated peptide of claim 9, wherein the peptide comprises a region of azurin selected from the group consisting of residues 36-128, residues 36-88 and residues 88-113.

11. The isolated peptide of claim 10, wherein the peptide consists of a region of azurin selected from the group consisting of residues 36-128, residues 36-88 and residues 88-113.

12. The isolated peptide of claim 1, wherein the peptide comprises equivalent residues of a cupredoxin as a region of azurin selected from the group consisting of residues 36-128, residues 36-88 and residues 88-113.

13. A composition, comprising at least one cupredoxin, cytochrome c_{551}, or peptide of claim 1 in a pharmaceutical composition.

14. The composition of claim 13, wherein the pharmaceutical composition is formulated for intravenous administration.

15. The composition of claim 13, wherein the cupredoxin is from an organism selected from the group consisting of *Pseudomonas aeruginosa, Alcaligenes faecalis, Achromobacter xylosoxidan, Bordetella bronchiseptica, Methylomonas sp., Neisseria meningitidis, Neisseria gonorrhea, Pseudomonas fluorescens, Pseudomonas chlororaphis, Xylella fastidiosa* and *Vibrio parahaemolyticus.*

16. The composition of claim 15, wherein the cupredoxin is from *Pseudomonas aeruginosa* or *Neisseria gonorrhea.*

17. The composition of claim 13, wherein the cupredoxin or cytochrome c_{551} is selected from the group consisting of SEQ ID NOS: 1-17.

18. A method to treat a patient infected with a virus or bacteria, comprising administering to the patient a therapeutically effective amount of the composition of claim 13.

19. The method of claim 18, wherein the patient is infected with a pathogen selected from the group consisting of HIV-1, Herpes simplex virus (HSV), Ebola virus, cytomeglovirus (CMV), parainfluenza viruses types A, B and C, hepatitis virus A, B, C, and G, the delta hepatitis virus (HDV), mumps virus, measles virus, respiratory syncytial virus, bunyvirus, arena virus, Dhori virus, poliovirus, rubella virus, dengue virus; SIV and *Mycobacterium tuberculosis.*

20. The method of claim 19, wherein the patient is infected with HIV-1.

21. The method of claim 18, wherein the patient is human.

22. The method of claim 18, wherein the composition is administered by a mode selected from the group consisting of intravenous injection, intramuscular injection, subcutaneous injection, inhalation, topical administration, transdermal patch, suppository, and oral.

23. The method of claim 22, wherein the mode of administration is by intravenous injection.

24. The method of claim 18, wherein the composition is administered within 0 minutes to 1 week of the administration of at least one drug selected from the group consisting of an anti-viral drug, anti-bacterial drug and an anti-HIV drug.

25. The method of claim 24, wherein the composition is administered at about the same time as another anti-HIV drug.

26. A composition comprising at least two isolated polypeptides selected from the group consisting of a cupredoxin, a cytochrome c_{551}, and variant, derivative or structural equivalent of a cupredoxin or a cytochrome c_{551}.

27. The composition of claim 26 in a pharmaceutical composition.

28. A kit comprising the composition of claim 13 in a vial.

29. The kit of claim 28 , wherein the kit is designed for intravenous administration.

30. A method, comprising contacting the cells with a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof; contacting mammalian cells with a bacterium or virus; and measuring the growth of infection of the virus or bacterium.

31. The method of claim 30, wherein the step of contacting the cells with a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof occurs before the step of contacting the cells with a bacterium or virus.

32. The method of claim 30, wherein the step of contacting the cells with a cupredoxin or cytochrome c_{551}, or variant, derivative or structural equivalent thereof occurs after the step of contacting the cells with a bacterium or virus.

33. The method of claim 30, wherein the cells are human cells.

34. The method of claim 30, wherein the cells are selected from the group consisting of lymphoma cells and peripheral blood mononuclear cells.

35. The method of claim 30, wherein the virus or bacterium is selected from the group consisting of HIV-1, Herpes simplex virus (HSV), Ebola virus, cytomeglovirus (CMV), parainfluenza viruses types A, B and C, hepatitis virus A, B, C, and G, the delta hepatitis virus (HDV), mumps virus, measles virus, respiratory syncytial virus, bunyvirus, arena virus, Dhori virus, poliovirus, rubella virus, dengue virus, SIV and *Mycobacterium tuberculosis*.

36. The method of claim 35, wherein the virus is HIV-1.

37. An expression vector, which encodes the peptide of claim 1.

38. An isolated peptide that is a variant, derivative or structural equivalent of a cupredoxin; and that can bind a protein selected from the group consisting of CD4, gp120, ICAM3 and DC-SIGN.