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UNITED STATES OF AMERICA
Before the
SECURITIES AND EXCHANGE COMMISSION

In the Matter of

IMMUNOTECH LABORATORIES, INC.

Administrative Proceeding
File No. 3-16321

DIVISION OF ENFORCEMENT'S MOTION TO PROVIDE EXHIBITS INADVERTENTLY NOT ATTACHED TO BRIEF

On February 3, 2015, the Division of Enforcement (the "Division") filed an Opposition to Petitioner Immunotech Laboratories, Inc.'s Opening Brief in Support of Its Petition for Termination of Trading Suspension ("Division Opposition"). On April 21, 2015, the Office of the Secretary for the U.S. Securities and Exchange Commission notified the Division that the Division Opposition failed to provide exhibits cited to in the Division's Brief. Pages 4 and 5 of the Division Opposition cite to two press releases issued by Immunotech Laboratories, Inc. on October 9 and 21, 2014 pertaining to its purported relationship with the Zimbabwean company, Uldic Investment Pvt. Ltd. The Division Opposition cites these press releases as Exhibits 4 and 5 respectively. In addition, footnote 2 of the Division Opposition cites to patents labeled as Exhibits 2 and 3. Attached to this motion are Exhibits 2-5 that were not previously provided.

Respectfully submitted,
DIVISION OF ENFORCEMENT
By its attorneys:

Deena R. Bernstein
Amy Gwiazda
Lauchlan Wash
U.S. Securities and Exchange Commission
33 Arch Street, 23rd Floor
Boston, MA 02110
(617) 573-8813 (Bernstein)
bernsteind@sec.gov

Date: April 22, 2015

CERTIFICATE OF SERVICE

I hereby certify that, on April 22, 2015, I served copies of the foregoing Motion to Provide Exhibits not Previously Provided, by facsimile transmission and UPS upon the following parties:

The Commission
Office of the Secretary
Securities and Exchange Commission
100 F. Street, N.E.
Washington, D.C. 20549
(fax) 202-772-9324

Counsel for Respondent
Adam S. Tracy
Securities Compliance Group, Inc.
520 W. Roosevelt Road #210
Wheaton, IL 60187
(fax) 630-689-9471

Lauchlan Wash

EXHIBIT 2

United States Patent	**7,479,538**
Zhabilov	**January 20, 2009**

Irreversibly-inactivated pepsinogen fragment and pharmaceutical compositions comprising the same for detecting, preventing, and treating HIV

Abstract

An isolated antiviral peptide is characterized by the amino acid sequence GDEPLENYLDTEYF and a significant in vitro binding affinity for HIV-1 gp 120 and gp 41, and human CD4 cells. The peptide exhibits anti-retroviral activity in vivo, particularly anti-HIV-1 activity.

Inventors: **Zhabilov; Harry H.** (San Marino, CA)
Assignee: **The Zhabilov Trust** (San Marino, CA)
Family ID: **36588580**
Appl. No.: **11/177,427**
Filed: **July 11, 2005**

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US 20060104992 A1	May 18, 2006

Current U.S. Class:	**530/327**; 435/174; 435/183; 435/5; 435/7.1; 530/300
Current CPC Class:	A61K 38/10 (20130101); C07K 14/47 (20130101); C07K 7/06 (20130101); G01N 2333/16 (20130101)
Current International Class:	A61K 38/04 (20060101); A61K 38/10 (20060101); A61K 38/17 (20060101); C07K 7/04 (20060101); C07K 7/08 (20060101); G01N 33/53 (20060101)
Field of Search:	;530/300

References Cited [Referenced By]

U.S. Patent Documents

5002766	March 1991	Ransberger et al.
5436143	July 1995	Hyman
5872210	February 1999	Medabalimi
6165794	December 2000	Craik et al.
6534310	March 2003	Craik et al.
6979566	December 2005	Friedman et al.
7244606	July 2007	Chou et al.
2002/0192797	December 2002	Dash et al.
2004/0005557	January 2004	Padigaru et al.
2004/0005691	January 2004	Chou et al.
2004/0038330	February 2004	Nagaoka

Foreign Patent Documents

678202	Dec 1995	AU
07316191	Dec 1995	JP

Other References

Xin et al. J. Biol. Chem. 1989, vol. 264, pp. 4482-4489. cited by examiner .
Yoshimasu et al. Protein Expression and Purification, 2002, vol. 25, pp. 229-236. cited by examiner .
U.S. Appl. No. 10/336,512, filed Jan. 29, 2004, Zhabilov. cited by other.

Primary Examiner: Campell; Bruce
Assistant Examiner: Li; Bao Qun
Attorney, Agent or Firm: Cislo & Thomas, LLP

Claims

What is claimed is:

1. An isolated peptide with antiviral efficacy consisting of the amino acid sequence of SEQ ID NO: 1, said peptide having substantial in vitro binding affinity for human immunodeficiency virus type-1 (HIV-1), gp 120, HIV-1 gp 41 and HIV-1 infected human CD4 cells.

2. A composition including a carrier and a peptide with antiviral efficacy, said peptide consisting of the amino acid sequence of SEQ ID NO: 1 and having substantial in vitro binding affinity for HIV-1 gp 120, HIV-1 gp41 and HIV-1 infected human CD4 cells.

3. A complex of the peptide of claim 1 with HIV-1 gp41, HIV-1 gp120, or HIV-1 infected human CD4+ cells.

4. A complex of the peptide of claim 1 with HIV-1 gp41.

5. A complex of the peptide of claim 1 with HIV-1 gp120.

6. A composition comprising a carrier and the complex of claim 4.

7. A composition comprising a carrier and the complex of claim 5.

Description

BACKGROUND OF THE INVENTION

Typically, infection with the human immunodeficiency virus, HIV-1, eventually causes acquired immunodeficiency syndrome (AIDS) and an associated syndrome, AIDS-related complex (ARC). Neutralizing this virus has proved difficult, largely because its structure obstructs immune system access to viral epitopes and its genetic material is highly variable. Accordingly, researchers have been seeking prophylactic and therapeutic methods for preventing or controlling HIV which are not dependent upon antibody-mediated immunity.

The HIV retrovirus replicates in certain immune system cells, specifically the CD4+ subset of T-lymphocytes (pre-Th cells arising in the thymus). In the usual course of a cell-mediated immune response to an intracellular pathogen such as a virus, dendritic cells (antigen-presenting cells) carrying antigen fragments and secreted cytokines activate these CD4+ T-cells. Activated cells, called T-helper or Th cells, in turn secrete their own cytokines and stimulate macrophages. CD4+Th cells also propagate cellular immune response by binding chemotactic cytokines (chemokines, CCs) to their CC surface receptors. It is by this route that HIV-1 infection of these cells is enabled because, in addition to binding chemokines, these CC receptors act together with the CD4+ surface glycoprotein as coreceptors for HIV-1 and mediate entry of the virus into the CD4+Th cell. There, the virus usurps the native genetic material for viral replication while destroying cell functions essential for building immunity; the increasing destruction of these cells appears to be responsible for the eventual collapse of the cell-mediated immune system often seen in terminal AIDS patients.

It has been recognized that denying entry into CD4+ cells to the HIV-1 virus could at least slow the progress of the infection and alleviate, if not cure, the disease and/or its symptoms. The complex mechanism by which the virus crosses the cell membrane has been widely investigated. Broadly, the entry of human immunodeficiency virus into, for example, CD4+ Th1 cells (T-helper type 1 cells, is dependent upon a sequential interaction of the gp120/gp41 subunits of the viral envelope glycoprotein gp160 with the CD4+Th1 cell surface glycoprotein and the cell surface receptor CCR5. On binding of gp120 with its cell surface binding sites, a conformational change in the latent gp41 subunit through an intermediate state to an active state is initiated, inducing fusion of the viral and cellular membranes and transport of the virus into the cell (Nature 387:426-30, 1997).

Accordingly, numerous binding experiments have been conducted in an effort to find antiviral ligands that will effectively compete with the HIV-1 for CD4+ gp and/or CCR5 binding sites, or that will preferentially block gp120 and/or gp41 binding domains. In one example, a reported structure (X-ray crystallography) comprising a HIV-1 gp120 core complexed with a two-domain fragment of human CD4 and an antigen-binding fragment of a neutralizing antibody that blocks chemokine-receptor binding, is said to reveal a CD4-gp120 interface, a conserved binding site for the chemokine receptor, evidence for a conformational change on CD4 binding, the nature of a CD4-induced antibody epitope, and specific mechanisms for viral immune evasion, "which should guide efforts to intervene" (Nature 393 (6686):632-1, 1998). Also, it has been shown that inhibition of the change in structure of gp41 from its intermediate to active state with peptides used as competitors for critical cell receptors may reduce viral load, and that while gp120 masks epitopes on the gp41 subunit in its latent state, gp41 may be vulnerable to neutralizing antibodies in its transient or intermediate state (Molecular Membrane Biology 16:3-9, 1999). In another study, disclosed in US Patent Application Publication US 2004/0018639 A1, filed Jan. 3, 2003, published Jan. 29, 2004, by Zhabilov et al., the content of which is incorporated herein in its entirety by reference, a protein designated "Thymus Factor" ("TF") is stated to have the ability to bind to a fragment of HIV-1 gp41 in gel electrophoresis, and that this binding property can be used to assay TF activity or in the treatment of HIV.

BRIEF DESCRIPTION OF THE DRAWINGS

Some aspects of the present invention are generally shown by way of reference to the accompanying drawings in which:

FIG. 1 illustrates the porcine pepsinogen sequence (Seq. No. 1 bolded), and major and minor sequences of this pepsinogen;

FIG. 2 is a photograph of an electrophoresis gel showing an inactivated pepsinogen fragment ("IPF") in the 45.0 kDa band;

FIG. 3 is a Biacore graph showing a HPLC (High Performance Liquid Chromatography) chromatogram of an isolated IPF in accordance with the present invention;

FIGS. 4, 5, 6, and 7 illustrate exemplary binding of four samples of IPF with gp41, gp120, human CD4, and human serum at 3 different dilutions; and

FIG. 8 is a photograph of an electrophoresis agarose gel showing bound IPF and gp41.

SUMMARY OF THE DISCLOSURE

Some embodiments of the present invention are generally directed to providing an isolated antiviral peptide characterized by the amino acid sequence GDEPLENYLDTEYF (SEQ ID:NO 1) and a significant in vitro binding affinity for HIV-1 gp 120, gp 41 and human CD4 cells. The peptide has anti-retroviral activity in vivo, particularly anti-HIV-1 activity. The peptide, referred to herein as IPF (Inactivated Pepsinogen Fragment), was isolated from porcine pepsinogen, purified, and irreversibly inactivated for use in HIV-1 prophylactic, therapeutic and diagnostic procedures.

Other embodiments of the present invention are generally directed to providing pharmaceutical compositions comprising IPF and methods for preventing, treating, and diagnosing HIV-1 infections and HIV-1 related conditions such as AIDS (Acquired Immune Deficiency Syndrome) and ARC (AIDS Related Complex) with these compositions.

DETAILED DESCRIPTION OF THE INVENTION

Pepsins (of which there are several isozymes) are the principal proteases in gastric secretions of adult mammals. They belong to the family of aspartic proteases and are synthesized and secreted by cells in the gastric mucosa as inactive enzyme-precursors consisting of a signal peptide, an activation peptide and an occluded active enzyme. En route to the lumen of the stomach for protein digestion, the signal peptide is cleaved to yield the inactive proenzyme pepsinogen, which, on exposure to a low gastric pH (<4), cleaves in turn to yield mature, catalytically active pepsin.

Porcine pepsin was one of the first enzymes to be studied, and is perhaps the best-understood aspartic protease. It has 327 amino acid (aa) residues, and a molecular mass of 34kDa (PNAS (U.S.) 70:3437-39 1973). Proteolytic activity of pepsin is at its highest at a pH of about 1.8 to 3.5; it is inactivated at a pH of about 5 and irreversibly inactivated (denatured) at a pH of about 6-7. Owing to their importance, amino acid residues associated with the substrate binding (active) site have been a research focal point. However, it is apparently still not clear how much functional activity, if any, is influenced by the remainder of the peptide.

The family of aspartic proteases (aspartases) is characterized by aspartic acid residues at their active (catalytic) sites. Human pepsin, for example, has two active site aspartate residues (coded "D" or "Asp"). This family also includes the HIV protease (and its numerous variants), comprising two identical chains each having a single active-site aspartate residue. Essential for maturation of the newly synthesized virus, which is expressed as a polyprotein, this protease has become a popular target for researchers attempting to block HIV replication.

The peptide of the present invention, characterized by the amino acid sequence GDEPLENYLDTEYF (-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr-Leu-Asp-Thr-Glu-Tyr-Phe-), has been shown to bind in vitro with the gp41 and gp120 subunits of HIV-1 and human CD4 cells, and is expected to have anti-retroviral activity in vivo, particularly inhibition of HIV-1 entry into human CD4+ cells.

The exemplified peptide was obtained from porcine pepsinogen (FIG. 1) by isolation from a 45 kDa band of IPF preparation under gel electrophoresis (FIG. 2, Examples hereinbelow). It can also be derived from pepsinogen from any other source containing this sequence, or from any other peptides or proteins containing this sequence whereby suitable source pepsinogens are readily available commercially. Common laboratory methods and reagents for selectively cleaving intact proteins and for isolating and sequencing the cleaved peptides, such as the Erdman degradation process, may be used. The peptide may also be produced by peptide synthesis, using conventional methods. Moreover, genetically engineered constructs expressing the sequence of interest are generally preferred, although chemical syntheses may also be used. The peptides in the IPF fractions may be isolated and concentrated by any one of several techniques well-known to those skilled in the art, such as ammonium sulfate precipitation. The produced peptide isolate may be purified by standard processes such as gel filtration and RP-HPLC, and inactivated, as discussed supra, by exposure to a neutral-to-alkaline environment of about pH 6.5 or greater or as otherwise known in the art. The peptide may also be alkylated to increase immunogenicity if desired, for example, by the process described for methylation of TF in U.S. Patent Application Publication US 2004/0018639 A1, supra. A HPLC chromatogram of the purified, inactivated IPF product of the invention is shown in FIG. 3.

Homologues or analogues of the sequence which conserve at least critical binding site amino acid structures and functions and also conserve any distal structural/functional residues essential for binding activity, as described herein, may be substituted for the IPF of SEQ ID:NO 1. Variants of the sequence, including chemically modified derivatives, having a high sequence similarity will be generally preferred, provided that binding activity is not significantly adversely affected. Residues superfluous to the disclosed function of the peptide of the invention may be deleted or added with the same proviso. Modified sequences may be evaluated for conserved binding activity by, for example, following the binding assays described herein or in the literature. Numerous databanks are accessible for protein sequence analysis, such as those which combine sequence similarity with fold recognition to predict functional equivalents. Binding properties (affinity, specificity, etc.) may also be evaluated by the binding assays described below or other conventional assays, as known in the art.

IPF demonstrates binding in vitro with nonglycolysed fragment 579-601 of the HIV-1 envelope protein gp41 subunit (FIGS. 4 and 8), with gp120 HIV-1 subunit (FIG. 5), with human CD4+ cells (FIG. 6), and with human serum (FIG. 7) under gel electrophoresis. The spontaneous binding of IPF with the gp41 subunit is a particularly important biological property. Separately, under simple agarose electrophoresis, IPF and gp41 move in opposite directions. However, when they are mixed prior to electrophoresis, gp41 changes direction and takes the direction of IPF. Quantitative analysis showed that the binding capacity ratio of IPF to gp41 was 1:0.66. That is, three molecules of IPF bound two molecules of gp41 to form a complex which may function in vivo as, for example, a superantigen with significant anti-HIV-1 biological activity. Such antigen can be used as a bioassay reagent, in the production of mono- or polyclonal antibodies, in the manufacture of vaccines, and in other applications wherein antigens are conventionally employed. While the mechanism of these binding events is not completely understood at this time, it is contemplated that exposure of HIV-1 to the IPF of the present invention will effectively block gp41 and gp120 domains essential for viral entry into CD4+ cells and inhibit viral infection, in vivo and in vitro. It is also contemplated that the IPF of the present invention will effectively compete with HIV-1 for its CD4+ cell surface binding sites and inhibit virus entry into these cells, in vivo and in vitro. Various in vitro protocols are known in the art for predicting in vivo antiviral activity of compounds for inhibiting replication of HIV, and these protocols may be used in connection with the practice of the present invention. Exemplary art-recognized anti-HIV screening assays are cited in U.S. Pat. No. 5,869,522, issued 9 Feb. 1999 to Boyd et al., including those described in J.Virol.Methods, 33:87-100,1991; J.Natl.Cancer Inst., 81:577-586, 1992; and J.Med.Chem. 35:1978-1986, 1992, and Boyd, M. R., in AIDS Etiology: Diagnosis, Treatment, and Prevention, pp305-319 (Lippincott, 1988, DeVita, V. T., Jr., et al., eds). In accordance with one aspect of the present invention, IPF is used to diagnose viral infection, particularly HIV-1 infection. Bioassays suitable for this purpose are well-known and routine. Typical of these are assays based on competitive binding between, for example, a known amount of a viral protein and a biological sample to be tested for the same viral protein, using an excess of antiviral reagent capable of specifically binding with the known protein, such as an antibody. A mixture of these is incubated and the amount of bound complex calculated and compared to that in a control mixture lacking the sample. The presence, if any, and amount of the viral protein in the sample can then be determined. There are numerous variations on this process, such as sandwich assays, assays with immobilized reagent, assays using labeled reagent (e.g., ELISA, RIA, FIA), and so on. Whatever the variation, whether for detecting or quantifying complex, or for additional reagents, or other modification, they all require a binding agent for the unknown sample. Any of these routine binding assays for quantifying or identifying an unknown sample may thus be used in the practice of the present invention by substituting IPF as the antiviral binding agent for samples to be tested for HIV-1 gp120, gp41, or infected CD4+T- cells.

In accordance with another aspect of the present invention, IPF is used as a prophylactic or therapeutic to prevent or to treat HIV infections. (Herein the term "HIV infections" refers to AIDS and ARC in addition to viral infection per se unless otherwise noted). For in vivo use, IPF may be prepared for administration by mixing it at the desired degree of purity with a pharmaceutically-acceptable carrier suitable for the route of administration, as well-known in the art. Although IPF is desirably administered with an adjuvant in some applications, in situations where a series of IPF doses are administered, boosters with IPF may not require adjuvant. Intramuscular or subcutaneous injections are presently the contemplated route for both therapeutic and prophylactic administration of IPF. However, intravenous delivery, delivery via catheter or other surgical tubing, or other parenteral route may also be used. Alternative routes include oral routes for administering tablets, liquid formulations and the like, as well as inhalation routes. Liquid formulations reconstituted from powder formulations may be utilized. IPF may also be administered via microspheres, liposomes, or other microparticulates, and via delivery systems or sustained release formulations dispersed in certain tissues including blood.

The dosage of IPF administered will depend upon the properties of the formulation employed, e.g., its binding activity and in vivo plasma half-life, the concentration of IPF in the formulation, the administration route, the site and rate of dosage, the clinical tolerance of the patient involved, the patient's condition, and other considerations, as known in the art. Different dosages may be utilized during a series of sequential treatments. The practitioner may administer an initial dose and then boost with relatively smaller doses of IPF. The dosages of IPF may be combined with other HIV antivirals, such as AZT.

The following is an example of a contemplated IPF formulation, dosage and administration schedule:

The patient is administered an intramuscular injection containing 8 mg of IPF (preferably 2 ml of a formulation containing 6 mg/ral of IPF in a pharmaceutically acceptable solution) or 57 .mu.g of IPF protein per kg body weight of the patient. Each treatment course consists of 16 injections, with two injections on consecutive days per week for 8 weeks. Three months after the last injection, if the patient's condition warrants, the treatment regimen is repeated. The treatment regimen may be repeated until satisfactory results are obtained, e.g., a halt or delay in the progress of the infection or disease, an alleviation of the infection or disease, or a cure is obtained. Preferably, in this application, IPF will be formulated with an aluminum hydroxide (Al(OH)3) adjuvant. Aluminum hydroxide is a widely used adjuvant, especially in commercial products such as vaccines. It is well suited for strong antigens. Many sources of aluminum hydroxide are available. The adjuvant is commercially available under the trade name Alhydrogel.RTM. by Accurate Chemical & Scientific Co. of Westbury, N.Y. In one example, the final 1 ml of the final IPF formulation may contain: 4 mg IPF (purity > 96% .+-.0.290); 2.26 mg 0.016M AlPO.sub.4 (or 0.5 mg A1.sup.+3); 4.1 mg 0.004M CH.sub.3COONa; and 12.9 mg C.sub.6H.sub.5O.sub.7 (sodium citrate); pH 6.2. In one regimen, 2 ml of this formulation makes up one vial with the dosage per patient per day being 16 vials. During the regimen, the patient should be monitored to assess the effectiveness of the regimen. CD+4 cell counts are useful and common methodology for evaluating HIV infection, as are assays for antibody or T-cell titers.

EXAMPLES

Isolation and Purification of Irreversibly-Inactive Pepsin Fraction

The following Examples show the isolation, purification, and characterization of IPF from active pig pepsinogen. Also illustrated is IPF binding activity.

Example I

Isolation and Inactivation of Pepsinogen Fragment

All the buffers and solutions used in this section were sterilized by filtration. If needed, the buffers (0.2 N or 0.1 N HCl) were used to adjust the solutions. All the chemicals, including the distilled water, for the preparation of the buffers and solutions were USP Grade. The ratio of the pepsin to the buffers was 1:4 (weight/weight).

IPF was isolated from active pepsin (Sigma 1:10000) by ammonium sulfate precipitation with centrifugation at 4.degree. C. The lyophilized pepsin powder was dissolved in 0.14M sodium chloride (NaCl), 0.05M sodium acetate (CH.sub.3COONa . 3H.sub.2O), 0.05M sodium citrate (C.sub.6H.sub.5O.sub.7Na.sub.2.2H.sub.2O), and 0.20N HCl (pH 2.8-3.2) buffer. The pH of the active pepsin suspension was then increased to 6.2-6.6 and the suspension was incubated for 30 minutes. The suspension was then precipitated with a saturated solution of (NH.sub.4).sub.2SO.sub.4. After degradation, the mixture was centrifuged (8000 RPM at 4.degree. C.) for 60 minutes and the supernatant discarded. The pellet was dissolved in a minimum quantity of 0.14M NaCl, and the resulting solution was dialyzed for 18 hr against dialysis buffer: 0.1M NaCl, 0.1M sodium acetate, and 0.02M thimerozal USP, pH 6.8.

Example II

Purification and Recovery of Irreversibly Inactivated Pepsinogen Fragment

The purification of IPF included the following steps: dialysis, centrifugation, gel filtration, and reversed phase HPLC.

After dialysis, the low molecular weight dialysate was centrifuged at 15,000 rpm at 4.degree. C. for 60 minutes (Beckman rotor) with precipitation of the residual ammonium sulfate. The product was purified by gel filtration to recover purified IPF from the crude mixture, and then purified by filtration on Bio-gel P10 or Sephadex G-75 gels (from Pharmacia Uppsala, Sweden), or 0.2.mu. SFCA membrane (Nalgene Labware, Rochester, N.Y.). Further purification was achieved by reversed phase high-performance liquid chromatography in an RP-HPLC system GOLD (Beckman) on C-18 columns (RP Ultrasphere 10 mm Spherical 80 A Preparative 21.2.times.150 mm) using gradient 30% acetonitrile diluted in sterile water, HPLC-grade at 15% methanol HPLC-grade mobile phase. Detection 254 nm; flow rate 0.850 ml/min., solvent at pH 6.8. The final purification step included sterile filtration with Nalgen filters 0.45.mu.. The HPLC elution profile of the product showed one isolated peak, IPF (see FIG. 3).

Example III

Determination of Molecular Weight

Molecular weight was determined by silver stained 13% non-reducing SDS-PAGE using the Laemmli method (Nature 227-680, 1970). The molecular weight standard demonstrated one peptide with a molecular weight of 45.000 KD (FIG. 2). This band was isolated, and HPLC chromatogram (FIG. 3) confirmed a single peptide in the band.

Example IV

Assessment of Binding Activity

Samples of IPF (#18, 19, 20, and 21) were used to detect binding with gp120, gp41, CD4+ cells, and serum from a healthy patient. New chips were coated with these proteins and Biacore assays for binding activity were performed. These samples were diluted to 1:2000, 1:500 and 1:100. The results are shown in FIGS. 4, 5, 6, and 7. Sample #21 bound to all target proteins better than the other samples. The assay used a Biacore (Biacore AB, Uppsala, Sweden) system based on sensor chips which provide surface conditions for attaching molecules of interest, a microfluidic flow system for delivering samples to the surface, and a surface plasma response (SPR) which detects mass concentration at the surface. SPR-based biosensors monitor interactions by measuring the mass of molecules bound to the surface. This response is expressed by resonance units (RU), whereby a change in concentration of 1 pg/mm is equivalent to a change of 0.0001 in the angle of intensity minimum, which equals one RU. The exact conversion factor between RU depends upon the properties of the sensor surface and the nature of the molecule responsible for the change in concentration. The assays demonstrate the formation of superantigen for provoking immune response.

Example V

UV Absorption

Circular dichroism (CD) provides information about the secondary structure of optically active materials. The far-UV or amide region (170-250nm) is dominated by contributions of the peptide bonds, whereas CD bands in the near-UV region (250-300nm) originate from the aromatic amino acids. The UV region of IPF was in the range of 252-260 nm.

A person skilled in the art would appreciate that exemplary embodiments described hereinabove are merely illustrative of the general principles of the present invention. Other modifications or variations may be employed that are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations may be utilized in accordance with the teachings herein. Accordingly, the drawings and description are illustrative and not meant to be a limitation thereof.

Moreover, all terms should be interpreted in the broadest possible manner consistent with the context. In particular, the terms "comprises" and "comprising" should be interpreted as referring to elements, components, or steps in a non-exclusive manner, indicating that the referenced elements, components, or steps may be present, or utilized, or combined with other elements, components, or steps that are not expressly referenced. Thus, it is intended that the invention cover all embodiments and variations thereof as long as such embodiments and variations come within the scope of the appended claims and their equivalents.

SEQUENCE LISTINGS

1

1 1 14 PRT Sus domesticus misc_feature Peptide sequence isolated from porcine pepsinogen 1 Gly Asp Glu Pro Leu Glu Asn Tyr Leu Asp Thr Glu Tyr Phe 1 5 10

* * * * *

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EXHIBIT 3

United States Patent **8,066,982**
Zhabilov **November 29, 2011**

Irreversibly-inactivated pepsinogen fragment and pharmaceutical compositions comprising the same for detecting, preventing and treating HIV

Abstract

An isolated antiviral peptide is characterized by the amino acid sequence GDEPLENYLDTEYF and a significant in vitro binding affinity for HIV-1 gp 120 and gp 41 and human CD4 cells. The peptide exhibits anti-retroviral activity in vivo, particularly anti-HIV-1 activity.

Inventors: **Zhabilov; Harry H.** (San Marino, CA)
Assignee: **The Zhabilov Trust** (San Marino, CA)
Family ID: **36588580**
Appl. No.: **12/321,262**
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Related U.S. Patent Documents

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11177427	Jul 11, 2005	7479538	
60635938	Dec 15, 2004		
60626882	Nov 12, 2004		

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Current International Class:	A61K 48/00 (20060101); C07K 5/00 (20060101)

References Cited [Referenced By]

U.S. Patent Documents

2009/0175838	July 2009	Newell et al.
2010/0034839	February 2010	Newell et al.

Other References

US. Appl. No. 11/973,920, filed Nov. 26, 2009, Agadjanyan et al. cited by other .
Rebecca L. O'Brien et al., "Heat Shock Protein Hsp60-reactive cells: A Large, Diversified T-lymphocyte Subset with Highly Focused Specificity" 5 pgs, Jan. 2, 1992. cited by other .
Srivastava, P. (2002). Interactions of heat shock proteins with peptides and antigen presenting cells Annu. Rev. immunol. 20: 395-425. cited by other .
Suzue K. and Young RA. Heat shock proteins as immunological carriers and vaccines. PubMed PMID 8856990. In: Stress-inducible Cellular Response, ed. U. Fiege, Birkhauser (1996) 77: 451-465. cited by other .
Murray P. and Young RA. Stress and Immunological recognition in host-pathogen interaction. ,J Bacteriol (1992) vol. 174, No. 13, p. 4193-4196. cited by other .
Suto, R. and Srivastava, P.K 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. Science Sep. 15, 1995; 269; 1585-8. cited by other .
Vabulas, R. M., Wagner, H. and Schild, H. (2002). Heat shock proteins as ligands of toll-like receptors. Curr. Topics Microbiol.Immunol. 270; 169. cited by other .
Sigal LJ., Crotty S., Andino R. and Rock KL. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cell requires presentation of exogenous antigen. Nature (1999) 398:77 80. cited by other .
Derky CS. Task force on recurrent respiratory papillomatosis. Arch Otolaryngol Head Neck Surg (1995) 121: 1386-1391. cited by other .
Panjwani, N. N., Popova, L, Febbraio, M and Srivastava, P. K. (2001) CD91 is common receptor for heat shock proteins gp96, HSP 90, HSP70 and calreticulin. Immunity 14:303. cited by other .
Srivastava, P. K., Deleo, A. B. and Old, L. J. Tumor rejection antigens of chemically induced sarcomas of inbred mice. Proc. Nat. Acad. Sci. USA. vol. 83:3407-May 11, 1986. cited by other .
Pockley, G. A. (2001) Heat Shock proteins in health and disease: therapeutic targets or therapeutic agents? Exp. Rev. Mol. Med. 21 September, http://www.ermm.cbcu.cam.ac.uk/01003556h.htm. cited by other .
Parmiani, G. et al., (2004) Heat Shock Proteins and Their Use as Anticancer Vaccines. Clinical Cancer Research. vol. 10, 8142-8146. 15 December. cited by other .
Gritti, I., Banfi, G., and Roi, G.S., Pepsinogens: Physiology, Pharmacology Pathophysiology and Exercise. (2000) Pharmacological Research. vol. 41. 3 November. cited by other .
Sogawa K et al.: "Molecular Cloning of Complementary DNA to Swine Pesinogen

Messenger RNA", Journal of Biological Chemistry, vol. 256, No. 23, 1981, pp. 12561-12565. cited by other .
Tanaka T et al.: "N-terminal portion acts as an initiator of the inactivation of pepsin at neutral pH." Protein Engineering Sep. 2001, p. 669-675, vol. 14, No. 9. cited by other .
Jiang S et al.: "Peptide and Non-Peptide HIV Fusion INhibitors" Current Pharamecutical Design, Bentham Silence Publishers, vol. 8. No. 8, Jan. 2002, p. 563-580. cited by other .
Yu F.I. et al.: "Fluorogenic Peptide Substrates for Assay for Asparty Proteinases" Analytical Biochemistry, vol. 234, Mar. 1996, pp. 113-188. cited by other .
Supplementary European Search Report, EP 05 85 0002, Oct. 1, 2010. cited by other.

Primary Examiner: Li; Bao
Attorney, Agent or Firm: Cislo & Thomas, LLP

Parent Case Text

CROSS-REFERENCE TO RELATED APPLICATIONS

This utility patent application is a divisional application of, and claims priority from, U.S. patent application Ser. No. 11/177,427 filed on Jul. 11, 2005 now U.S. Pat. No. 7,479,538, which is hereby incorporated in its entirety by reference. U.S. patent application Ser. No. 11/177,427 in turn claims priority from U.S. Provisional Application Nos. 60/626,882 (filed on Nov. 12, 2004) and 60/635,938 (filed on Dec. 15, 2004), the disclosures of which are hereby incorporated in their entirety by reference.

Claims

What is claimed is:

1. A method for inhibiting HIV-1 entry into human CD4+ cells comprising the step of contacting at least one HIV-1 envelope protein subunit selected from the group consisting of the HIV-1 gp41 subunit and the HIV-1 gp120 subunit with an isolated irreversibly inactivated peptide consisting essentially of the amino acid sequence of SEQ ID NO. 1, wherein said peptide binds to at least one HIV-1 envelope protein subunit through SEQ ID NO. 1, thereby inhibiting viral entry into said human CD4+ cells.

2. The method of claim 1, further comprising the step of administering to a patient a pharmaceutical composition comprising the isolated irreversibly inactivated peptide consisting essentially of the amino acid sequence of SEQ ID NO. 1.

3. The method of claim 1, further comprising the step of alkylating the isolated irreversibly inactivated peptide consisting essentially of the amino acid sequence of SEQ ID NO. 1 before the step of contacting the at least one HIV-1 envelope protein subunit selected from the group consisting of the HIV-1 gp41 subunit and the HIV-1 gp120 subunit of the HIV-1 envelope protein to increase immunogenicity.

4. The method of claim 1, further comprising the steps of isolating an irreversibly-inactivated pepsinogen fraction from active pepsin by ammonium sulfate precipitation; purifying said isolated irreversibly-inactivated pepsinogen fraction to obtain an inactivated pepsinogen fragment (IPF) consisting essentially of a peptide set forth in SEQ ID NO. 1; contacting at least one HIV-1 envelope protein subunit selected from the group consisting of the HIV-1 gp41 subunit and the HIV-1 gp120 subunit; and binding said IPF peptide to the at least one HIV-1 envelope protein subunit through SEQ ID NO. 1 to thereby inhibit HIV-1 entry into the human CD4+ cells.

5. A method tar inhibiting HIV-1 entry into human CD4+ cells comprising contacting a HIV-1 gp120 subunit of an HIV-1 envelope protein with a composition comprising an isolated irreversibly inactivated peptide consisting of the amino acid sequence of SEQ ID NO. 1 to bind the gp120 subunit envelope protein and thereby inhibit viral entry into said human CD4+ cell.

6. The method of claim 5, further comprising the step of administering to a patient a pharmaceutical composition comprising the isolated irreversibly inactivated peptide consisting of the amino acid sequence of SEQ ID NO. 1.

7. The method of claim 5, further comprising the step of alkylating the isolated irreversibly inactivated peptide consisting of the amino acid sequence of SEQ ID NO. 1 before the step of contacting the HIV-1 gp120 subunit with a composition comprising the isolated irreversibly inactivated peptide consisting of the amino acid sequence of SEQ ID NO. 1.

8. The method of claim 5, further comprising the steps of: isolating an irreversibly-inactivated pepsinogen fraction from active pepsin by ammonium sulfate precipitation; purifying said isolated pepsinogen fraction to obtain an inactivated pepsinogen fragment (IPF) set forth in SEQ ID NO. 1; contacting the HIV-1 gp120 subunit of an HIV-1 envelope protein with said IPF peptide; and binding said IPF peptide to the HIV-1 gp120 subunit of the HIV-1 envelope protein to thereby inhibit HIV-1 entry into the human CD4+ cells.

Description

BACKGROUND OF THE INVENTION

Typically, infection with the human immunodeficiency virus, HIV-1, eventually causes acquired immunodeficiency syndrome (AIDS) and an associated syndrome, AIDS-related complex (ARC). Neutralizing this virus has proved difficult, largely because its structure obstructs immune system access to viral epitopes and its genetic material is highly variable. Accordingly, researchers have been seeking prophylactic and therapeutic methods for preventing or controlling HIV which are not dependent upon antibody-mediated immunity.

The HIV retrovirus replicates in certain immune system cells, specifically the CD4+ subset of T-lymphocytes (pre-Th cells arising in the thymus). In the usual course of a cell-mediated immune response to an intracellular pathogen such as a virus, dendritic cells (antigen-presenting cells) carrying antigen fragments and secreted cytokines activate these CD4+ T-cells. Activated cells, called T-helper or Th cells, in turn secrete their own cytokines and stimulate macrophages. CD4+Th cells also propagate cellular immune response by binding chemotactic cytokines (chemokines, CCs) to their CC surface receptors. It is by this route that HIV-1 infection of these cells is enabled because, in addition to binding chemokines, these CC receptors act together with the CD4+ surface glycoprotein as coreceptors for HIV-1 and mediate entry of the virus into the CD4+Th cell. There, the virus usurps the native genetic material for viral replication while destroying cell functions essential for building immunity; the increasing destruction of these cells appears to be responsible for the eventual collapse of the cell-mediated immune system often seen in terminal AIDS patients.

It has been recognized that denying entry into CD4+ cells to the HIV-1 virus could at least slow the progress of the infection and alleviate, if not cure, the disease and/or its symptoms. The complex mechanism by which the virus crosses the cell membrane has been widely investigated. Broadly, the entry of human immunodeficiency virus into, for example, CD4+ Th1 cells (T-helper type 1 cells, is dependent upon a sequential interaction of the gp120/gp41 subunits of the viral envelope glycoprotein gp160 with the CD4+Th1 cell surface glycoprotein and the cell surface receptor CCR5. On binding of gp120 with its cell surface binding sites, a conformational change in the latent gp41 subunit through an intermediate state to an active state is initiated, inducing fusion of the viral and cellular membranes and transport of the virus into the cell (Nature 387:426-30, 1997).

Accordingly, numerous binding experiments have been conducted in an effort to find antiviral ligands that will effectively compete with the HIV-1 for CD4+ gp and/or CCR5 binding sites, or that will preferentially block gp120 and/or gp41 binding domains. In one example, a reported structure (X-ray crystallography) comprising an HIV-1 gp120 core complexed with a two-domain fragment of human CD4 and an antigen-binding fragment of a neutralizing antibody that blocks chemokine-receptor binding, is said to reveal a CD4-gp120 interface, a conserved binding site for the chemokine receptor, evidence for a conformational change on CD4 binding, the nature of a CD4-induced antibody epitope, and specific mechanisms for viral immune evasion, "which should guide efforts to intervene" (Nature 393 (6686):632-1, 1998). Also, it has been shown that inhibition of the change in structure of gp41 from its intermediate to active state with peptides used as competitors for critical cell receptors may reduce viral load, and that while gp120 masks epitopes on the gp41 subunit in its latent state, gp41 may be vulnerable to neutralizing antibodies in its transient or intermediate state (Molecular Membrane Biology 16:3-9, 1999). In another study, disclosed in U.S. Patent Application Publication US 2004/0018639 A1, filed Jun. 3, 2003, published Jan. 29, 2004, by Zhabilov et al. the content of which is incorporated herein in its entirety by reference, a protein designated "Thymus Factor" ("TF") is stated to have the ability to bind to a fragment of HIV-1 gp41 in gel electrophoresis, and that this binding property can be used to assay TF activity or in the treatment of HIV.

BRIEF DESCRIPTION OF THE DRAWINGS

Some aspects of the present invention are generally shown by way of reference to the accompanying drawings in which:

FIG. 1 illustrates the porcine pepsinogen sequence (Seq. No. 1 bolded), and major and minor sequences of this pepsinogen;

FIG. 2 is a photograph of an electrophoresis gel showing an inactivated pepsinogen fragment "IPF" in the 45.0 kDa band;

FIG. 3 is a Biacore graph showing an HPLC (High Performance Liquid Chromatography) chromatogram of an isolated IPF in accordance with the present invention;

FIGS. 4, 5, 6, and 7 illustrate exemplary binding of four samples of IPF with gp41, gp120, human

CD4, and human serum at 3 different dilutions; and

FIG. 8 is a photograph of an electrophoresis agarose gel showing bound IPF and gp41.

SUMMARY OF THE DISCLOSURE

Some embodiments of the present invention are generally directed to providing an isolated antiviral peptide characterized by the amino acid sequence GDEPLENYLDTEYF (SEQ ID NO. 1) and a significant in vitro binding affinity for HIV-1 gp 120 and gp 41 and human CD4 cells. The peptide has anti-retroviral activity in vivo, particularly anti-HIV-1 activity. The peptide, referred to herein as IPF (Inactivated Pepsinogen Fragment), was isolated from porcine pepsinogen, purified and irreversibly inactivated for use in HIV-1 prophylactic, therapeutic and diagnostic procedures.

Other embodiments of the present invention are generally directed to providing pharmaceutical compositions comprising IPF and methods for preventing, treating, and diagnosing HIV-1 infections and HIV-1 related conditions such as AIDS (Acquired Immune Deficiency Syndrome) and ARC (AIDS Related Complex) with these compositions.

DETAILED DESCRIPTION OF THE INVENTION

Pepsins (of which there are several isozymes) are the principal proteases in gastric secretions of adult mammals. They belong to the family of aspartic proteases and are synthesized and secreted by cells in the gastric mucosa as inactive enzyme-precursors consisting of a signal peptide, an activation peptide and an occluded active enzyme. En route to the lumen of the stomach for protein digestion, the signal peptide is cleaved to yield the inactive proenzyme pepsinogen, which, on exposure to a low gastric pH (<4), cleaves in turn to yield mature, catalytically active pepsin.

Porcine pepsin was one of the first enzymes to be studied, and is perhaps the best-understood aspartic protease. It has 327 amino acid (aa) residues, and a molecular mass of 34 kDa (PNAS (U.S.) 70:3437-39 1973). Proteolytic activity of pepsin is at its highest at a pH of about 1.8 to 3.5; it is inactivated at a pH of about 5 and irreversibly inactivated (denatured) at a pH of about 6-7. Owing to their importance, amino acid residues associated with the substrate binding (active) site have been a research focal point. However, it is apparently still not clear how much functional activity, if any, is influenced by the remainder of the peptide.

The family of aspartic proteases (aspartases) is characterized by aspartic acid residues at their active (catalytic) sites. Human pepsin, for example, has two active site aspartate residues (coded "D" or "Asp"). This family also includes the HIV protease (and its numerous variants), comprising two identical chains each having a single active-site aspartate residue. Essential for maturation of the newly synthesized virus, which is expressed as a polyprotein, this protease has become a popular target for researchers attempting to block HIV replication.

The peptide of the present invention, characterized by the amino acid sequence GDEPLENYLDTEYF (SEQ ID NO: 1) (-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Try-Leu-Asp-Thr-Glu-Try-Phe-), has been shown to bind in vitro with the gp41 and gp120 subunits of HIV-1 and human CD4 cells, and is expected to have anti-retroviral activity in vivo, particularly inhibition of HIV-1 entry in to human CD4+ cells.

The exemplified peptide was obtained from porcine pepsinogen (FIG. 1) by isolation from a 45 kDa band of IPF preparation under gel electrophoresis (FIG. 2, Examples hereinbelow). It can also be derived from pepsinogen from any other source containing this sequence, or from any other peptides or proteins containing this sequence whereby suitable source pepsinogens are readily available commercially. Common laboratory methods and reagents for selectively cleaving intact proteins and for isolating and sequencing the cleaved peptides, such as the Erdman degradation process, may be used. The peptide may also be produced by peptide synthesis, using conventional methods. Moreover, genetically engineered constructs expressing the sequence of interest are generally preferred, although chemical syntheses may also be used.

The peptides in the IPF fractions may be isolated and concentrated by any one of several techniques well-known to those skilled in the art, such as ammonium sulfate precipitation. The produced peptide isolate may be purified by standard processes such as gel filtration and RP-HPLC, and inactivated as discussed supra by exposure to a neutral-to-alkaline environment of about pH 6.5 or greater or as otherwise known in the art. The peptide may also be alkylated to increase immunogenicity if desired, for example, by the process described for methylation of TF in U.S. Patent Application Publication US 2004/0018639 A1, supra. A HPLC chromatogram of the purified, inactivated IPF product of the invention is shown in FIG. 3.

Homologues or analogues of the sequence which conserve at least critical binding site amino acid structures and functions and also conserve any distal structural/functional residues essential for binding activity as described herein may be substituted for the IPF of SEQ ID NO. 1. Variants of the sequence, including chemically modified derivatives, having a high sequence similarity will be generally preferred, provided that binding activity is not significantly adversely affected. Residues superfluous to the disclosed function of the peptide of the invention may be deleted or added with the same proviso. Modified sequences may be evaluated for conserved binding activity by, for example, following the binding assays described herein or in the literature. Numerous databanks are accessible for protein sequence analysis, such as those which combine sequence similarity with the fold recognition to predict functional equivalents. Binding properties (affinity, specificity, etc.) may also be evaluated by the bind assays described below or other conventional assays, as known in the art.

IPF demonstrates binding in vitro with nonglycolysed fragment 579-601 of the HIV-1 envelope protein gp41 subunit (FIGS. 4 and 8), with gp120 HIV-1 subunit (FIG. 5), with human CD4+ cells (FIG. 6), and with human serum (FIG. 7) under gel electrophoresis. The spontaneous binding of IPF with the gp41 subunit is a particularly important biological property. Separately, under simple agarose electrophoresis, IPF and gp41 move in opposite directions. However, when they are mixed prior to electrophoresis, gp41 changes direction and takes the direction of IPF. Quantitative analysis showed that the binding capacity ratio of IPF to gp41 was 1:0.66. That is, three molecules of IPF bound two molecules of gp41 to form a complex which may function in vivo as, for example, a superantigen with significant anti-HIV-1 biological activity. Such antigen can be used as a bioassay reagent, in the production of mono- or polyclonal antibodies, in the manufacture of vaccines, and in other applications wherein antigens are conventionally employed. While the mechanism of these binding events is not completely understood at this time, it is contemplated that exposure of HIV-1 to the IPF of the present invention will effectively block gp41 and gp120 domains essential for viral entry into CD4+ cells and inhibit viral infection, in vivo and in vitro. It is also contemplated that the IPF of the present invention will effectively compete with HIV-1 for its CD4+ cell surface binding sites and inhibit virus entry into these cells, in vivo and in vitro. Various in vitro protocols are known in the art for predicting in vivo antiviral activity of compounds for inhibiting replication of HIV, and these protocols may be used in connection with the practice of the present invention. Exemplary art-recognized anti-HIV screening assays are cited in U.S. Pat. No. 5,869,522, issued 9 Feb. 1999 to Boyd et al., including those described in J. Virol. Methods, 33:87-100, 1991; J. Natl. Cancer Inst., 81:577-586, 1992; and J. Med. Chem. 35:1978-1986, 1992, and Boyd, M. R., in AIDS Etiology: Diagnosis, Treatment, and Prevention, pp 305-319 (Lippincott, 1988, DeVita, V. T., Jr., et al., eds).

In accordance with one aspect of the present invention, IPF is used to diagnose viral infection, particularly HIV-1 infection. Bioassays suitable for this purpose are well-known and routine. Typical of these are assays based on competitive binding between, for example, a known amount of a viral protein and a biological sample to be tested for the same viral protein, using an excess of antiviral reagent capable of specifically binding with the known protein, such as an antibody. A mixture of these is incubated and the amount of bound complex calculated and compared to that in a control mixture lacking the sample. The presence, if any, and amount of the viral protein in the sample can then be determined. There are numerous variations on this process, such as sandwich assays, assays with immobilized reagent, assays using labeled reagent (e.g., ELISA, RIA, FIA), and so on. Whatever the variation, whether for detecting or quantifying complex, or for additional reagents, or other modification, they all require a binding agent for the unknown sample. Any of these routine binding assays for quantifying or identifying an unknown sample may thus be used in the practice of the present invention by substituting IPF as the antiviral binding agent for samples to be tested for HIV-1 gp120, gp41, or infected CD4+T-cells.

In accordance with another aspect of the present invention, IPF is used as a prophylactic or therapeutic to prevent or to treat HIV infections. (Herein the term "HIV infections" refers to AIDS and ARC in addition to viral infection per se unless otherwise noted). For in vivo use, IPF may be prepared for administration by mixing it at the desired degree of purity with a pharmaceutically-acceptable carrier suitable for the route of administration, as well-known in the art. Although IPF is desirably administered with an adjuvant in some applications, in situations where a series of IPF doses are administered, boosters with IPF may not require adjuvant. Intramuscular or subcutaneous injections are presently the contemplated route for both therapeutic and prophylactic administration of IPF. However, intravenous delivery, delivery via catheter or other surgical tubing, or other parenteral route may also be used. Alternative routes include oral routes for administering tablets, liquid formulations and the like, as well as inhalation routes. Liquid formulations reconstituted from powder formulations may be utilized. IPF may also be administered via microspheres, liposomes, or other microparticulates, and via delivery systems or sustained release formulations dispersed in certain tissues including blood.

The dosage of IPF administered will depend upon the properties of the formulation employed, e.g., its binding activity and in vivo plasma half-life, the concentration of IPF in the formulation, the administration route, the site and rate of dosage, the clinical tolerance of the patient involved, the patient's condition, and other considerations, as known in the art. Different dosages may be utilized during a series of sequential treatments. The practitioner may administer an initial dose and then boost with relatively smaller doses of IPF. The dosages of IPF may be combined with other HIV antivirals, such as AZT.

The following is an example of a contemplated IPF formulation, dosage and administration schedule:

The patient is administered an intramuscular or injection containing 8 mg of IPF (preferably 2 ml of a formulation containing 6 mg/ral of IPF in a pharmaceutically acceptable solution) or 57 .mu.g of IPF protein per kg body weight of the patient. Each treatment course consists of 16 injections, with two injections on consecutive days per week for 8 weeks. Three months after the last injection, if the patient's condition warrants, the treatment regimen is repeated. The treatment regimen may be repeated until satisfactory results are obtained, e.g., a halt or delay in the progress of the infection or disease, an alleviation of the infection or disease, or a cure is obtained. Preferably, in this application, IPF will be formulated with an aluminum hydroxide (Al(OH.sub.3) adjuvant. Aluminum hydroxide is a widely used adjuvant, especially in commercial products such as vaccines. It is well suited for strong antigens. Many sources of aluminum hydroxide are available. The adjuvant is commercially available under the trade name of Alhydorgel.RTM. by Accurate Chemical & Scientific Co. of Westbury, N.Y. In one example, the final 1 ml of the final IPF formulation may contain: 4 mg IPF (purity>96%.+-.0.290); 2.26 mg 0.016M AlPO.sub.4 (or 0.5 mg Al.sup.+3); 4.1 mg 0.004M CH.sub.3COONa; and 12.9 mg C.sub.6H.sub.5O.sub.7 (sodium citrate); pH 6.2. In one regimen, 2 ml of this formulation makes up one vial with the dosage per patient per day is 16 vials. During the regimen, the patient should be monitored to assess the effectiveness of the regimen. CD+4 cell counts are useful and common methodology for evaluating HIV infection, as are assays for antibody or T-cell titers.

EXAMPLES

Isolation and Purification of Irreversibly-Inactive Pepsin Fraction

The following Examples show the isolation, purification, and characterization of IPF from active pig pepsinogen. Also illustrated is IPF binding activity.

Example I

Isolation and Inactivation of Pepsinogen Fragment

All the buffers and solutions used in this section were sterilized by filtration. If needed, the buffers (0.2 N or 0.1 N HCl) were used to adjust the solutions. All the chemicals, including the distilled water, for the preparation of the buffers and solutions were USP Grade. The ratio of the pepsin to the buffers was 1:4 (weight/weight).

IPF was isolated from active pepsin (Sigma 1:10000) by ammonium sulfate precipitation with centrifugation at 4.degree. C. The lyophilized pepsin powder was dissolved in 0.14M sodium chloride (NaCl), 0.05M sodium acetate (CH.sub.3COONa.3H.sub.2O), 0.05M sodium citrate (C.sub.6H.sub.5O.sub.7Na.sub.2.2H.sub.2O), and 0.20N HCl (pH 2.8-3.2) buffer. The pH of the active pepsin suspension was then increased to 6.2-6.6 and the suspension was incubated for 30 minutes. The suspension was then precipitated with a saturated solution of (NH.sub.4).sub.2SO.sub.4. After degradation, the mixture was centrifuged (8000 RPM at 4.degree. C.) for 60 minutes and the supernatant discarded. The pellet was dissolved in a minimum quantity of 0.14M NaCl, and the resulting solution was dialyzed for 18 hr against dialysis buffer: 0.1M NaCl, 0.1M sodium acetate, and 0.02M thimerozal USP, pH 6.8.

Example II

Purification and Recovery of Irreversibly Inactivated Pepsinogen Fragment

The purification of IPF included the following steps: dialysis, centrifugation, gel filtration and reversed-phase HPLC.

After dialysis, the low molecular weight dialysate was centrifuged at 15,000 rpm at 4.degree. C. for 60 minutes (Beckman rotor) with precipitation of the residual ammonium sulfate. The product was purified by gel filtration to recover purified IPF from the crude mixture, and then purified by filtration on Bio-gel P10 or Sephadex G-75 gels (from Pharmacia Uppsala, Sweden), or 0.2.mu. SFCA membrane (Nalgene Labware, Rochester, N.Y.). Further purification was achieved by reversed phase high-performance liquid chromatography in an RP-HPLC system GOLD (Beckman) on C-18 columns (RP Ultrasphere 10 mm Spherical 80A Preparative 21.2.times.150 mm) using gradient 30% acetonitrile diluted in sterile water, HPLC-grade at 15% methanol HPLC-grade mobile phase. Detection 254 nm; flow rate 0.850 ml/min., solvent at pH 6.8. The final purification step included sterile filtration with Nalgen filters 0.45.mu.. The HPLC elution profile of the product showed one isolated peak, IPF (see FIG. 3).

Example III

Determination of Molecular Weight

Molecular weight was determined by silver stained 13% non-reducing SDS-PAGE using the Laemmli method (Nature 227-680, 1970). The molecular weight standard demonstrated one peptide with a molecular weight of 45.000 KD (FIG. 2). This band was isolated, and HPLC chromatogram (FIG. 3) confirmed a single peptide in the band.

Example IV

Assessment of Binding Activity

Samples of IPF (#18, 19, 20, and 21) were used to detect binding with gp120, gp41, CD4+ cells, and serum from a healthy patient. New chips were coated with these proteins and Biacore assays for binding activity were performed. These samples were diluted to 1:2000, 1:500 and 1:100. The results are shown in FIGS. 4, 5, 6, and 7. Sample #21 bound to all target proteins better than the other samples.

The assay used a Biacore (Biacore AB, Uppsala, Sweden) system based on sensor chips which provide surface conditions for attaching molecules of interest, a microfluidic flow system for delivering samples to the surface, and a surface plasma response (SPR) which detects mass concentration at the surface. SPR-based biosensors monitor interactions by measuring the mass of molecules bound to the surface. This response is expressed by resonance units (RU), whereby a change in concentration of 1 pg/mm is equivalent to a change of 0.0001 in the angle of intensity minimum, which equals one RU. The exact conversion factor between RU depends upon the properties of the sensor surface and the nature of the molecule responsible for the change in concentration. The assays demonstrate the formation of superantigen for provoking immune response.

Example V

UV Absorption

Circular dichroism (CD) provides information about the secondary structure of optically active materials. The far-UV or amide region (170-250 nm) is dominated by contributions of the peptide bonds, whereas CD bands in the near-UV region (250-300 nm) originate from the aromatic amino acids. The UV region of IPF was in the range of 252-260 nm.

A person skilled in the art would appreciate that exemplary embodiments described hereinabove are merely illustrative of the general principles of the present invention. Other modifications or variations may be employed that are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations may be utilized in accordance with the teachings herein. Accordingly, the drawings and description are illustrative and not meant to be limitations thereof.

Moreover, all terms should be interpreted in the broadest possible manner consistent with the text. In particular, the terms "comprises" and "comprising" should be interpreted as referring to elements, components, or steps in a non-exclusive manner, indicating that the referenced elements, components, or steps may be present, or utilized, or combined with other elements, components, or steps that are not expressly referenced. Thus it is intended that the invention cover all embodiments and variations thereof as long as such embodiments and variations come within the scope of the appended claims and their equivalents.

SEQUENCE LISTINGS

1

22114PRTSus scrofa 1Gly Asp Glu Pro Leu Glu Asn Tyr Leu Asp Thr Glu Tyr Phe1 5 10270PRTSus scrofa 2Met Lys Trp Leu Leu Leu Leu Ser Leu Val Val Leu Ser Glu Cys Leu1 5 10 15Val Lys Val Pro Leu Val Arg Lys Lys Ser Leu Arg Gln Asn Leu Ile 20 25 30Lys Asn Gly Lys Leu Lys Asp Phe Leu Lys Thr His Lys His Asn Pro 35 40 45Ala Ser Lys Tyr Phe Pro Glu Ala Ala Ala Leu Ile Gly Asp Glu Pro 50 55 60Leu Glu Asn Tyr Leu Asp65 70311PRTSus scrofa 3Ile Gly Asp Glu Pro Leu Glu Asn Tyr Leu Asp1 5 10470PRTSus scrofa 4Thr Glu Tyr Phe Gly Thr Ile Gly Ile Gly Thr Pro Ala Gln Asp Phe1 5 10 15Thr Val Ile Phe Asp Thr Gly Ser Ser Asn Leu Trp Val Pro Ser Val 20 25 30Tyr Cys Ser Ser Leu Ala Cys Ser Asp His Asn Gln Glu Asn Pro Asp 35 40 45Asp Ser Ser Thr Phe Glu Ala Thr Ser Gln Glu Leu Ser Ile Thr Tyr 50 55 60Gly Thr Gly Ser Met Thr65 7054PRTSus scrofa 5Thr Glu Tyr Phe1670PRTSus scrofa 6Gly Ile Leu Gly Tyr Asp Thr Val Gln Val Gly Gly Ile Ser Asp Thr1 5 10 15Asn Gln Ile Phe Gly Leu Ser Glu Thr Glu Pro Gly Ser Phe Leu Tyr 20 25 30Tyr Ala Pro Phe Asp Gly Ile Leu Gly Leu Ala Tyr Pro Ser Ile Ser 35 40 45Ala Ser Gly Ala Thr Pro Val Phe Asp Asn Leu Trp Asp Gln Gly Leu 50 55 60Val Ser Gln Asp Leu Phe65 7078PRTSus scrofa 7Ser Gly Ala Thr Pro Glx Thr Glu1 5870PRTSus scrofa 8Ser Val Tyr Leu Ser Ser Asn Asp Asp Ser Gly Ser Val Val Leu Leu1 5 10 15Gly Gly Ile Asp Ser Ser Tyr Tyr Thr Gly Ser Leu Asn Trp Val Pro 20 25 30Val Ser Val Glu Gly Tyr Trp Gln Ile Thr Leu Asp Ser Ile Thr Met 35 40 45Asp Gly Glu Thr Ile Ala Cys Ser Gly Gly Cys Gln Ala Ile Val Asp 50 55 60Thr Gly Thr Ser Leu Leu65 70920PRTSus scrofamisc_feature(2)..(2)Xaa can be any naturally occurring amino acid 9Asn Xaa Val Pro Val Ser Val Glu Gly Tyr Xaa Gln Ile Thr Leu Asp1 5 10 15Ser Ile Thr Xaa 201027PRTSus scrofa 10Leu Gly Gly Ile Asp Ser Ser Tyr Tyr Thr Gly Ser Leu Asn Trp Val1 5 10 15Pro Val Ser Val Glu Gly Tyr Trp Gln Ile Thr 20 251128PRTSus scrofa 11Ser Tyr Tyr Thr Gly Ser Leu Asn Ile Arg Val Pro Val Ser Val Glu1 5 10 15Gly Tyr Trp Gln Ile Thr Leu Asp Ser Ile Thr Met 20 251225PRTSus scrofa 12Ser Tyr Tyr Thr Gly Ser Leu Asn Trp Val Pro Val Ser Val Glu Gly1 5 10 15Tyr Trp Gln Ile Thr Leu Asp Ser Ile 20 251325PRTSus scrofa 13Asn Trp Val Pro Val Ser Val Glu Gly Tyr Trp Gln Ile Thr Leu Asp1 5 10 15Ser Ile Thr Met Asp Gly Arg Thr Ile 20 251470PRTSus scrofa 14Thr Gly Pro Thr Ser Ala Ile Ala Ile Asn Ile Gln Ser Asp Ile Gly1 5 10 15Ala Ser Glu Asn Ser Asp Gly Glu Met Val Ile Ser Cys Ser Ser Ile 20 25 30Asp Ser Leu Pro Asp Ile Val Phe Thr Ile Asn Gly Val Gln Tyr Pro 35 40 45Leu Ser Pro Ser Ala Tyr Ile Leu Gln Asp Asp Asp Ser Cys Thr Ser 50 55 60Gly Phe Glu Gly Asn Met65 701536PRTSus scrofa 15Val Pro Thr Ser Ser Gly Glu Leu Trp Ile Leu Gly Asp Val Phe Ile1 5 10 15Arg Gln Tyr Tyr Thr Val Phe Asp Arg Ala Asn Asn Lys Val Gly Leu 20 25 30Ala Pro Val Ala 351614PRTSus scrofa 16Gly Asp Glu Pro Leu Glu Asn Tyr Leu Ile Asp Thr Glu Trp1 5 101720PRTSus scrofamisc_feature(2)..(2)Xaa can be any naturally occurring amino acid 17Asn Xaa

Val Pro Val Ser Val Glu Gly Tyr Xaa Gln Ile Thr Leu Asp1 5 10 15Ser Ile Thr Xaa 20187PRTSus scrofa 18Ser Gly Ala Thr Pro Val Phe1 51927PRTSus scrofa 19Leu Gly Gly Ile Ile Ser Ser Tyr Tyr Thr Gly Ser Leu Asn Trp Val1 5 10 15Pro Val Ser Val Glu Gly Tyr Trp Gln Ile Thr 20 252027PRTSus scrofa 20Ser Tyr Tyr Thr Gly Ser Leu Asn Trp Val Pro Val Ser Val Glu Gly1 5 10 15Tyr Trp Gln Ile Thr Leu Ser Asp Ile Thr Met 20 252125PRTSus scrofa 21Ser Ala Tyr Thr Gly Ser Leu Asn Trp Val Pro Val Ser Val Glu Gly1 5 10 15Tyr Trp Gln Ile Thr Leu Asp Ser Ile 20 252225PRTSus scrofa 22Asn Trp Val Pro Val Ser Val Glu Gly Tyr Trp Gln Ile Thr Leu Asp1 5 10 15Ser Ile Thr Met Asp Gly Arg Thr Ile 20 25

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BODY:

Immunotech Laboratories, Inc. Enters into Negotiations with Uldic Investment Pvt. Ltd. for Market Development Monrovia, California (FSCwire) - **Immunotech** Laboratories, Inc. (OTCMARKETS: IMMB) **Immunotech** Laboratories, Inc.(the Company) and **Immunotech** Laboratories, BG (IMMB-BG) the Company announced today that they have entered into negotiations with Uldic Investment Pvt. Ltd. (Uldic or the Representative/Distributor), located in Zimbabwe, to pursue the development of market opportunities and Clinical Testing for the Companys HIV/AIDS and Hepatitis C virus treatment (ITV-1 Treatment) in Africa, initially focusing on clinical testing for Hepatitis C in Mauritius Island and Malawi. About **Immunotech** Laboratories, Inc. Headquartered in Monrovia, CA, **Immunotech** Laboratories is a drug development company committed to the commercialization of its proprietary proteins for the treatment of debilitating infectious diseases. The Company strives to become a leader in immuno-therapeutic treatment and prevention of HIV/AIDS, Cancer and other immuno related disorders. For more information visit:

http://www.immunotechlab.com (http://www.immunotechlab.com/) Safe Harbor Statement: This news release contains forward-looking statements that involve risks and uncertainties associated with financial projections, budgets, milestone timelines, clinical development, regulatory approvals, and other risks described by **Immunotech** Laboratories, Inc. from time to time in its periodic reports filed with the SEC. IPF is not approved by the US Food and Drug Administration or by any comparable regulatory agencies elsewhere in the world. While **Immunotech** Laboratories believes that the forward-looking statements and underlying assumptions contained therein are reasonable, any of the assumptions could be inaccurate, including, but not limited to, the ability of **Immunotech** Laboratories to establish the efficacy of IPF in the treatment of any disease or health condition, the development of studies and strategies leading to commercialization of IPF in the United States, the obtaining of funding required to carry out the development plan, the completion of studies and tests on time or at all, and the successful outcome of such studies or tests. Therefore, there can be no assurance that the forward-looking statements included in this release will prove to be accurate. In light of the significant uncertainties inherent in the forward-looking statements included herein, **Immunotech** Laboratories or any other person that the objectives and plans of **Immunotech** Laboratories will be achieved should not regard the forward-looking statements as a representation. Contact: **Immunotech** Laboratories, Inc, 818-409-8988 ir@immunotechlab.com (mailto:ir@immunotechlab.com) GIC 813-213-8156 Houston, Texas To view this press release as a PDF file, click onto the following link: http://www.fscwire.com/sites/default/files/news_release_pdf/immunotech10092014.pdf Source: **Immunotech** Laboratories Inc. (OTC Pink:IMMB) http://www.immunotechlab.com/ Maximum News Dissemination by FSCwire. http://www.fscwire.com Copyright(C)2014 Filing Services Canada Inc.

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HEADLINE: Enters into Agreement to Market Potential **Ebola** Virus Disease ("EVD") Treatment and Implement Strategy for Company's ITV 1 Infectious Diseases Treatment in Africa

BODY:

IMMUNOTECH LABORATORIES INC ("IMMB-0") - Enters into Agreement to Market Potential **Ebola** Virus Disease - ("EVD") Treatment and Implement Strategy for Company's ITV-1 - Infectious Diseases Treatment in Africa

Immunotech Laboratories, Inc. ("**Immunotech**" or the "Company") and wholly-owned subsidiary **Immunotech** Laboratories, BG (IMMB-BG) today announced that they have successfully completed negotiations with Uldic Investment Pvt. Ltd. (Uldic), located in Zimbabwe, to pursue the development of market opportunities related to the deadly **Ebola** virus, and to conduct human clinical trials using the Company's HIV/AIDS and Hepatitis C virus treatment, Immune Therapeutic Vaccine-1 (ITV-1), in Sub-Saharan West Africa.

With the establishment of an African operation, **Immunotech** hopes to fulfill the Company's vision of bringing a therapy based on the patented Inactivated Pepsin Fraction (IPF) protein developed by **Immunotech** for infectious diseases such as HIV/AIDS, Hepatitis C and a new potential initiative, the **Ebola** virus. In parts of Africa, approved experimental treatments are permitted, and with the **Ebola** outbreak, **Immunotech** expects that it can market its treatment for infectious diseases through the Company's new agreement with Uldic.

ITV-1 is a suspension of Inactivated Pepsin Fraction (IPF), which studies have shown is promising in combination with protease inhibitors in the treatment of the HIV/AIDS virus. IPF is a platform technology that can be used to facilitate a broad range of applications. It is free from neurological, gastrointestinal and hematological side effects seen in the anti-retrovirals in use today. IPF has not shown itself to be subject to viral resistance, and it is cost effective.

The Company says that the immune system has components that bind and present antigens to cells that are capable of initiating a response to those antigens. CD1d CD 56 molecules are a family of highly conserved antigen-presenting proteins that bind lipids and glycolipids, resulting in activation of natural killer T-cells (NKT cells) to elicit protective immunity against the immunogen.

Immunotech has isolated IPF which is the most extensively studied CD 56 ligand to date. The compounds has their ability to stimulate human NKT-cell lines, secretion of key cytokines such as IFN- IL 2 and IL-12, and activate autologous dendritic cells, as well as binding to CD1d and the invariant T-cell receptor. A lead compound, IPF, emerged from these studies and this protein exhibits a stronger adjuvant effect in various HIV vaccine platforms in mice. IPF also provides a protective adjuvant effect with a candidate HIV and HCV vaccine.

While the majority of the Company's studies have focused on the potential of the IPF as a vaccine adjuvant, it is foreseeable that the compounds could also be used as a potential immuno-therapeutic to treat infectious diseases.

About **Immunotech** Laboratories, Inc.Headquartered in Monrovia, CA, **Immunotech** Laboratories is a drug development company committed to the commercialization of its proprietary proteins for the treatment of debilitating infectious diseases. The Company strives to become a leader in immuno-therapeutic treatment and the prevention of HIV/AIDS, Cancer and other immuno-related disorders.

For more information visit: http://www.immunotechlab.com

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CONTACT: TEL: 818-409-8988 Billy Ray E-mail: ir@immunotechlab.com

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