COMPOSITIONS AND METHODS FOR EVALUATING VIRAL RECEPTOR/CO-RECEPTOR USAGE AND INHIBITORS OF VIRUS ENTRY USING RECOMBINANT VIRUS ASSAYS

Throughout this application, various publications are referenced by author and date within the text. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. All patents, patent applications and publications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

Background of the Invention

Virus entry is an attractive new target for anti-viral treatment, and about 10 drugs that are designed to block virus attachment or membrane fusion are currently being evaluated in preclinical or clinical studies (Richman, 1998; PhRMA, 1999; Stephenson, 1999). Enveloped animal viruses attach to and enter the host cell via the interaction of viral proteins in the virion membrane (envelope proteins) and cell surface proteins (virus receptors). Receptor recognition and binding are mediated by the surface envelope protein.

Summary of the Invention

Accordingly, it is an object of the invention to provide a rapid, sensitive phenotypic assay to measure the susceptibility of a virus to inhibitors of viral entry.

A further object of the invention is to provide a retroviral vector system that produces virus particles containing viral envelope proteins derived from a variety of sources and the identification of cell lines that express viral receptors and are permissive for viral replication.

Another object of the invention is to provide an expression vector for viral envelope that is capable of accepting patient-derived segments encoding envelope genes.

Another object of the invention is to provide a bio-safe vector that represents most of the HIV-1 viral genome, but carries a luciferase reporter gene in place of the envelope region.

A further object of the invention is to the phenotypic assay which reduces the likelihood of forming recombinant infectious HIV-1, by providing a viral expression vector that carries a deletion in a transcriptional regulatory region (the 3′ copy of U3) of the HIV-1 genome.

Another object of the invention is to provide an assay capable of identifying and determining receptor/co-receptor tropism, which quickly and accurately identifies patients that are infected with strains of a tropic virus.

These and other objects may be achieved by the present invention by: a method for identifying whether a compound inhibits entry of a virus into a cell which comprises: (a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus; (b) co-transfecting into a first cell (i) the nucleic acid of step (a), and (ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal, such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient; (c) contacting the viral particles produced in step (b) with a second cell in the presence of the compound; wherein the second cell expresses a cell surface receptor to which the virus binds; (d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and (e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of the compound, wherein a reduced amount of signal measured in the presence of the compound indicates that the compound inhibits entry of the virus into the second cell.

In another aspect, the invention provides a method for identifying a cell surface receptor which is bound by a virus upon infection of a cell by the virus which comprises: (a) obtaining viral particles which comprise (i) a viral nucleic acid and (ii) an indicator nucleic acid which produces a detectable signal; (b) contacting a cell which expresses a cell surface receptor with the viral particles from step (a); and (c) measuring the amount of detectable signal produced within the cell, wherein production of the signal indicates

the cell surface receptor expressed by the cell is bound by the virus, thereby identifying the cell surface receptor as being bound by the virus upon infection of the cell.

In another aspect, the invention provides a method for identifying whether an antibody inhibits entry of a virus into a cell which comprises: (a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus; (b) co-transfecting into a first cell (i) the nucleic acid of step (a), and (ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal, such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient; (c) contacting the viral particles produced in step (b) with a second cell in the presence of the antibody, wherein the second cell expresses a cell surface receptor to which the virus binds; (d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and (e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of the compound, wherein a reduced amount of signal measured in the presence of the antibody indicates that the antibody inhibits entry of the virus into the second cell.

In another aspect, the invention provides a method for determining susceptibility of a virus to a compound which inhibits viral cell entry which comprises: (a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus; (b) co-transfecting into a first cell (i) the nucleic acid of step (a), and (ii) a viral expression

vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal, such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient; (c) contacting the viral particles produced in step (b) with a second cell in the presence of the compound, wherein the second cell expresses a cell surface receptor to which the virus binds; (d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and (e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of the compound, wherein a reduced amount of signal measured in the presence of the compound indicates that the virus is susceptible to the compound.

In another aspect, the invention provides a method for determining resistance of a virus to a compound which inhibits viral entry into a cell which comprises: (a) determining susceptibility of a virus to a compound described herein, wherein a nucleic acid encoding a viral envelope protein is obtained from a patient at a first time; (b) determining susceptibility of the virus to the compound described herein, wherein the nucleic acid encoding the viral envelope protein is obtained from the patient at a later second time; and (c) comparing the susceptibilities determined in steps (a) and (b), wherein a decrease in susceptibility at the later second time indicates resistance of the virus to the compound.

In another aspect, the invention provides a method for identifying a mutation in a virus that confers resistance to

a compound that inhibits viral entry into a cell which comprises: (a) determining the nucleic acid sequence or the amino acid sequence of the virus prior to any treatment of the virus with the compound; (b) obtaining a virus resistant to the compound; (c) determining the nucleic acid sequence or the amino acid sequence of the resistant virus from step (b); and (d) comparing the nucleic acid sequence or the amino acid sequences of steps (a) and (c), respectively, so as to identify the mutation in the virus that confers resistance to the compound.

Brief Description of the Drawings

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1A. Structure of envelope expression and viral expression vectors.

The HIV envelope expression vector (pHIUVenv) is modified to accept envelope sequences that have been amplified from patient plasma samples. The designations a/b and c/d, refer to restriction endonuclease sites positioned at the 5' and 3' end of the HIV-1 envelope polyprotein (gp160). The HIV expression vector (pHIVlucÄU3) encodes all HIV proteins except the envelope polyprotein. A portion of the envelope gene has been deleted to accommodate a indicator gene cassette, in this case, "Firefly Luciferase" that is used to monitor the ability of the virus to replicate in the presence or absence of anti-viral drugs. The 3' U3 region has been partially deleted to prevent transcription from the 5' LTR in infected cells. Virus produced in this system is limited to a single round of replication.

Figure 1B. Cell Based Entry Assay

Drug susceptibility, co-receptor tropism and virus neutralization testing are performed by co-transfecting a host cell with pHIVenv and pHIVlucÄU3 . The host cell produces HIV particles that are pseudo-typed with HIV

envelope sequences derived from the test virus or patient sample. Virus particles are collected (~48 h) after transfection and are used to infect target cells that express HIV receptors (e.g. CD4) and co-receptors (e.g. CXCR4, CCR5). After infection (~72 h) the target cells are lysed and luciferase activity is measured. HIV must complete one round of replication to successfully infect the target host cell and produce luciferase activity. If the virus is unable to enter the target cell, luciferase activity is diminished. This system can be used to evaluate susceptibility to entry inhibitors, receptor and co-receptor tropism, and virus neutralization.

Figure 2. HIV envelope expression vectors.

HIV envelope sequences are amplified from patient samples and inserted into expression vectors using restriction endonuclease sites (5′ a/b and 3′c/d). Envelope transcription is driven by the immediate early gene promoter of human cytomegalovirus (CMV). Envelope RNA is polyadenylated using an simian virus 40 (SV40) polyadenylation signal sequence (A+). An intron located between the CMV promoter and the HIV envelope sequences is designed to increase envelope mRNA levels in transfected cells. FL- express full-length envelope proteins (gp120, gp41) ÄCT- express envelope proteins (gp120, gp21) lacking the C-terminal cytoplasmic tail domain of gp41+CT- express envelope proteins (gp120, gp41) containing a constant pre-defined gp41 cytoplasmic tail domain gp120- express gp120 proteins derived from the patient together with a constant pre-defined gp41. gp41- express a constant pre-defined gp120

together with gp41 proteins derived from the patient.

Figure 3A. Co-receptor Tropism Screening Assay.

In this figure, the assay is performed using two cell lines. One cell line expresses CD4 and CCR5 (top six panels). The other cell line expresses CD4 and CXCR4 (bottom six panels). The assay is performed by infecting cells with a large number of recombinant virus stocks derived from cells transfected with pHIVenv and pHIVluc?U3 vectors. Tthe example shown represents the analysis of 96 viruses formatted in a 96 well plate Infections are performed in the absence of drug (no drug), or in the presence of a drug that preferentially inhibits either R5 tropic (CCR inhibitor) or X4 tropic (CXCR4 inhibitor) viruses. Co-receptor tropism is assessed by comparing the amount of luciferase activity produced in each cell type, both in the presence and absence of drug (see Figure 3B for interpretation of assay results).

Figure 3B. Determining co-receptor tropism.

In this figure, the results of the assay are interpreted by comparing the ability of each sample virus to infect (produce luciferase activity) in cells expressing CD4/CCR5 (R5 cells) or cells expressing CD4/CXCR4 (X4 cells). The ability of a CCR5 or CXCR4 inhibitor to specifically block infection (inhibit luciferase activity) is also evaluated. X4 tropic viruses (green panels)- infect X4 cells but not R5 cells. Infection of X4 cells is blocked by the CXCR4 inhibitor . R5

tropic viruses (blue panels)- infect R5 cells but not X4 cells. Infection of R5 cells is blocked by the CCR5 inhibitor. Dual tropic or X4/R5 mixtures (yellow panels)- infect X4 and R5 cells. Infection of R5 cells is blocked by the CCR5 inhibitor and infection of X4 cells is blocked by the CXCR4 inhibitor. Non-viable viruses (red panels)- do not replicate in either X4 or R5 cells.

Figure 4A. Measuring Entry Inhibitor Susceptibility: Fusion Inhibitor.

In this figure, susceptibility to the fusion inhibitor T-20 is demonstrated. Cells expressing CD4, CCR5 and CXCR4 were infected in the absence of T-20 and over a wide range of T-20 concentrations (x-axis log10 scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of T-20 to the amount of luciferase produced in the absence of T-20. R5 tropic, X4 tropic and dual tropic viruses were tested. Drug susceptibility is quantified by determining the concentration of T-20 required to inhibit 50% of viral replication (IC50, shown as vertical dashed lines). Viruses with lower IC50 values are more susceptible to T-20 than viruses with higher IC50 values. NL4-3: well-characterized X4 tropic strain JRCSF: well-characterized R5 tropic strain 91US005.11: R5 tropic isolate obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP) 92HT593.1: Dual tropic (X4R5) isolate obtained from the NIH ARRRP.92HT599.24: X4 tropic isolate obtained from the NIH

ARRRP.

Figure 4B. Measuring Entry Inhibitor Susceptibility: Drug Resistance Mutations.

In this figure, reduced susceptibility to the fusion inhibitor T-20 conferred by specific drug resistance mutations in the gp41 envelope protein is demonstrated. Cells expressing CD4, CCR5 and CXCR4 were infected in the absence of T-20 and over a wide range of T-20 concentrations (x-axis log10 scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of T-20 to the amount of luciferase produced in the absence of T-20. Isogenic viruses containing one or two specific mutations in the gp41 transmembrane envelope protein were tested (highlighted in red in the figure legend). Drug susceptibility is quantified by determining the concentration of T-20 required to inhibit 50% of viral replication (IC50, shown as vertical dashed lines). Viruses with lower IC50 values are more susceptible to T-20 than viruses with higher IC50 values.

No mutation (wildtype sequence): GIV

Single mutations: GIV, DIM, SIV

Double mutations: DIM, SIM, DTV

Figure 5A.

Measuring Entry Inhibitor Susceptibility: CCR5 Inhibitor

In this figure, susceptibility to a CCR5 inhibitor (merck

compound) is demonstrated. Cells expressing CD4 and CCR5 (R5 cells) were infected in the absence of the CCR5 inhibitor and over a wide range of CCR5 inhibitor concentrations (x-axis log10 scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of CCR5 inhibitor to the amount of luciferase produced in the absence of CCR5 inhibitor. R5 tropic, X4 tropic and dual tropic viruses were tested. Drug susceptibility is quantified by determining the concentration of CCR5 inhibitor required to inhibit 50% of viral replication (IC50, shown as vertical dashed lines). Viruses with lower IC50 values are more susceptible to the CCR5 inhibitor than viruses with higher IC50 values. The X4 tropic virus did not infect the R5 cells. NL4-3: well-characterized X4 tropic strain JRCSF: well-characterized R5 tropic strain 92HT593.1: Dual tropic (X4R5) isolate obtained from the NIH ARRRP.

Figure 5B.

Measuring Entry Inhibitor Susceptibility: CXCR4 Inhibitor.

In this figure, susceptibility to a CXCR4 inhibitor (AMD3100) is demonstrated. Cells expressing CD4 and CXCR4 (X4 cells) were infected in the absence of the CXCR4 inhibitor and over a wide range of CXCR4 inhibitor concentrations (x-axis log10 scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of CXCR4 inhibitor to the amount of luciferase produced in the absence of CXCR4 inhibitor. R5 tropic, X4 tropic and dual tropic viruses were tested. Drug susceptibility is quantified by determining the concentration of CXCR4 inhibitor required to inhibit 50% of

viral replication (IC50, shown as vertical dashed lines). Viruses with lower IC50 values are more susceptible to the CCR5 inhibitor than viruses with higher IC50 values. The R5 tropic virus did not infect the X4 cells.

NL4-3: well-characterized X4 tropic strain

JRCSF: well-characterized R5 tropic strain

92HT593.1: Dual tropic (X4R5) isolate obtained from the NIH ARRRP.

Figure 6. Entry Inhibitor Susceptibility: Fusion Inhibitor

This figure demonstrates that the amplicons corresponding to the full length envelope sequence or cytoplasmic-tail deleted envelope sequence are generated. The lane numbers correspond to the co-receptor tropism shown next to each number on the right of the gels.

Figure 7. Reduced Susceptibility: Fusion Inhibitor

Scatter plots are shown which indicate the results from FACS (fluorescence activated cell sorting) assays using antibodies against either CCR5 or CXCR4 (shown on Y axis). The cell lines express the co-receptors listed below the plots and the CD4 fluorescence is shown along the X-axis. The anti-CXCR4 antibody binds most strongly with the cells which express the corresponding co-receptor, CXCR-4.

Figure 8. Entry Inhibitor Susceptibility: CCR5 Inhibitor

Inhibition is shown following administration of co-receptor antagonists.

Figure 9. Entry inhibitor susceptibility: CXCR4 Inhibitor Map and amino acid sequence is shown for a peptide which is an inhibitor of fusion between a viral membrane and a cell membrane.

Figure 10. Inhibition by Co-Receptor Antagonists

Figure 11. T20 Resistance Mutations

Figure 12. Identifying Entry Inhibitor Resistance Mutations

Figure 13. Fusion Inhibitor Peptides

The invention in its particular features can become more apparent from the following detailed description considered with reference to the accompanying figures and examples. The following description discusses the means and methods to carry out the present invention pertaining to a phenotypic assay relating to identifying and evaluating inhibitors of viral entry, including for example, and not as a limitation to the present invention, HIV-1 and inhibitors to HIV-1 viral entry.

Detailed Description of the Invention

This invention provides a method for identifying whether a compound inhibits entry of a virus into a cell which comprises: (a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus; (b) co-transfecting into a first cell (i) the nucleic acid of step (a), and (ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal, such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient; (c) contacting the viral particles produced in step (b) with a second cell in the presence of the compound, wherein the second cell expresses a cell surface receptor to which the virus binds; (d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and (e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of the compound, wherein a reduced amount of signal measured in the presence of the compound indicates that the compound inhibits entry of the virus into the second cell.

In one embodiment of this invention, the indicator nucleic acid comprises an indicator gene. In another embodiment of this invention, the indicator gene is a luciferase gene.

In one embodiment of this invention, the cell surface receptor is CD4. In one embodiment of this invention, the cell surface receptor is a chemokine receptor. In one embodiment of this invention, the cell surface receptor is

CXCR4 or CCR5.

In one embodiment of this invention, the patient is infected with the HIV-1 virus, a hepatitis virus (such as the HCV or HBV virus), or any other virus.

In one embodiment of this invention, the nucleic acid of step (a) comprises DNA encoding gp120 and gp41.

In one embodiment of this invention, the viral expression vector comprises HIV nucleic acid.

In one embodiment of this invention, the viral expression vector comprises an HIV gag-pol gene.

In one embodiment of this invention, the viral expression vector comprises DNA encoding vif, vpr, tat, rev, vpu, and nef.

In one embodiment of this invention, the first cell is a mammalian cell.

In one embodiment of this invention, the mammalian cell is a human cell.

In one embodiment of this invention, the human cell is a human embryonic kidney cell.

In one embodiment of this invention, the human embryonic kidney cell is a 293 cell.

In one embodiment of this invention, the second cell is a human T cell.

In one embodiment of this invention, the second cell is a

human T cell leukemia cell line.

In one embodiment of this invention, the second cell is a peripheral blood mononuclear cell.

In one embodiment of this invention, the second cell is an astroglioma cell.

In one embodiment of this invention, the astroglioma cell is a U87 cell.

In one embodiment of this invention, the second cell is a human osteosarcoma cell.

In one embodiment of this invention, the human osteosarcoma cell is an HT4 cell.

In one embodiment of this invention, the compound binds to the cell surface receptor.

In one embodiment of this invention, the compound is a ligand of the cell surface receptor.

In one embodiment of this invention, the compound comprises an antibody.

In one embodiment of this invention, the compound inhibits membrane fusion.

In one embodiment of this invention, the compound is a peptide, a peptidomimetic, an organic molecule, or a synthetic compound.

In one embodiment of this invention, the compound binds the viral envelope protein.

This invention provides for a method for making a composition which comprises admixing the compound identified by the

screening method (method for identifying a compound) described herein with a carrier.

In one embodiment of this invention, the carrier is saline, polyethylene glycol, a buffer solution, a starch, or an organic solvent.

The invention provides for a method for identifying a cell surface receptor which is bound by a virus upon infection of a cell by the virus which comprises: (a) obtaining viral particles which comprise (i) a viral nucleic acid and (ii) an indicator nucleic acid which produces a detectable signal; (b) contacting a cell which expresses a cell surface receptor with the viral particles from step (a); and (c) measuring the amount of detectable signal produced within the cell, wherein production of the signal indicates the cell surface receptor expressed by the cell is bound by the virus, thereby identifying the cell surface receptor as being bound by the virus upon infection of the cell.

The invention also provides for a method for identifying whether an antibody inhibits entry of a virus into a cell which comprises: (a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus; (b) co-transfecting into a first cell (i) the nucleic acid of step (a), and (ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal, such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient; (c) contacting the viral particles produced in step (b) with a second cell in the presence of the antibody, wherein the second cell expresses a cell surface receptor to which the virus binds; (d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and (e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence

of the compound, wherein a reduced amount of signal measured in the presence of the antibody indicates that the antibody inhibits entry of the virus into the second cell.

The invention provides for a method for determining susceptibility of a virus to a compound which inhibits viral cell entry which comprises: (a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus; (b) co-transfecting into a first cell (i) the nucleic acid of step (a), and (ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal, such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient; (c) contacting the viral particles produced in step (b) with a second cell in the presence of the compound, wherein the second cell expresses a cell surface receptor to which the virus binds; (d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and (e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of the compound, wherein a reduced amount of signal measured in the presence of the compound indicates that the virus is susceptible to the compound.

The invention provides a method for determining resistance of a virus to a compound which inhibits viral entry into a cell which comprises: (a) determining susceptibility of a virus to a compound according to the method of claim 33, wherein a nucleic acid encoding a viral envelope protein is obtained from a patient at a first time; (b) determining susceptibility of the virus to the compound according to the method of claim 33, wherein the nucleic acid encoding the viral envelope protein is obtained from the patient at a later second time; and (c) comparing the susceptibilities determined in steps (a) and (b), wherein a decrease in susceptibility at the later second time indicates resistance

of the virus to the compound.

The invention provides for a method for identifying a mutation in a virus that confers resistance to a compound that inhibits viral entry into a cell which comprises: (a) determining the nucleic acid sequence or the amino acid sequence of the virus prior to any treatment of the virus with the compound; (b) obtaining a virus resistant to the compound; (c) determining the nucleic acid sequence or the amino acid sequence of the resistant virus from step (b); and (d) comparing the nucleic acid sequence or the amino acid sequences of steps (a) and (c), respectively, so as to identify the mutation in the virus that confers resistance to the compound.

In one embodiment of this invention, the virus obtained in step (b) is the virus of step (a) grown in the presence of the compound until resistance is developed.

In one embodiment of this invention, the virus obtained in step (b) is isolated from a patient which has been undergoing treatment with the compound.

In a preferred embodiment, this invention provides a means and method for accurately and reproducibly measuring the susceptibility of HIV-1 to virus entry inhibitors.

In another preferred embodiment, this invention also provides a means and method for accurately and reproducibly measuring HIV-1 co-receptor tropism.

In a preferred embodiment, this invention provides a means and method for accurately and reproducibly measuring antibody mediated neutralization of HIV-1.

In a preferred embodiment, this invention further provides a means and method for discovering, optimizing and characterizing novel or new drugs that target various defined and as yet undefined steps in the virus attachment and entry process.

In a preferred embodiment, this invention further provides a means and method for discovering, optimizing and characterizing HIV-1 vaccines (either preventative or therapeutic) that target various defined and as yet undefined steps in the virus attachment and entry process.

In a preferred embodiment, this invention provides a means and method for identifying amino acid substitutions/mutations in HIV-1 envelope proteins (gp41TM and gp120SU) that alter susceptibility to inhibitors of virus entry.

In a preferred embodiment, this invention provides a means and method for quantifying the affect that specific mutations in HIV-1 envelope have on virus entry inhibitor susceptibility.

In a preferred embodiment, this invention further provides a means and method for determining HIV-1 envelope amino acid substitutions/mutations that are frequently observed, either alone or in combination, in viruses that exhibit altered susceptibility to virus entry inhibitors.

In a preferred embodiment, this invention provides a means and method for identifying amino acid substitutions/mutations in HIV-1 envelope proteins (gp41TM and gp120SU) that alter receptor or co-receptor tropism.

In a preferred embodiment, this invention provides a means and method for quantifying the affect that specific mutations in HIV-1 envelope have receptor or co-receptor tropism.

In a preferred embodiment, this invention further provides a means and method for identifying HIV-1 envelope amino acid substitutions/mutations that are frequently observed, either alone or in combination, in viruses that exhibit CXCR4 or CCR5 co-receptor tropism.

In a preferred embodiment, this invention provides a means and method for identifying amino acid substitutions/mutations in HIV-1 envelope proteins (gp41TM and gp120SU) that alter antibody mediated neutralization.

In a preferred embodiment, this invention provides a means and method for quantifying the affect that specific mutations in HIV-1 envelope have on antibody mediated neutralization.

In a preferred embodiment, this invention further provides a means and method for identifying HIV-1 envelope amino acid substitutions/mutations that are frequently observed, either alone or in combination, in viruses that exhibit antibody medicated virus neutralization.

In a preferred embodiment, this invention further provides a means and method to identify antibodies that are frequently observed in patient samples viruses that are capable of neutralizing HIV-1.

In a preferred embodiment, this invention further provides a

means and method for identification of viruses that require CD4 binding for infection.

In a preferred embodiment, this invention further provides a means and method for the identification of viruses that do not require CD4 binding for infection.

In a preferred embodiment, this invention also provides a means and method for identifying the incidence of patient samples that exhibit CD4 independent infection.

In a preferred embodiment, this invention further provides a means and method for identification of viruses that require CD8 binding for infection.

In a preferred embodiment, this invention also provides a means and method for identifying the incidence of patient viruses that exhibit CD8 dependent infection.

In a preferred embodiment, this invention further provides the means and method for the identification of viruses that require the CXCR4 chemokine receptor binding, the CCR5 chemokine receptor binding, or either CXCR4 or CCR5 binding (dual tropic) for infection.

In a preferred embodiment, this invention further provides a means and method for identifying the incidence of viruses that require the CXCR4 chemokine receptor binding, the CCR5 chemokine receptor binding, or either CXCR4 or CCR5 binding (dual tropic) for infection.

In a preferred embodiment, this invention further provides a

means and method for identifying HIV-1 envelope amino acid substitutions/mutations that are frequently observed, either alone or in combination, in viruses that exhibit (a) altered susceptibility to virus entry inhibitors, (b) CXCR4 or CCR5 co-receptor tropism, and (c) antibody medicated virus neutralization.

In a preferred embodiment, this invention provides a means and method for using virus entry inhibitor susceptibility to guide the treatment of HIV-1.

In a preferred embodiment, this invention further provides a means and method for using virus entry inhibitor susceptibility to guide the treatment of patients failing antiretroviral drug treatment.

In a preferred embodiment, this invention further provides the means and methods for using virus entry inhibitor susceptibility to guide the treatment of patients newly infected with HIV-1.

In a preferred embodiment, this invention provides a means and method for using HIV-1 co-receptor tropism to guide the treatment of HIV-1 or to guide the treatment of patients failing antiretroviral drug treatment.

In a preferred embodiment, this invention further provides the means and method for using HIV-1 co-receptor tropism to guide the treatment of patients newly infected with HIV-1.

In a preferred embodiment, this invention further provides a means and method for measuring antibody mediated

neutralization of HIV-1 to monitor the initial protective antibody response following vaccination.

In a preferred embodiment, this invention further provides a means and method for measuring antibody mediated neutralization of HIV-1 to monitor the initial therapeutic antibody response following vaccination.

In a preferred embodiment, this invention further provides a means and method for measuring antibody mediated neutralization of HIV-1 over time to monitor the durability of a protective antibody response following vaccination.

In a preferred embodiment, this invention further provides a means and method for measuring antibody mediated neutralization of HIV-1 to develop and optimize vaccination prime-boost schedules that maximize vaccination potency and durability.

For example, in the case of HIV-1, the SU protein (gp120-SU) is tightly associated with the transmembrane envelope protein (gp41-TM) that anchors the complex to the virus membrane. The envelope proteins gp120 and gp41 are derived by cleavage of gp160, the uncleaved precursor product of the envelope gene. The binding of HIV-1 to its cellular receptor (CD4) and co-receptor (either CCR5 or CXCR4) promotes conformational changes in the TM protein resulting in the fusion of the viral and cellular membrane and entry of the virus core into the cytoplasm (Retroviruses, 1997). Although the new HIV entry inhibitors target either viral envelope proteins (gp120/gp41) or host proteins (CD4, CCR5, CXCR4), the majority of resistance-associated mutations in HIV-1 are

expected to be located in the viral envelope gene; e.g. one likely way viruses might evolve is to shift co-receptor utilization. Entry blockers constitute a novel class of anti-retroviral drugs, and the potential for broad activity against current multi-drug resistant HIV-1 variants is high. Among the class of potential viral entry blockers are fusion inhibitors, receptor/co-receptor antagonists and vaccines.

Nonetheless, inhibitors of viral entry are likely to generate drug resistant viruses (through mutation of the envelope gene), thus complicating patient treatment similar to that observed for protease inhibitor (PRI) and reverse transcriptase inhibitor (RTI) treatment for HIV. In fact, FDA approval of any new drug that blocks viral entry will require the evaluation of resistance data. The need for a diagnostic assay that measures susceptibility to entry blockers has been documented in the case of the fusion inhibitor T-20. Viruses exhibiting reduced susceptibility to T-20 have been reported after passage in vitro in the presence of the drug. At this time, convenient phenotypic assays that are capable of measuring susceptibility to drugs that block viral entry are not available. Consequently physicians will soon be faced with the challenge of tailoring therapy in the absence of the tools necessary to address drug susceptibility. Therefore, a reliable assay that accurately measures susceptibility to drugs that inhibit viral entry from infected patients would be extremely valuable.

For example, recent World Health Organization estimates indicate that worldwide more than 33 million people are infected with HIV-1, the causative agent for the AIDS pandemic. Nearly one million people are infected in the

United States and 300,000 are currently receiving anti-viral therapy (CDC, 1999; WHO 1999). Combating AIDS has become the common goal of an unprecedented effort of governmental agencies, academic laboratories, and the pharmaceutical/biotechnology industry. Fourteen anti-viral drugs have been approved by the FDA for treatment of HIV-1 infection (carpenter et al., 2000) and more than 20 additional drugs are currently being evaluated in clinical trials (PHRMA, 1999). The approved drugs inhibit HIV-1 replication by interfering with the enzymatic activities of either protease (PR) or reverse transcriptase (RT). PR inhibitors (PRIs) block the proper formation of viral proteins that are necessary for virus infection and replication, while RT inhibitors (RTIs) block the virus from copying its genetic material. Due to sub-optimal potency, current PRIs and RTIs are most often used in combination to suppress viral replication (Carpenter et al., 2000).

What is desired, therefore, is to provide a rapid, accurate safe viral assay capable of evaluating:

33. the activity of inhibitors of viral attachment and entry (including fusion, receptor and co-receptor inhibitors);

34. receptor/co-receptor viral tropism to facilitate viral entry inhibitor drug design and treatment;

35. changes in drug susceptibility of patient viruses to inhibitors of attachment and entry; and

36. viral neutralizing activity generated in response to

vaccination using viral envelope protein antigens.

The methods of this invention can be used for any viral disease that may be responsive to a viral entry inhibitor and where anti-viral drug susceptibility and resistance to a viral entry inhibitor is a concern including, for example, including, but not limited to other lentiviruses (e.g. HIV-2), other retroviruses (e.g. HTLV-1 and 2), hepadnaviruses (e.g. human hepatitis B virus), flaviviruses (e.g. human hepatitis C virus) and herpesviruses (e.g. human cytomegalovirus).

Entry blockers constitute a novel class of anti-retroviral drugs, and the potential for broad activity against current multi-drug resistant HIV-1 variants is high. Among the class of potential viral entry blockers are fusion inhibitors, receptor/co-receptor antagonists and vaccines.

Fusion Inhibitors

Compounds designed to competitively inhibit the conformational change of TM, designated fusion inhibitors, are potent inhibitors of HIV-1 replication. Although their activity has been demonstrated both in cell culture systems and HIV-1 infected patients (Wild et al., 1992; Judice et al., 1997; Kilby et al., 1998), no fusion inhibitor has yet been approved for the treatment of HIV-1 infection in the U.s. Drugs within this class, such as t-20 and t-1249 (Trimeris Inc., USA), are the subject of advanced clinical investigations.

Receptor/Co-receptor Antagonists

In addition to fusion inhibitors, which act after HIV-1 has

interacted with its receptors, efforts are in progress to develop drugs that prevent HIV-1 from interacting with CD4 or either of its two principal co-receptors. The ability of such reagents to inhibit HIV-1 infection has been demonstrated in cell culture systems and animal models. Lead compounds targeting either gp120, CD4, the CCR5 co-receptor used by macrophage tropic viruses (R5), or the CXCR4 co-receptor used by T-cell tropic viruses (X4) have been identified (Allaway et al., 1993; Reimann et al., 1995; Baba et al., 1999; Bridger et al., 1999).

Currently, no co-receptor antagonists are approved for the treatment of HIV-1 infection in the U.S. Drugs within these classes, such as PRO 542 (Progenics Inc., USA), 5a8 (Tanox, USA), TAK-779 (Takeda Inc., Japan), and AMD-3100 (Anormed Inc., Canada), are the subjects of preclinical or early stage clinical investigations. Therefore, an assay capable of identifying and determining receptor/co-receptor tropism, which quickly and accurately identifies patients that are infected with strains of a tropic virus (e.g. HIV-1), would facilitate viral entry inhibitor drug design and treatment.

Vaccines

Vaccines have also proven to be an effective strategy in the fight against pathogenic viral infections in humans, and several vaccine candidates to prevent HIV-1 infection are in clinical development. The envelope proteins gp120 and gp41 are the most obvious candidates in the intense search for an HIV-1 vaccine, and many of the 11 vaccine candidates in clinical evaluation are envelope-based (PhRMA, 1999). It is generally thought that an effective envelope vaccine may elicit the generation of neutralizing antibodies that block

viral infection (Mascola et al., 2000). Therefore, a sensitive high-throughput assay that reliably measures the efficacy of such neutralizing antibodies and does not require prolonged cultivation of virus is urgently needed. Such an assay could significantly aid the search for an effective AIDS vaccine. This is particularly true, considering that late-stage clinical trials encompass large patient populations numbering in the thousands. Since neutralizing antibodies should prevent successful infection of target cells, a envelope receptor assay would be beneficial to serve as a virus neutralization assay.

Unfortunately, most of these drug combinations are effective for only a limited time in large part due to the emergence of drug resistant viruses. The lack of proofreading functions inherent to RT and RNA polymerase II, coupled with high level, error-prone replication allows viruses such as HIV-1 to mutate readily (Coffin, 1995). This high mutation frequency contributes to the ability of HIV-1 to evade successful long-term drug therapy, resulting in viral load rebound. Resistance-associated mutations to all of the 14 approved drugs as well as to many investigational compounds have been described (Schinazi et al., 1999). Consequently, multi-drug resistant HIV-1 variants pose an increasing problem in the care of infected patients. To achieve long-term clinical benefit, it is desirable to select those drugs that maximally suppress viral replication and avoid the drugs to which a patient's virus is resistant (DHHs, 2000). Long-term solutions can rely on drug resistance tests that can guide physicians in selecting the most effective drugs against the patient's virus. The need for resistance testing has been affirmed in recent guidelines from the DHHs (DHHs,

2000), recommending that resistance tests be routinely used when treating HIV-1 infected patients. Susceptibility tests can also assist in the development of new drugs that target resistant viruses. A recent FDA advisory committee (November 1999) recommended that resistance testing be used in the development of new anti-viral drugs for HIV-1.

Several strategies have been applied to the assessment of antiviral drug susceptibility. Genotypic tests analyze mutations in the underlying nucleotide sequence, or genotype, and attempt to correlate these mutations with drug resistance (Rodriguez-Rosado et al., 1999; Schinazi et al, 1999). However, the relationship between genotype and phenotype is complex and not easily interpreted, and the results of these tests are not quantitative. The use of genotypic drug susceptibility data requires interpretation either by experts (Baxter et al., 1999) or computer algorithms and are not always predictive of treatment outcome (Piketty et al., 1999).

Phenotypic drug susceptibility assays directly measure and quantify the ability of viruses to replicate in the presence of drug. Early phenotypic tests required prolonged virus cultivation and consequently were slow, labor intensive, and not easily automated for high throughput (Japour et al., 1993). As a result, these early phenotypic tests were considered impractical for patient management. The development of recombinant virus assays (Shi and Mellors, 1997; Hertogs et al., 1998) simplified phenotypic testing and increased throughput. However, a major disadvantage of these assays is a lengthy turnaround time of 4-8 weeks. More recently, recombinant virus assays have been developed and

others that are capable of measuring drug susceptibility during a single round of replication (Zennou et al., 1998; Petropoulos et al., 2000), resulting in a dramatic reduction in turnaround time to 8-10 days. Patients failing anti-retroviral therapy can benefit from phenotypic assays. Such assays are attractive tools for patient management because they provide a direct and rapid measure of drug susceptibility.

The assay of this invention can be used with other viral infections arising from infections due to other viruses within these families as well as viral infections arising from viruses in other viral families. In addition, the drug susceptibility and resistance test of this invention is useful for screening for compounds to treat viral diseases for which there is no currently available therapy.

The structure, life cycle and genetic elements of the viruses which could be tested in the drug susceptibility and resistance test of this invention would be known to one of ordinary skill in the art. It is useful to the practice of this invention, for example, to understand the life cycle of a retrovirus, as well as the viral genes required for retrovirus rescue and infectivity. Retrovirally infected cells shed a membrane virus containing a diploid RNA genome. The virus, studded with an envelope glycoprotein (which serves to determine the host range of infectivity), attaches to a cellular receptor in the plasma membrane of the cell to be infected. After receptor binding, the virus is internalized and uncoated as it passes through the cytoplasm of the host cell. Either on its way to the nucleus or in the nucleus, the reverse transcriptase molecules resident in the

viral core drive the synthesis of the double-stranded DNA provirus, a synthesis that is primed by the binding of a tRNA molecule to the genomic viral RNA. The double-stranded DNA provirus is subsequently integrated in the genome of the host cell, where it can serve as a transcriptional template for both mRNAs encoding viral proteins and virion genomic RNA, which will be packaged into viral core particles. On their way out of the infected cell, core particles move through the cytoplasm, attach to the inside of the plasma membrane of the newly infected cell, and bud, taking with them tracts of membrane containing the virally encoded envelope glycoprotein gene product. This cycle of infection--reverse transcription, transcription, translation, virion assembly, and budding repeats itself over and over again as infection spreads.

The viral RNA and, as a result, the proviral DNA encode several cis-acting elements that are vital to the successful completion of the viral lifecycle. The virion RNA carries the viral promoter at its 3' end. Replicative acrobatics place the viral promoter at the 5' end of the proviral genome as the genome is reverse transcribed. Just 3' to the 5' retroviral LTR lies the viral packaging site. The retroviral lifecycle requires the presence of virally encoded transacting factors. The viral-RNA-dependent DNA polymerase (pol)--reverse transcriptase is also contained within the viral core and is vital to the viral life cycle in that it is responsible for the conversion of the genomic RNA to the integrative intermediate proviral DNA. The viral envelope glycoprotein, env, is required for viral attachment to the uninfected cell and for viral spread. There are also transcriptional trans-activating factors, so called transactivators, that can serve to modulate the level of

transcription of the integrated parental provirus. Typically, replication-competent (non-defective) viruses are self-contained in that they encode all of these trans-acting factors. Their defective counterparts are not self-contained.

In the case of a DNA virus, such as a hepadnavirus, understanding the life cycle and viral genes required for infection is useful to the practice of this invention. The process of HBV entry has not been well defined. Replication of HBV uses an RNA intermediate template. In the infected cell the first step in replication is the conversion of the asymmetric relaxed circle DNA (rc-DNA) to covalently closed circle DNA (cccDNA). This process, which occurs within the nucleus of infected liver cells, involves completion of the DNA positive-strand synthesis and ligation of the DNA ends. In the second step, the cccDNA is transcribed by the host RNA polymerase to generate a 3.5 kB RNA template (the pregenome). This pregenome is complexed with protein in the viral core. The third step involves the synthesis of the first negativesense DNA strand by copying the pregenomic RNA using the virally encoded P protein reverse transcriptase. The P protein also serves as the minus strand DNA primer. Finally, the synthesis of the second positive-sense DNA strand occurs by copying the first DNA strand, using the P protein DNA polymerase activity and an oligomer of viral RNA as primer. The pregenome also transcribes mRNA for the major structural core proteins.

Design and Methods

37) Construction of an Expression Vector for a Viral Envelope Protein That Is Capable of Accepting Patient-

derived Segments Encoding the Envelope Protein.

In one embodiment, an envelope expression vector capable of expressing HIV-1 envelope proteins in transfected cells was constructed. Similar expression vectors have been described, including a plasmid (pAmphoEnv) constructed to express amphotropic murine leukemia virus (A-MLV) envelope protein as described in U.S. Patent No. 5,837,464 and (Petropoulos et al., 2000). The pAmphoEnv vector uses the immediate early gene promoter of human cytomegalovirus (CMV) and the SV40 polyadenlyation signal sequence to produce A-MLV envelope mRNA in transfected cells. The pAmphoEnv plasmid is modified by deleting the A-MLV envelope gene and introducing restriction enzyme cleavage sites that can enable the insertion of viral envelope fragments derived from a variety of isolates, such as HIV-1. In the case of, HIV-1, the envelope open reading frame spans approximately 2,600 nucleotides and encodes the envelope polyprotein, gp160. The gp160 polyprotein is cleaved by a cellular furin-like protease to produce two subunits, gp41 and gp120. HIV-1 envelope expression vectors can be constructed in stages as follows:

(a) Replacing the A-MLV Envelope Nucleic Acid Sequences from the Envelope Expression Vector (pAmphoEnv) with a Multiple Cloning Site Polylinker:

The A-MLV envelope nucleic acid sequences can be deleted from the pAmphoEnv vector by restriction enzyme digestion. The digested vector can be re-circularized by ligation to a duplex oligonucleotide polylinker containing four unique internal restriction sites (a, b, c, d) for insertion of

envelope sequences. The ligation reaction can be used to transform *Escherichia Coli* and molecular clones containing the correct polylinker sequence can be identified and confirmed by restriction mapping and DNA sequencing, respectively. The introduction of multiple unique cloning sites into the vector can facilitate the insertion of HIV-1 envelope sequences. Restriction sites within the polylinker can be chosen based on their infrequent occurrence in HIV-1 envelope sequences (LANL HIV-1 database, www.lanl.gov). This vector can be referred to as pCX. The functionality of the pCX vector can be demonstrated by inserting a reporter gene or indicator nucleic acid, such as firefly luciferase, into the pCX multiple cloning site and measuring a signal from the indicator nucleic acid or reporter gene activity in transfected cells. As used herein, "indicator nucleic acid" refers to a nucleic acid encoding a protein, DNA or RNA structure that either directly or through a reaction gives rise to a measurable or noticeable signal, e.g. color or light of measurable wavelength, or generation of a specific DNA or RNA structure used as an indicator which could be amplified using any one of a number of quantitative amplification assays.

(b) Inserting Viral Envelope Sequences into the pCX Envelope Expression Vector:

Using mutagenic primers for PCR amplification, viral envelope fragments are generated that contain two unique restriction sites (a, b and c, d, respectively) adjacent to the initiation and termination codons of, for example, the HIV-1 envelope open reading frame. Introduction of two unique restriction sites at each end of the envelope open reading

frame can improve chances of cloning HIV-1 envelope fragments harboring internal restriction sites for any one of the enzymes found in the multiple cloning site of the pCX vector.

In the case of HIV-1, two well-characterized molecular clones of HIV-1 with known differences in the envelope gene, NL4-3 (a syncytium-inducing, T-cell tropic, laboratory strain) and JR-CSF (a non-syncytium-inducing, macrophage-tropic, primary isolate) can be used as template for PCR amplification. The 2,600 nucleotide amplification products can be digested with two restriction enzymes (each enzyme cleaving at one end of the fragment; e.g. a and c or b and d) and subsequently inserted into the pCX vector by ligation and transformation of *Escherichia Coli*. Molecular clones containing the appropriate envelope sequences can be identified by restriction mapping and confirmed by DNA sequencing. The resulting plasmids, pHIVenv (NL4-3) and pHIVenv (JR-CSF), can be used to express HIV-1 envelope proteins in transfected cells (**Figure 1A**). The functionality of the envelope expression vectors, such as the pHIVenv vectors, can be demonstrated by measuring viral envelope synthesis in transfected cells (Western Blot), and by their ability to pseudotype envelope deficient retrovirus vectors. High titer virus stocks using the human embryonic kidney 293 cell line has been demonstrated (Petropoulos et al., 2000), however the present invention is not restricted to those cell lines. Other suitable cell lines used as a first cell for transfection of nucleic acid obtained from the patient encoding a viral envelope protein include, by way an example and not as limitation to the present invention, 5.25; HOX; U87; MT2; PM1; CEM; etc. The cell line optimally will be engineered to express one or more co-receptors.

(c) Modifying the pCX Vector to Improve the Efficiency of Cloning Viral Envelope Sequences:

To improve the cloning efficiency of viral envelope fragments, the pCX expression vector can be modified by inserting a bacterial killer gene cassette (e.g. control of cell death b gene (ccdB) or a member of the hok-killer gene family) under the control of the *Escherichia Coli* lac promoter into the multiple cloning site (the et al., 1990; Bernard and Couturier, 1992; Bernard et al., 1993). This modified vector is referred to as pCXccdB. Transcription of the ccdB killer gene is repressed in bacterial strains that express the laci^q repressor, such as JM109. This or an equivalent strain can be used to propagate plasmids carrying the ccdB killer gene that are under the control of the lac promoter. Conversely, in this system bacterial strains that do not overexpress the laci^q repressor, such as DH5á and Top10, cannot maintain plasmids that express the ccdB gene. Transformants can be killed due to the ccdB activity. DH5á and Top10 cells can be purchased from several vendors (Life Technologies or Invitrogen). Using this selective cloning approach, the parental expression vector is propagated in a laci^q bacterial strain. The vector is digested with two restriction enzymes that both remove the ccdB gene cassette, and, in the case of HIV-1, are compatible with the insertion of HIV-1 envelope sequences (a, b, c, d). Following ligation of the vector and envelope fragments, a strain of bacteria lacking laci^q is transformed. Once transformed, bacteria containing plasmids in which the viral envelope inserts have replaced the ccdB killer gene can grow. Bacteria containing plasmids that retain or reconstitute the ccdB killer gene can not survive. In this way, the population of transformed

bacteria is enriched for plasmids that contain viral envelope inserts, but is lacking in the parental vector containing the ccdB gene. The construction of the pCXccdB vector is not essential for the success of phase I of this project, but it is expected to significantly improve the efficiency of cloning HIV-1 envelope sequences derived from patient samples; thus, the probability of maintaining the heterogeneity of viral sequences can be improved. The structure of the pCXccdB vector can be confirmed by restriction mapping and DNA sequencing.

(d) Inserting Viral Envelope Sequences into the pCXccdB Expression Vector:-

The functionality of the pCXccdB vector can be evaluated by setting up ligation reactions containing viral envelope sequences and incompletely digested pCXccdB vector DNA. Following bacterial transformation, plasmid DNA can be prepared from individual bacterial clones and analyzed by restriction digestion for the presence of viral envelope fragments and the absence of ccdB sequences. The feasibility of this approach is tested by amplifying the envelope region from a total of 13 available HIV-1 clones (pCRII-91US005.11, pCRII-91006.10, pCRII-92US657.1, pCRII-92US711.14, pCRII-91US712.4, pCRII-92US714.1, pCRII-91HT652.11, pCRII-92BR020.4, pCRII-91HT651.1A, pCRII-92HT593.1, pCRII-92HT594.10, pCRII-92HT596.4, pCRII-92HT599.24), obtainable through the AIDS research reagent reference program (ARRRP), Rockville, Maryland. Each fragment can be inserted into pCXccdB and the structure of the resulting pHIVenv expression vectors can be confirmed by restriction mapping and/or DNA sequencing. The functionality of each pHIVenv vector can be demonstrated by

measuring HIV-1 envelope protein synthesis in transfected cells (Western Blot), and by their ability to pseudotype envelope-deficient retrovirus vectors.

2) Construction of a Bio-safe Viral Expression Vector Comprising Indicator Nucleic Acid in Place of the Region Encoding the Envelope Protein.

A bio-safe viral vector is constructed to evaluate inhibitors of viral entry according to similar means and methods as described in U.S. Patent No. 5,837,464 and Petropoulos et al., 2000 used to evaluate inhibitors of PR and RT. The viral expression vector of the present invention can be co-transfected into cells together with the envelope expression vectors (described above) to produce high titer virus stocks. Such virus stocks can be evaluated for susceptibility to inhibitors of virus entry, including antiviral drugs and neutralizing antibodies. In the case of HIV-1, the viral expression vector can be generated from NL4-3, a well-characterized infectious molecular clone of HIV-1. The 5' long terminal repeat (LTR) which controls viral gene expression can be modified so that transcription of the viral genes in transfected cells is driven by the CMV immediate early promoter (Naviaux et al., 1996). Most of the envelope gene can be deleted, but important control elements such as the rev responsive element (RRE) and accessory protein coding regions (rev, tat) are retained . In place of the deleted envelope sequences, an indicator nucleic acid, such as a firefly luciferase reporter gene cassette that is under the control of CMV promoter-enhancer sequences (**Figures 1B and 3**) is inserted. Virus infection can be monitored by measuring

luciferase activity in infected cells. It is conceivable, although unlikely, that inter-plasmid recombination between the retroviral vector and, for example, the pHIVenv sequences in transfected cells may lead to the generation of infectious HIV-1. In effort to generate a biosafe vector, introduction of several genetic alterations in the HIV genome can be done. For example, deletion of most of the envelope gene, while retaining the important control sequence, RRE, and also deletion of the transcriptional enhancer sequences in the U3 region of the 3' LTR of the vector (**Figure 2**) can be accomplished. During the replication of the retroviral genome, the U3 region located at the 3' end of the virus genome serves as the template for the U3 region of the 5' LTR of the provirus in infected cells. Such proviruses lack the strong promoter element in the U3 region of the 5' LTR and thus are unable to produce retroviral RNA in infected cells. This self-inactivating (SIN) strategy has been used successfully for several retroviral vector systems, including HIV-1 (Hwang et al., 1997; Miyoshi et al, 1998). In the assay of the present invention, viral gene expression is not required in infected cells because virus infection is measured by a detectable signal produced by the indicator nucleic acid, such as the production of luciferase activity, driven by its own separate promoter (**Figure 1B**). Deletion of envelope sequences and the transcriptional enhancer region (U3) can be accomplished by standard molecular cloning procedures, and each deletion can be verified by DNA sequence analysis.

Functionality of this vector, for example in the case of HIV-1, designated pHIVlucÄU3, can be demonstrated by co-transfection of 293 cells with the pHIVenv vector described above. Efficient transcomplementation of viral proteins

produced by both vectors in the transfected cells can lead to the production of viral particles. Virus particles can be harvested from the culture supernatants and analyzed by Western-blotting. Virus titers can be quantitated by routine applications of either p24 ELISA, quantitative PCR or TaqMan assays.

It is not necessary to produce a self-inactivating viral expression vector to carry out the present invention, but it is desirable to improve assay reproducibility and biosafety.

3) Identification of Suitable Cell Lines Which Express Receptors and Co-receptors and Support Viral Infection.

Different mammalian cell lines that have been described previously and are known to support infection of a particular virus can be evaluated. As discussed herein for one embodiment relating to HIV-1, the assay can be performed by (a) co-transfecting a first cell with pHIVenv and pHIVlucÄU3, (b) harvesting virus after transfection, (c) using this virus to infect a second cell, both in the presence and absence of virus entry inhibitors, and (d) measuring luciferase production in infected cells.

Table 1 lists representative examples of such cell lines evaluated for HIV-1 infection, including the cell line and its associated receptor/co-receptor. Several of these cell lines can be obtained from public cell repositories.

Viral particles harvested from transfected 293 cell cultures can be used to infect a variety of different cell lines. In the case of HIV-1, the pHIVlucÄU3 vector contains deletions in the envelope gene and the U3 promoter-enhancer as described

above, therefore infection of a permissive cell line with virus particles produced by this vector is restricted to a single round of replication. This includes (a) virus attachment and entry, mediated by the viral envelope proteins, produced in trans by the pHIVenv vector as described, (b) the conversion of single stranded viral RNA into double stranded DNA by RT, and (c) integration of viral DNA into the host cell genome (provirus formation). The active transcription of viral genes by RNA polymerase II that normally occurs in infected cells following proviral integration can be restricted by deleting essential viral promoter-enhancer sequences in the pHIVlucÄU3 vector. However, this restriction can not interfere with luciferase gene expression in infected cells since this gene is driven independently of viral gene expression using an internal CMV promoter (**Figure 1B**). The amount of luciferase activity produced following infection can be used as a measure of viral infectivity.

HIV-1 attachment and entry into host cells requires interaction with a primary receptor (CD4) and one of several co-receptors, most often CCR5 or CXCR4. Cell lines can be screened that are known to express various combinations of CD4, CCR5 and CXCR4. Specifically, cell lines listed in Table 1 that express (a) CD4 plus CCR5, (b) CD4 plus CXCR4, and (c) CD4 plus CCR5 plus CXCR4 are evaluated. Cell lines that express the CD4 receptor alone, or either the CCR5 or CXCR4 co-receptor alone, may serve as useful controls and can be used to evaluate HIV-1 isolates that do not require CD4 binding or that use co-receptors other than CCR5 and CXCR4. The principal criterion for judging cell line suitability can be infectivity as measured by luciferase production (10^4-10^6 relative light units). In addition, cell lines can be

evaluated based on growth rates, viability, stability and other parameters as deemed necessary. Cell lines can be selected that are easy to maintain and for example, produce large amounts of luciferase activity following infection, which can be inf the ected by different envelope receptor tropisms, e.g. CD4/CXCR4 and CD4/CCR5. Additional well-characterized cell lines that support, for example, HIV replication and express the HIV-1 receptor and co-receptors (e.g. CEM-NKr-CCR5; release category a) are available through public repositories such as the ARRRP.

Further, cell lines can be enhanced using standard procedures, such as promoting infection by the addition of polybrene to cells (Porter et al., 1998). For example, in the case of HIV, other potential cell lines can be identified for use with the present invention by infection with HIV-1 laboratory strains and comparing the recombinant virus infectivity titers to those obtained with infectious HIV-1, or by transfecting cells directly with the viral expression plasmids described herein, and scoring for virus production. Accumulation of viral transcripts can be checked by using a quantitative RT-PCR assay. Cell lines suitable for other viruses can be identified in a similar manner.

The present invention can optimize assay conditions and allow for high-throughput testing of patient samples using automation. Sample preparation methods can be optimized to efficiently capture viral genomic and envelope RNAs. RT-PCR conditions can be optimized to enable amplification of patient-derived viral envelope sequences, such as HIV-1 envelope sequences (~2,600 base pairs) at low viral loads (~500 copies per ml).

4) Demonstration of the Utility of the Assay

The utility of the assay of the present invention is demonstrated by the results achieved from: (1) testing for dose-dependent inhibition of viral entry in the presence of well-characterized inhibitors; and the (2) testing for dose-dependent inhibition of infection in the presence of well-characterized HIV-1 neutralizing antibodies.

The following applications for the virus entry assay of the present invention were evaluated:

i) detecting inhibition of HIV-1 replication by inhibitors of virus attachment and entry (including fusion, receptor and co-receptor inhibitors);

ii) measuring changes in susceptibility to HIV-1 attachment and entry inhibitors; and

iii) detecting neutralization activity of antibodies generated in response to vaccines targeted against HIV-1 envelope proteins.

In a preferred embodiment, the assay can be performed by (a) co-transfecting a first cell with pHIVenv and pHIVlucÄU3 vectors, (b) harvesting virus after approximately 48 h after transfection, (c) using this virus to infect a second cell, both in the presence and absence of virus entry inhibitors and (d) measuring luciferase production approximately 48-72 hrs. after infection. Dose-dependent inhibition of HIV-1 replication can be evaluated against a wide range of virus

entry inhibitor concentrations using a 96-well format. The appropriate concentration range can be determined empirically for each inhibitor. The data can be plotted as the percent inhibition of luciferase activity vs. drug concentration (log_{10}). Data analysis can be performed using computer software. Inhibition curves are used to determine 50% inhibitory concentrations (IC_{50}) for specific drugs or antibodies (**Figure 6**).

Envelope proteins derived from a variety of well-characterized HIV-1 isolates are evaluated using pHIVenv vectors constructed as described above. To define envelope co-receptor tropism, in the case of HIV-1, infection using cells expressing CD4 plus CXCR4 and CD4 plus CCR5 is evaluated as described above. A wide variety of compounds that are known to inhibit HIV-1 entry (Table 2), including non-specific agents such as sulfonated polyanions (dextran sulfate and heparin) can be used with the assay of the present invention. Chemokines such as Rantes and SDF-1, the natural ligands for the CCR5 and CXCR4 chemokine receptors, respectively (see Alkhatib et al., 1996; Bleul et al., 1996) are also suitable for use with the present invention. Further, virus entry inhibitors such as T-20 and T1249 (Trimeris, Inc.), PRO 542 (Progenics), 5a8 (Tanox) were used to evaluate utility of the assay of the present invention.

Drug toxicity in target cells are evaluated using standard viability or cytotoxicity assays (e.g. dye exclusion, MTS, ATP).

HIV-1 mutants exhibiting reduced susceptibility to the fusion inhibitor T20 (Rimsky et al., 1998) and the genetic

determinants (mutations) that enable these viruses to replicate in the presence of drug map within the envelope protein (gp41-TM) have been described. To demonstrate that the assay of the present invention is capable of measuring changes in drug susceptibility (i.e. resistance), (a) pHIVenv vectors are generated that carry these mutant envelope genes, (b) first cells are co-transfected using these vectors and the pHIVlucÄU3 vector, (c) viruses bearing these mutant envelope proteins are harvested, and (d) the viruses are tested for infectivity in the presence of T20. Reduced drug susceptibility to T20 is evaluated by comparing the IC_{50} of viruses bearing mutant envelope proteins to those that lack the defined drug resistance mutations. Viruses bearing envelope proteins with drug resistance mutations can exhibit higher IC_{50} values than viruses bearing envelope proteins that lack drug resistance mutations, i.e. inhibition can require a higher drug concentration (equivalent to data presented in **Figure 8**). Drug resistance mutations can be introduced into envelope expression vectors (pHIVenv) using standard site directed mutagenesis techniques according to standard protocols (Petropoulos et al., 2000; Ziermann et al., 2000)

It is widely accepted that effective vaccines that protect against HIV-1 infection should elicit a strong humoral immune response characterized by broadly cross-reactive neutralizing antibodies. Consequently, the serum of vaccinated individuals is routinely evaluated for the presence of high titer neutralizing antibodies targeted against the immunogen. Most recently, using the HIV-1/simian immunodeficiency virus (SIV) chimeric virus macaque model (SHIV), Mascola and colleagues have shown that passive transfer of such neutralizing antibodies led to reduced viral load after

mucosal challenge (Mascola et al., 2000). The assay of the present invention can be used to rapidly and reliably determinine the viral neutralizing activity of antibodies generated in response to vaccines targeting envelope antigens, such HIV-1 envelope antigens. For example, the assay of the present invention can (a) generate pHIVenv vectors that express a variety of well-characterized envelope proteins, (b) co-transfect a first cell using these vectors and the pHIVlucÄU3 vector, (c) harvest viruses and incubate with serial dilutions of antibody preparations or vaccine serum (d) test these viruses for infectivity in a second cell. Data analysis and IC_{50} determinations can be performed as described previously and in the literature. In the case of HIV-1, viruses can be selected to represent different HIV-1 genetic backgrounds (e.g. clade A, B, C, D, E, F), different cell and co-receptor tropisms (macrophage/CCR5, T-cell/CXCR4), and different envelope properties (syncytium and non-syncytium inducing, laboratory adapted growth or primary isolate) (Table 2). It can be beneficial to prepare stocks of a defined titer from each virus to optimize assay sensitivity and reproducibility by using a virus input of approximately 20-100 $TCID_{50}$/well and making adjustments as necessary. Antibody preparations can be selected based on previously documented neutralization properties, either functional, such as their ability to neutralize primary isolates, or physical, such as their ability to bind specific gp120 or gp41 epitopes (Table 2). The performance of the assay of the present invention can be judged against the activity of these well-characterized antibody reagents in conventional virus neutralization assays as described in the scientific literature. Serum from a broadly representative group of HIV-1 infected individuals can be used to establish

an appropriate range of serum dilutions that can maximize assay sensitivity, yet minimize cytotoxicity. Cytoxicity can be evaluated using standard viability or cytotoxicity assays (e.g. dye exclusion, MTS, ATP).

The following examples are presented to further illustrate and explain the present invention and should not be taken as limiting in any regard.

EXAMPLE 1

Measuring Phenotypic Drug Susceptibility to Inhibitors of HIV-1 Entry

This example provides a means and method for accurately and reproducibly measuring susceptibility to inhibitors of HIV-1 attachment and entry (heretofore collectively referred to as entry). Based on this example, the means and method for measuring susceptibility to inhibitors of HIV-1 entry can be adapted to other viruses, including, but not limited to other lentiviruses (e.g. HIV-2), other retroviruses (e.g. HTLV-1 and 2), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus). This example further provides a means and method for measuring alterations (increases and decreases) in susceptibility to entry inhibitors.

Measurements of entry inhibitor susceptibility are carried out using adaptations of the means and methods for phenotypic drug susceptibility and resistance tests described in US Patent Number 5,837,464 (International Publication Number WO 97/27319) which is hereby incorporated by reference.

One vector, an example of the envelope expression vector, (pHIVenv) is designed to express the envelope polyprotein (gp160) encoded by patient derived HIV envelope sequences (**Figure 1**). Gp160 is subsequently cleaved by a cellular protease to generate the surface (gp120SU) and transmembrane (gp41TM) subunits that comprise the envelope protein on the surface of HIV-1 virus particles. A second vector, an example of the viral expression vector, (either pHIVluc or pHIVluc U3) is designed to express genomic and subgenomic viral RNAs and all HIV proteins except the envelope polyprotein **(Figures 1A-1B)**.

In this application, patient-derived segment(s) correspond to the coding region (~2.5 kB) of the HIV-1 envelope polyprotein (gp160) and represent either (a) envelope sequences amplified by the reverse transcription-polymerase chain reaction method (RT-PCR) using viral RNA isolated from virus derived from HIV-infected individuals, or (b) envelope sequences derived from molecular clones of HIV-1 that contain specific mutations introduced by site directed mutagenesis of a parental molecular clone (typically NL4-3).

Isolation of viral RNA was performed using standard procedures (e.g. RNAgents Total RNA Isolation System, Promega, Madison WI or RNAzol, Tel-Test, Friendswood, TX). The RT-PCR protocol was divided into two steps. A retroviral reverse transcriptase [e.g. Superscript II (Invitrogen, Life Technologies) Moloney MuLV reverse transcriptase (Roche Molecular Systems, Inc., Branchburg, NJ), or avian myeloblastosis virus (AMV) reverse transcriptase, (Boehringer Mannheim, Indianapolis, IN)] was used to copy viral RNA into first strand cDNA. The cDNA was then amplified to high copy

number using a thermostable DNA polymerase [e.g. Taq (Roche Molecular Systems, Inc., Branchburg, NJ), Tth (Roche Molecular Systems, Inc., Branchburg, NJ), PrimeZyme (isolated from Thermus brockianus, Biometra, Gottingen, Germany)] or a combination of thermostable polymerases as described for the performance of "long PCR" (Barnes, W.M., (1994) Proc. Natl. Acad. Sci, USA 91, 2216-2220) [e.g. Expand High Fidelity PCR System (Taq + Pwo), (Boehringer Mannheim. Indianapolis, IN) OR GeneAmp XL PCR kit (Tth + Vent), (Roche Molecular Systems, Inc., Branchburg, NJ), Advantage-2, (CloneTech).

Oligo-dT was used for reverse transcription of viral RNA into first strand cDNA. Envelope PCR primers, forward primer Xho/Pin and reverse primer Mlu/Xba (**Table 3**) were used to amplify the patient-derived segments. These primers are designed to amplify the ~2.5 kB envelope gene encoding the gp160 envelope polyprotein, while introducing Xho I and Pin AI recognition sites at the 5' end of the PCR amplification product, and Mlu I and Xba I sites at the 3' end of the PCR amplification product.

Patient derived segments (2.5 kB envelope sequence amplification product) were inserted into HIV-1 envelope expression vectors using restriction endonuclease digestion, DNA ligation and bacterial transformation methods as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319), with minor adaptations. The ~2.5 kB amplification product was digested with either Xho I or Pin AI at the 5' end and either Mlu I or Xba I at the 3' end. The resulting digestion products were ligated, using DNA ligase, into the 5' Xho I/Pin AI and 3' Mlu I/Xba I sites

of modified pCXAS or pCXAT expression vectors. The construction of the pCXAS and pCXAT vectors has been described (in the CPT of base patent, I believe it was example 6 US Patent Number 5,837,464 (International Publication Number WO 97/27319)). Modified pCXAS and pCXAT vectors contain a Pin AI restriction site in addition to the Xho I, MluI and Xba I restriction sites that exist in pCXAS and pCXAT. The Pin AI site was introduced between the Xho I and Mlu I sites by site directed mutagenesis, such that the four sites are located 5' to 3' in the following order; Xho I, Pin AI, Mlu I and Xba I. In a preferred embodiment, the 2.5 kB amplification products were digested with Pin AI and Mlu I and ligated into the 5' Pin AI site and the 3' Mlu I site of the modified pCXAS expression vector. Ligation reaction products were used to transform E. coli. Following a 24-36 h incubation period at 30-37° C, the expression vector plasmid DNA was purified from the E. coli cultures. To ensure that expression vector preparations adequately represents the HIV quasi-species present in the serum of a given patient, many (>100) independent E. coli transformants were pooled and used for the preparations of pHIVenv plasmid DNA. Vectors that are assembled in this manner for the purposes of expressing patient virus derived envelope proteins are collectively referred to as pHIVenv **(Figures 1 and 3)**.

The genomic HIV expression vectors pHIVluc and pHIVluc@U3 are designed to transcribe HIV genomic RNA and subgenomic mRNAs and to express all HIV proteins except the envelope polyprotein **(Figure 1B)**. In these vectors, a portion of the envelope gene has been deleted to accommodate a functional indicator gene cassette, in this case, "Firefly Luciferase"

that is used to monitor the ability of the virus to replicate in the presence or absence of anti-viral drugs. In pHIVluc@U3, a portion of the 3' U3 region has been deleted to prevent transcription of viral RNAs from the 5' LTR in infected cells.

Susceptibility assays for HIV-1 entry inhibitors were performed using packaging host cells consisting of the human embryonic kidney cell line 293 (Cell Culture Facility, UC San Francisco, SF, CA) and target host cells consisting of a human osteosarcoma (HOS) cell line expressing CD4 (HT4) plus CCR5, and CXCR4, or astrocytoma (U-87) cell lines expressing either CD4 and CCR5 or CD4 and CXCR4.

Drug susceptibility testing was performed using pHIVenv and pHIVluc or pHIVluc U3. Pseudotyped HIV particles containing envelope proteins encoded by the patient derived segment were produced by transfecting a packaging host cell (HEK 293) with resistance test vector DNA. Virus particles were collected (~48 h) after transfection and are used to infect target cells (HT4/CCR5/CXCR4, or U-87/CD4/CXCR4, or U-87/CD4/CCR5) that express HIV receptors (i.e. CD4) and co-receptors (i.e. CXCR4, CCR5). After infection (~72 h) the target cells are lysed and luciferase activity is measured. HIV must complete one round of replication to successfully infect the target host cell and produce luciferase activity. The amount of luciferase activity detected in the infected cells is used as a direct measure of "infectivity" **(Figure 1 and 2)**. If for any reason (e.g. lack of the appropriate receptor or co-receptor, inhibitory drug activity, neutralizing antibody binding), the virus is unable to enter the target cell, luciferase activity is diminished. Drug susceptibility is

assessed by comparing the infectivity in the absence of drug to infectivity in the presence of drug. Relative drug susceptibility can be quantified by comparing the susceptibility of the "test" virus to the susceptibility of a well-characterized reference virus (wildtype) derived from a molecular clone of HIV-1, for example NL4-3 or HXB2.

Packaging host cells were seeded in 10-cm-diameter dishes and were transfected one day after plating with pHIVenv and pHIVluc or pHIVlucÄU3. Transfections were performed using a calcium-phosphate co-precipitation procedure. The cell culture media containing the DNA precipitate was replaced with fresh medium, from one to 24 hours, after transfection. Cell culture media containing viral particles was typically harvested 2 days after transfection and was passed through a 0.45-mm filter. Before infection, target cells were plated in cell culture media. Entry inhibitor drugs were typically added to target cells at the time of infection (one day prior to infection on occasion). Typically, 3 days after infection target cells were assayed for luciferase activity using the Steady-Glo reagent (Promega) and a luminometer.

In one embodiment, the susceptibility to a fusion inhibitor drug (T-20, also referred to as DP178; Trimeris, Research Triangle Park, NC) was demonstrated **(Figure 6)**. Target cells (HT4/CCR5/CXCR4) expressing CD4, CCR5 and CXCR4 were infected in the absence of T-20 and over a wide range of T-20 concentrations (x-axis $\log_{10}$ scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of T-20 to the amount of luciferase produced in the absence of T-20. R5 tropic (JRCSF, 91US005.11), X4 tropic

(NL4-3, 92HT599.24) and dual tropic (92HT593.1) viruses were tested. Drug susceptibility is quantified by determining the concentration of T-20 required to inhibit viral replication by 50% (IC_{50}, shown as vertical dashed lines in **Figure 6**). Viruses with lower IC_{50} values are more susceptible to T-20 than viruses with higher IC_{50} values.

In still further embodiments, susceptibility to a wide variety of entry inhibitors can be measured. These inhibitors include, but are not limited to, the drugs and compound listed in Table 4 (anti-HIV drug table).

In a second embodiment, susceptibility to a CCR5 inhibitor belonging to the 4-(piperidin-1-yl) butane class of compounds (Dorn, C.P. et al., (2001), Finke, P.E. et al., (2001); Merck, West Point, PA) is demonstrated. Target cells (U-87/CD4/CCR5) expressing CD4 and CCR5 (R5 cells) were infected in the absence of the CCR5 inhibitor and over a wide range of CCR5 inhibitor concentrations (x-axis log_{10} scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of CCR5 inhibitor to the amount of luciferase produced in the absence of CCR5 inhibitor. R5 tropic (JRCSF), X4 tropic (NL4-3) and dual tropic viruses (92HT593.1) were tested. Drug susceptibility was quantified by determining the concentration of CCR5 inhibitor required to viral replication by 50% (IC_{50}, shown as vertical dashed lines in **Figure 8**). Viruses with lower IC_{50} values are more susceptible to the CCR5 inhibitor than viruses with higher IC_{50} values. The X4 tropic virus did not infect the U-87/CD4/CCR5 target cells.

In a third embodiment, susceptibility to a CXCR4 inhibitor (AMD3100; AnorMED) was demonstrated. Target cells (U-87/CD4/CXCR4) expressing CD4 and CXCR4 were infected in the absence of the CXCR4 inhibitor and over a wide range of CXCR4 inhibitor concentrations (x-axis $\log_{10}$ scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of CXCR4 inhibitor to the amount of luciferase produced in the absence of CXCR4 inhibitor. R5 tropic (JRCSF), X4 tropic (NL4-3) and dual tropic (92HT593.1) viruses were tested. Drug susceptibility is quantified by determining the concentration of CXCR4 inhibitor required to inhibit viral replication by 50% (IC_{50}, shown as vertical dashed lines in **Figure 9**). Viruses with lower IC_{50} values are more susceptible to the CCR5 inhibitor than viruses with higher IC_{50} values. The R5 tropic virus did not infect the U-87/CD4/CXCR4 target cells.

Susceptibility to a CD4 inhibitor (e.g. murine monoclonal antibody 5A8; Tanox, Houston, TX) can be measured. Target cells (e.g. HT4/CCR5/CXCR4, U-87/CD4/CXCR4, or U-87/CD4/CCR5) expressing CD4 and one or both co-receptors can be infected in the absence of the CD4 inhibitor drug and over a wide range of CD4 inhibitor drug concentrations (x-axis $\log_{10}$ scale). The percent inhibition of viral replication (y-axis) can be determined by comparing the amount of luciferase produced in infected cells in the presence of CD4 inhibitor to the amount of luciferase produced in the absence of CD4 inhibitor. R5 tropic (e.g. JRCSF), X4 tropic (e.g. NL4-3) and dual tropic (e.g. 92HT593.1) viruses can be tested. Drug susceptibility can be quantified by determining the concentration of CD4 inhibitor required to inhibit viral

replication by 50% (IC_{50}). Viruses with lower IC_{50} values are more susceptible to the CD4 inhibitor than viruses with higher IC_{50} values.

EXAMPLE 2

Discovery, optimization and characterization of new and novel inhibitors of virus entry.

In one embodiment, the virus entry assay can be used to identify new compounds/chemical entities that inhibit virus entry. Target cells (e.g. HT4/CCR5/CXCR4, U-87/CD4/CXCR4, or U-87/CD4/CCR5) expressing CD4 and one or both co-receptors can be infected in the presence of individual members of large chemical libraries (high throughput screening, HTS). The ability of a compound to inhibit viral replication (a "hit") can be determined by comparing the amount of luciferase produced in infected target cells in the presence of a specific compound to the amount of luciferase produced in the absence of the compound.

In a further embodiment, the virus entry assay can be used to optimize the antiviral activity of lead compounds identified by HTS. Chemical modified derivatives of lead compounds can be tested to identify specific derivatives that have enhanced virus entry inhibitory activity. Target cells (e.g. HT4/CCR5/CXCR4, U-87/CD4/CXCR4, or U-87/CD4/CCR5) expressing CD4 and one or both co-receptors can be infected in the absence of the inhibitor candidate and over a wide range of inhibitor candidate concentrations (x-axis $\log_{10}$ scale). The percent inhibition of viral replication (y-axis) can be determined by comparing the amount of luciferase produced in infected cells in the presence of the candidate

inhibitor to the amount of luciferase produced in the absence of candidate inhibitor. Drug susceptibility can be quantified by determining the concentration of inhibitor candidate required to inhibit viral replication by 50% (IC_{50}). Derivatized compounds with lower IC_{50} values are more potent inhibitors of virus entry (have greater antiviral activity) than derivatives with higher IC_{50} values.

In yet a further embodiment, the virus entry assay can be used to characterize the mechanism of action of new virus entry inhibitor drug candidates, and the antiviral activity against a spectrum of viruses that may differ in susceptibility. Target cells (e.g., HT4/CCR5/CXCR4, U-87/CD4/CXCR4, or U-87/CD4/CCR5) expressing CD4 and one or both co-receptors can be infected in the absence of the new entry inhibitor drug candidate and over a wide range of entry inhibitor drug concentrations (x-axis $\log_{10}$ scale). The percent inhibition of viral replication (y-axis) can be determined by comparing the amount of luciferase produced in infected cells in the presence of new entry inhibitor to the amount of luciferase produced in the absence of the new entry inhibitor. R5 tropic (e.g. JRCSF), X4 tropic (e.g. NL4-3) and dual tropic (e.g. 92HT593.1) viruses can be tested. Drug susceptibility can be quantified by determining the concentration of CD4 inhibitor required to inhibit viral replication by 50% (IC_{50}).

To determine whether the new entry inhibitor acts by blocking the CCR5 or CXCR4 co-receptors, the R5 tropic viruses are tested against the new inhibitor in U-87/CD4/CCR5 cells and X4 tropic viruses are tested against the new inhibitor using U-87/CD4/CXCR4 cells. Inhibition of R5 virus infection is

indicative of CCR5 co-receptor antagonism and conversely, inhibition of X4 virus infection is indicative of CXCR4 co-receptor antagonism. Inhibition of R5 and X4 virus infection may be indicative of either CD4 antagonism or the inhibition of membrane fusion.

To characterize the activity of a new inhibitor against viruses that exhibit resistance, or have reduced susceptibility, to other virus entry inhibitors of the same class, or different class, selected panels of drug resistant viruses can be tested in the virus entry assay using the new entry inhibitor drug. The panel may include viruses with varying levels of susceptibility to CCR5 inhibitors, CXCR4 inhibitors, CD4 inhibitors, and membrane fusion inhibitors. The panel may include viruses with one or more specific mutations that are associated with reduced susceptibility/resistance to one or more entry inhibitors.

EXAMPLE 3

Identifying Envelope Amino Acid Substitutions/Mutations that Alter Susceptibility to Virus Entry Inhibitors.

This example provides a means and method for identifying mutations in HIV-1 envelope that confer reduced susceptibility/resistance to virus entry inhibitors. This example also provides a means and method for quantifying the degree of reduced susceptibility to entry inhibitors conferred by specific envelope mutations.

Envelope sequences derived from patient samples, or individual clones derived from patient samples, or envelope sequences engineered by site directed mutagenesis to contain

specific mutations, are tested in the entry assay to quantify drug susceptibility based on a well-characterized reference standard (e.g. NL4-3, HXB2).

In one embodiment, susceptibility to longitudinal patient samples (viruses collected from the same patient at different timepoints) is evaluated. For example, susceptibility to entry inhibitors is measured prior to initiating therapy, before or after changes in drug treatment, or before or after changes in virologic (RNA copy number), immunologic (CD4 T-cells), or clinical (opportunistic infection) markers of disease progression.

Genotypic analysis of patient HIV samples

Envelope sequences representing patient sample pools, or clones derived from patient pools, can be analyzed by any broadly available DNA sequencing methods. In one embodiment of the invention, patient HIV sample sequences are determined using viral RNA purification, RT/PCR and dideoxynucleotide chain terminator sequencing chemistry and capillary gel electrophoresis (Applied Biosystems, Foster City, CA). Envelope sequences of patient virus pools or clones are compared to reference sequences, other patient samples, or to a sample obtained from the same patient prior to initiation of therapy, if available. The genotype is examined for sequences that are different from the reference or pre-treatment sequence and correlated to differences in entry inhibitor susceptibility.

Entry inhibitor susceptibility of site directed mutants

Genotypic changes that correlate with changes in fitness are evaluated by constructing envelope expression vectors

(pHIVenv) containing the specific mutation on a defined, drug susceptible, genetic background (e.g. NL4-3 reference strain). Mutations may be incorporated alone and/or in combination with other mutations that are thought to modulate the entry inhibitor susceptibility. Envelope mutations are introduced into pHIVenv vectors using any of the broadly available methods for site-directed mutagenesis. In one embodiment of this invention the mega-primer PCR method for site-directed mutagenesis is used (Sarkar, G. and Summer, S.S., 1990). A pHIVenv vector containing a specific envelope mutation or group of mutations are tested using the virus entry assay described in Example 1. Drug susceptibility of the virus containing envelope mutations is compared to the drug susceptibility of a genetically defined drug susceptible virus that lacks the specific mutations under evaluation. Observed changes in entry inhibitor susceptibility are attributed to the specific mutations introduced into the pHIVenv vector.

In one embodiment of the invention, reduced susceptibility to the fusion inhibitor T-20 conferred by specific drug resistance mutations in the gp41 envelope protein is demonstrated **(Figure 7)**. Cells expressing CD4, CCR5 and CXCR4 were infected in the absence of T-20 and over a wide range of T-20 concentrations (x-axis log10 scale). The percent inhibition of viral replication (y-axis) was determined by comparing the amount of luciferase produced in infected cells in the presence of T-20 to the amount of luciferase produced in the absence of T-20. Isogenic viruses containing one or two specific mutations in the gp41 transmembrane envelope protein were tested (highlighted in red in the figure legend; Rimsky et al., J. Virol. 72: 986-

993). Drug susceptibility is quantified by determining the concentration of T-20 required to inhibit viral replication by 50% (IC_{50}, shown as vertical dashed lines). Viruses with lower IC_{50} values are more susceptible to T-20 than viruses with higher IC_{50} values.

In one embodiment, drug resistance mutations were introduced into well-characterized X4 tropic (NL4-3) and R5 tropic (JRCSF) viruses. T20 susceptibility was measured using the virus entry assay **(Figure 7)**. The fold change (FC) in T-20 susceptibility for each virus was determined by dividing the IC50 of the test virus by the IC50 of the HXB2 strain of HIV-1. T-20 sensitivity of similar mutant viruses has been reported in the scientific literature (Rimsky et al.,). In this embodiment, viruses with one mutation within the GIV motif of gp41 (DIV, GIM, SIV) were less susceptible to T20 than the wildtype virus (GIV) **(Figure 11)**. Viruses with two mutations within the GIV motif (DIM, SIM, DTV) were less susceptible to T20 than viruses with one, or no mutations in the GIV motif **(Figure 11)**.

In another embodiment, mutations that may confer reduced (or increased) susceptibility to the entry inhibitor are identified by sequencing the envelope genes of the sensitive and resistant viruses. The deduced amino acid sequences of the sensitive and resistant viruses are compared to identify candidate drug resistance mutations. The ability of a specific mutation to confer altered drug susceptibility is confirmed or disproved by introducing the mutation into a drug sensitive virus and measuring the susceptibility of the mutant virus in the virus entry assay. In the example represented here, a short stretch of amino acid sequences

within the first heptad repeat (HR-1) of the HIV-1 gp41 transmembrane envelope protein is aligned for viruses exhibiting different T-20 susceptibilities **(Figure 11)**. Highlighted amino acids represent mutations known to confer reduced susceptibility to T-20.

Similar phenotypic and genotypic analyses can be used to identify envelope amino acid sequences that (a) alter/influence susceptibility to CCR5 or CXCR4 inhibitors, (b) specify X4, R5 and dual tropism, and (c) elicit neutralizing antibodies.

In one embodiment, reduced susceptibility to co-receptor (CCR5, CXCR4) inhibitors conferred by specific envelope amino acid sequences/mutations is demonstrated.

In a further embodiment, reduced susceptibility to receptor (CD4) inhibitors conferred by specific envelope amino acid sequences/mutations is demonstrated.

EXAMPLE 4

Determining HIV-1 Co-receptor and Receptor Tropism

This example provides a means and method for determining HIV-1 co-receptor tropism. This example also provides a means and method for determining HIV-1 receptor tropism.

In one embodiment, viruses that use the CCR5 co-receptor are identified. In a related embodiment, viruses that use the CXCR4 co-receptor are identified. In a further related embodiment, viruses that use CCR5 and CXCR4 are identified. In a further related embodiment, viruses that use co-

receptors other than CCR5 or CXCR4 are identified.

In another embodiment, viruses that use the CD4 receptor are identified. In a related embodiment, viruses that use CD8 are identified. In a further related embodiment, viruses that do not require CD4 or CD8 to infect cells are identified.

In this embodiment, the assay is performed using two cell lines. One cell line expresses CD4 plus CCR5 (U-87/CD4/CCR5), also referred to as R5 cells in this application. The other cell line expresses CD4 and CXCR4 (U-87/CD4/CXCR4) also referred to as X4 cells in this application. The virus entry assay is performed by infecting individual cell cultures with recombinant virus stocks derived from cells transfected with pHIVenv and pHIV luc or pHIVlucDU3 vectors. pHIVenv vectors contain patient virus derived sequences and express HIV-1 envelope proteins (gp120SU, gp41TM). In this embodiment viruses are evaluated in using R5 and X4 target cells cultured in 96 well plates **(Figure 3A)**. Typically, R5 and X4 cells are plated one day prior to infection. Infection with each virus stock is performed in the absence of drug (no drug), in the presence of inhibitory concentrations of a drug that preferentially inhibits R5 tropic viruses (CCR inhibitor, e.g. a piperidin 1yl butane compound), and in the presence of inhibitory concentrations of a drug that preferentially inhibits X4 tropic viruses (CXCR4 inhibitor, e.g. AMD3100). Co-receptor tropism is assessed by comparing the amount of luciferase activity produced in each cell type, both in the presence and absence of drug. In this embodiment, the results of the assay are interpreted by comparing the ability of each virus

to preferentially infect (produce luciferase activity) R5 cells or X4 cells, or both X4 and R5 cells if the virus is dual tropic. The ability of the CCR5 or CXCR4 inhibitor to specifically block infection (inhibit luciferase activity) is also evaluated **(Figure 3B)**. In this embodiment, X4 tropic viruses infect X4 cells but not R5 cells and infection of X4 cells is blocked by the the CXCR4 inhibitor (AMD3100). In this embodiment, R5 tropic viruses infect R5 cells but not X4 cells and infection of R5 cells is blocked by the CCR5 inhibitor (piperidin-1yl butane compound). In this embodiment, dual tropic, or mixtures of X4 and R5 tropic viruses, infect both X4 and R5 cells and infection of R5 cells is blocked by the CCR5 inhibitor and infection of X4 cells is blocked by the CXCR4 inhibitor. In this embodiment, non-viable viruses do not replicate in either X4 or R5 cells (luciferase activity is not produced).

In another embodiment, the assay is performed using three or more cell lines. One cell line expresses CD4 plus CCR5 (U-87/CD4/CCR5), also referred to as R5 cells in this application. The other cell line expresses CD4 and CXCR4 (U-87/CD4/CXCR4) also referred to as X4 cells in this application. Additional cell lines express CD4 plus other candidate HIV-1 co-receptors, including, but not limited to, BONZO, BOB, etc. See Table 1. These additional cell lines express other candidate co-receptors, but do not express CCR5 or CXCR4. The virus entry assay is performed by infecting individual cell cultures with recombinant virus stocks derived from cells transfected with pHIVenv and pHIV luc or pHIVlucDU3 vectors. pHIVenv vectors contain patient virus derived sequences and express HIV-1 envelope proteins (gp120SU, gp41TM). In this embodiment viruses are evaluated

in using cells cultured in 96 well plates. Infection with each virus stock is performed in R5 cells, X4 cells and the cell lines expressing CD4 plus the candidate co-receptors. Co-receptor tropism is assessed by comparing the amount of luciferase activity produced in each cell type. In this embodiment, the results of the assay are interpreted by comparing the ability of each virus to preferentially infect (produce luciferase activity) R5 cells or X4 cells, or the cell line that expresses the candidate co-receptor. In this embodiment, X4 tropic viruses infect X4 cells but not R5 cells. In this embodiment, R5 tropic viruses infect R5 cells but not X4 cells. In this embodiment dual tropic, or mixtures of X4 and R5 tropic viruses, infect both X4 and R5 cells. In this embodiment, the infection of cell lines expressing alternative candidate co-receptors (neither CCR5 or CXCR4) is attributed to tropism for the alternative co-receptor. In this embodiment, non-viable viruses do not replicate in either X4 or R5 cells.

In another embodiment, the assay is performed using four cell lines. One cell line expresses CD4 plus CCR5 (U-87/CD4/CCR5), also referred to as R5 cells in this application. A second other cell line expresses CD4 and CXCR4 (U-87/CD4/CXCR4) also referred to as X4 cells in this application. A third cell line expresses CD8 plus CCR5 (U-87/CD8/CCR5), also referred to as CD8/R5 cells in this application. A fourth cell line expresses CD8 and CXCR4 (U-87/CD8/CXCR4) also referred to as CD8/X4 cells in this application. The virus entry assay is performed by infecting individual cell cultures with recombinant virus stocks derived from cells transfected with pHIVenv and pHIV luc or pHIVlucDU3 vectors. pHIVenv vectors contain patient virus

derived sequences and express HIV-1 envelope proteins (gp120SU, gp41TM). In this embodiment viruses are evaluated in using cells cultured in 96 well plates. Infection with each virus stock is performed in R5 cells, X4 cells, CD8/R5 cells and CD8/X4 cells. Co-receptor tropism is assessed by comparing the amount of luciferase activity produced in each cell type. In this embodiment, the results of the assay are interpreted by comparing the ability of each virus to preferentially infect (produce luciferase activity) R5 cells, X4 cells, CD8/R5 cells, or CD8/X4 cells. In this embodiment, CD4 tropic viruses infect X4 cells and/or R5 cells. In this embodiment, CD8 tropic viruses infect CD8/R5 cells and/or CD8/X4 cells. In this embodiment, dual tropic (CD4 and CD8 receptor use) viruses infect X4 cells and/or R5 cells plus CD8/X4 and/or CD8/R5 cells. In this embodiment, the infection of cell lines expressing CD8 but not CD4 is attributed to CD8 receptor tropism. In this embodiment, non-viable viruses do not replicate in either X4 or R5 cells.

In a further related embodiment, the assay is performed using two cell lines. One cell line expresses CD4 plus CCR5 and CXCR4 (HT4/CCR5/CXCR4). A second cell line expresses CD8 plus CCR5 and CXCR4 (HOS/CD8/CCR5/CXCR4). The virus entry assay is performed by infecting individual cell cultures with recombinant virus stocks derived from cells transfected with pHIVenv and pHIV luc or pHIVlucDU3 vectors. pHIVenv vectors contain patient virus derived sequences and express HIV-1 envelope proteins (gp120SU, gp41TM). In this embodiment viruses are evaluated in using cells cultured in 96 well plates. Infection with each virus stock is performed in HT4/CCR5/CXCR4 cells and HOS/CD8/CCR5/CXCR4 cells. Co-receptor tropism is assessed by comparing the amount of

luciferase activity produced in each cell type. In this embodiment, the results of the assay are interpreted by comparing the ability of each virus to preferentially infect (produce luciferase activity) HT4/CCR5/CXCR4 cells or HOS/CD8/CCR5/CXCR4 cells. In this embodiment, CD4 tropic viruses infect HT4/CCR5/CXCR4 cells, but not HOS/CD8/CCR5/CXCR4 cells. In this embodiment, CD8 tropic viruses infect HOS/CD8/CCR5/CXCR4 cells but not HT$/CCR5/CXCR4 cells. In this embodiment, dual tropic (CD4 and CD8 receptor use) viruses infect both HT4/CCR5/CXCR4 cells and HOS/CD8/CCR5/CXCR4 cells. In this embodiment, the infection of cell lines expressing CD8 but not CD4 is attributed to CD8 receptor tropism. In this embodiment, non-viable viruses do not replicate in either X4 or R5 cells.

In another embodiment, the assay is performed using two cell lines. One cell line expresses CD4 plus CCR5 and CXCR4 (HT4/CCR5/CXCR4). A second cell line expresses CCR5 and CXCR4 but not CD4 or CD8 (HOS/CCR5/CXCR4). The virus entry assay is performed by infecting individual cell cultures with recombinant virus stocks derived from cells transfected with pHIVenv and pHIV luc or pHIVlucDU3 vectors. pHIVenv vectors contain patient virus derived sequences and express HIV-1 envelope proteins (gp120SU, gp41TM). In this embodiment viruses are evaluated in using cells cultured in 96 well plates. Infection with each virus stock is performed in HT4/CCR5/CXCR4 cells and HOS/CCR5/CXCR4 cells. CD4 and CD8 independent infection is assessed by comparing the amount of luciferase activity produced in each cell type. In this embodiment, the results of the assay are interpreted by comparing the ability of each virus to preferentially infect (produce luciferase activity) HT4/CCR5/CXCR4 cells or

HOS/CCR5/CXCR4 cells. In this embodiment, CD4 dependent viruses infect HT4/CCR5/CXCR4 cells, but not HOS/CCR5/CXCR4 cells. In this embodiment, CD4 independent viruses infect both HOS/CCR5/CXCR4 cells and HT4/CCR5/CXCR4 cells. In this embodiment, the infection of cell lines that lack CD4 expression is attributed to CD4 independent infection. In this embodiment, non-viable viruses do not replicate in either X4 or R5 cells.

EXAMPLE 5

Identifying HIV-1 Envelope Amino Acid Substitutions/Mutations that Alter Co-receptor and Receptor Tropism

This example provides a means and method for identifying HIV-1 envelope amino acid sequences that specify, or alter, co-receptor tropism (X4 vs. R5 vs. dual X4/R5). This example also provides a means and method for identifying HIV-1 envelope amino acid sequences that specify co-receptor usage other than CXCR4 or CCR5. The example also provides a means and method for identifying HIV-1 envelope sequences that specific, or receptor tropism (CD4 vs. CD8).

Envelope sequences derived from patient samples, or individual clones derived from patient samples, or envelope sequences engineered by site directed mutagenesis to contain specific mutations, are tested in the entry assay to determine co-receptor tropism as described in **Example 4**.

In one embodiment, co-receptor tropism of longitudinal patient samples (viruses collected from the same patient at different timepoints) is evaluated. For example, co-receptor tropism is evaluated prior to initiating therapy, before or

after changes in drug treatment, or before or after changes in virologic (RNA copy number), immunologic (CD4 T-cells), or clinical (opportunistic infection) markers of disease progression.

In another embodiment, co-receptor tropism is evaluated for samples collected from a large number of different patients. In a further embodiment, co-receptor tropism is evaluated for samples collected from a large number of patients representing different virus and patient populations. Such patient populations may include, but are not limited to, newly infected patients, chronically infected patients, patients with advanced disease, and patients undergoing antiretroviral therapy or immuno-therapy. Such virus populations may include, but are not limited to, viruses with distinct genetic characteristics (clade A, B, C, D, E, F, G), viruses susceptible to antiretroviral drugs, viruses with reduced susceptibility/resistance to antiretroviral drugs.

Genotypic analysis of patient HIV samples

Envelope sequences representing patient sample pools, or clones derived from patient pools, can be analyzed by any broadly available DNA sequencing methods. In one embodiment of the invention, patient HIV sample sequences are determined using viral RNA purification, RT/PCR and dideoxynucleotide chain terminator sequencing chemistry and capillary gel electrophoresis (Applied Biosystems, Foster City, CA). Envelope sequences of patient virus pools or clones are compared to reference sequences, other patient samples, or to a sample obtained from the same patient prior to initiation of therapy, if available. The genotype is

examined for sequences that are different from the reference or pre-treatment sequence and correlated to differences in entry inhibitor susceptibility.

Co-receptor and Receptor tropism of genetically characterized viruses

Envelope amino acid sequences that correlate co-receptor tropism are evaluated by constructing envelope expression vectors (pHIVenv) containing a specific mutation on a defined genetic background (e.g. NL4-3 for X4 tropism, JRCSF for R5 tropism). Mutations may be incorporated alone and/or in combination with other mutations that are thought to modulate co-receptor usage. Envelope mutations are introduced into pHIVenv vectors using any of the broadly available methods for site-directed mutagenesis. In one embodiment of this invention the mega-primer PCR method for site-directed mutagenesis is used (Sarkar, G. and Summer, S.S., 1990). A pHIVenv vector containing a specific envelope mutation or group of mutations are tested using the virus entry assay described in Example 1. Co-receptor tropism of the virus containing envelope mutations is compared to the co-receptor tropism of a genetically defined virus that lacks the specific mutations under evaluation. The ability of a specific mutation to confer altered co-receptor tropism is confirmed or disproved by introducing the mutation into well-characterized reference virus and evaluating the co-receptor tropism of the mutant virus in the virus entry assay as described in **Example 4**. Observed changes in co-receptor tropism are attributed to the specific mutations introduced into the pHIVenv vector.

In one embodiment of the invention, genetic determinants of

R5 tropism are identified by evaluating amino acid sequences within the V3 loop of the gp120 surface envelope protein. The amino acid sequences under evaluation are identified by comparing the amino acid sequences of large numbers of X4 tropic and R5 tropic viruses. Consistent differences between the X4 and R5 viruses are selected for evaluation. Isogenic viruses based on an well-characterized X4 parental clone (e.g NL4-3, HXB2) containing specific "R5 candidate" mutations in the V3 loop of the gp120 envelope protein are constructed by site directed mutagenesis and tested for co-receptor tropism as described in **Example 4**. Cells expressing CD4 plus CCR5 (e.g. U-87/CD4/CCR5) or CD4 plus CXCR4 (U-87/CD4/CXCR4) are infected in the absence of an R5 (peperidin-lyl butane compound) and X4 (AMD3100) inhibitor and in the presence of inhibitory concentrations of R5 and X4 drug concentrations. Amino acid substitutions that change the X4 tropic virus to an R5 tropic virus are characterized as genetic determinants of R5 tropism.

In a related embodiment of the invention, genetic determinants of X4 tropism are identified by evaluating amino acid sequences within the V3 loop of the gp120 surface envelope protein. The amino acid sequences under evaluation are identified by comparing the amino acid sequences of large numbers of X4 tropic and R5 tropic viruses. Consistent differences between the X4 and R5 viruses are selected for evaluation. Isogenic viruses based on an well-characterized R5 parental clone (e.g JRCSF) containing specific "X4 candidate" mutations in the V3 loop of the gp120 envelope protein are constructed by site directed mutagenesis and tested for co-receptor tropism as described in **Example 4**. Cells expressing CD4 plus CCR5 (e.g. U-87/CD4/CCR5) or CD4

plus CXCR4 (U-87/CD4/CXCR4) are infected in the absence of an R5 (peperidin-1yl butane compound) and X4 (AMD3100) inhibitor and in the presence of inhibitory concentrations of R5 and X4 drug concentrations. Amino acid substitutions that change the X4 tropic virus to an R5 tropic virus are characterized as genetic determinants of R5 tropism.

In a related embodiments of the invention, genetic determinants of X4 or R5 tropism are identified by evaluating amino acid sequences within the entire gp120 surface envelope protein.

In a related embodiment of the invention, genetic determinants of X4 or R5 tropism are identified by evaluating amino acid sequences within the gp41 transmembrane envelope protein.

In a related embodiment of the invention, genetic determinants that specify the use of co-receptors other than CCR5 and CXCR4 are identified by evaluating amino acid sequences within the V3 loop of the gp120 surface envelope protein. The amino acid sequences under evaluation are identified by comparing the amino acid sequences of viruses that are able to replicate on cells that do not express CXCR4 or CCR5, but do express other candidate co-receptors. Consistent differences in amino acid sequences between these non-X4, non R5 viruses and the X4 and R5 viruses are selected for evaluation. Isogenic viruses based on an well-characterized X4 (e.g. NL4-3) or R5 (e.g. JRCSF) parental clone containing specific "non-X4, non-R5 candidate" mutations in the V3 loop of the gp120 envelope protein are constructed by site directed mutagenesis and tested for co-

receptor tropism as described in **Example 4**. Cells expressing CD4 plus CCR5 (e.g. U-87/CD4/CCR5), CD4 plus CXCR4 (U-87/CD4/CXCR4), and CD4 plus other candidate co-receptors (U-87/CD4/X) are infected in the absence of an R5 (peperidin-1yl butane compound) and X4 (AMD3100) inhibitor and in the presence of inhibitory concentrations of R5 and X4 drug concentrations. Amino acid substitutions that confers tropism for a non-X4, non-R5 co-receptor are characterized as genetic determinants of tropism for the specific co-receptor.

In a related embodiments of the invention, genetic determinants of tropism for other co-receptors are identified by evaluating amino acid sequences within the entire gp120 surface envelope protein.

In a related embodiment of the invention, genetic determinants of tropism for other co-receptors are identified by evaluating amino acid sequences within the gp41 transmembrane envelope protein.

In another embodiment of the invention, genetic determinants that specify the use of CD8 (in addition to, or instead of CD4) as a receptor for HIV-1 are identified by evaluating amino acid sequences within the V3 loop of the gp120 surface envelope protein. The amino acid sequences under evaluation are identified by comparing the amino acid sequences of viruses that are able to replicate in cells that do not express CD4, but do express CD8. Consistent differences in amino acid sequences between these CD4 tropic viruses and CD8 tropic viruses are selected for evaluation. Isogenic viruses based on an well-characterized CD4 tropic (e.g. NL4-3, JRCSF)

parental clones containing specific "CD8 candidate" mutations in the V3 loop of the gp120 envelope protein are constructed by site directed mutagenesis and tested for CD8 receptor tropism as described in **Example 4**. Cells expressing CD4 plus CCR5 (e.g. U-87/CD4/CCR5), CD4 plus CXCR4 (U-87/CD4/CXCR4), CD8 plus CCR5 (e.g. U-87/CD8/CCR5), CD8 plus CXCR4 (U-87/CD8/CXCR4) are infected. Amino acid substitutions that enable replication in cells that express CD8 but not CD4 are characterized as genetic determinants of CD8 tropism.

In a related embodiments of the invention, genetic determinants of CD8 tropism are identified by evaluating amino acid sequences within the entire gp120 surface envelope protein.

In a related embodiment of the invention, genetic determinants of CD8 tropism are identified by evaluating amino acid sequences within the gp41 transmembrane envelope protein.

EXAMPLE 6

Measuring HIV-1 Antibody Neutralization

This example provides a means and method for evaluating antibody mediated neutralization of HIV-1, also referred to as virus neutralization in this application. This example also provides a means and method for evaluating the virus neutralization activity of antibodies within HIV-1 infected patients. This example also provides a means and method for evaluating the virus neutralizing activity of antibodies within individuals or animals vaccinated with therapeutic vaccines and vaccine candidates. This example also provides

a means and method for evaluating the virus neutralizing activity of antibodies within individuals or animals vaccinated with protective (preventative or prophylactic) vaccine and vaccine candidates. This example also provides a means and method for evaluating the virus neutralizing activity or preparations of specific monoclonal or polyclonal antibodies.

Envelope sequences derived from patient samples, or individual clones derived from patient samples, or envelope sequences engineered by site directed mutagenesis to contain specific mutations, are tested in the entry assay to evaluate antibody mediated neutralization.

In one embodiment, antibody mediated neutralization is evaluated in longitudinal patient samples (viruses collected from the same patient at different time points) is evaluated. For example, virus neutralization is evaluated prior to vaccination, during a course of vaccination, and at incremental time points after the course of vaccination is completed. In a related embodiment, the sera of animals including, but not limited to, mice, rats, rabbits, pigs, and cattle, are evaluated prior to inoculation with candidate vaccines, during a course of repeated inoculation, and at incremental time points after the course of inoculation is completed. In one embodiment, virus neutralization is evaluated for preventative vaccines and vaccine candidates. In another embodiment, virus neutralization is evaluated for therapeutic vaccines.

In another embodiment, virus neutralization is evaluated for samples collected from a large number of different patients.

In a further embodiment, virus neutralization is evaluated for samples collected from a large number of patients representing different patient populations and different virus populations. "Patient populations" may include, but are not limited to, newly infected patients, chronically infected patients, patients with advanced HIV/AIDS disease, patients with rapid disease progression, patients with slow disease progression (typically referred to as long term non-progressors), patients undergoing antiretroviral therapy or immuno-therapy (e.g. interleukin-2, or other cytokines), vaccinated and unvaccinated individuals. "Virus populations" may include, but are not limited to, viruses with distinct genetic characteristics and geographical origins (clade A, B, C, D, E, F, G), viruses susceptible to antiretroviral drugs, viruses with reduced susceptibility/resistance to antiretroviral drugs, primary isolates, isolates adapted for growth in cell culture (often referred to as lab-adapted viruses), syncytia inducing (SI) viruses, non-syncytia inducing (NSI) viruses, macrophage (M) tropic viruses, T-cell (T) tropic viruses and dual tropic (M and T) viruses.

Characterization of Patient Antibody (patient antibody vs. standard virus panel)

In this embodiment, the assay is performed using a target cell line that expresses the HIV-1 receptor CD4 plus the HIV-1 co-receptors CCR5 and CXCR4 (HT4/CCR5/CXCR4). Such a cell line is capable of evaluating the neutralizing activity of antibodies for both R5 and X4 tropic viruses. In a related embodiment, the assay is performed using two target cell lines. One cell line expresses CD4 plus CCR5 (U-87/CD4/CCR5) and is used to test R5 tropic viruses. Another cell line expresses CD4 plus CXCR4 (U-87/CD4/CXCR4)

and is used to evaluate X4 tropic viruses. The virus entry assay is performed by infecting individual target cell cultures with recombinant virus stocks derived from packaging host cells transfected with pHIVenv and pHIVluc or pHIVlucDU3 vectors. In this embodiment, pHIVenv vectors contain envelope sequences of specific, well-characterized viruses and express the HIV-1 envelope proteins (gp120SU, gp41TM). Such viruses represent a "standard virus panel" (see above description of virus population). Some, but not all, reasonable examples of viruses that may constitute a standard panel are listed in Table 4. A standard virus panel is used to compare the neutralizing antibody activity of sera obtained from many different patients and/or animals (see above description of patient population). In this embodiment viruses are evaluated using target cells cultured in 96 well plates. Typically, target cells are plated at 5,000 cells per well for HT4/CCR5/CXCR4 or 10,000 cells per well for U-87/CD4/CCR5 and U-87/CD4/CXCR4 one day prior to infection. Prior to target cell infection, each virus stock is pre-incubated with the sera or antibody preparation (typically for 1 h) that is being evaluated. The sera or antibody preparations are tested undiluted and at incrementally greater dilutions (typically four to five serial 10-fold dilutions). Infection of target cells with each virus stock is also performed in the absence of antibody (no antibody). Virus neutralization is assessed by comparing the amount of luciferase activity produced in target cells, both in the presence and absence of antibody. In this embodiment, the results of the assay are interpreted by comparing the ability of each antibody to preferentially block infection of target cells (reduce or eliminate luciferase activity). Virus neutralization activity is

quantified by noting the highest antibody dilution (most dilute) that is able to block target cell infection (e.g. the highest dilution that is able to reduce the luciferase activity produced in the absence of antibody by 50%).

Characterization of Patient HIV-1 (patient virus vs. standard antibody panel)

In this embodiment, the assay is performed using a target cell line that expresses the HIV-1 receptor CD4 plus the HIV-1 co-receptors CCR5 and CXCR4 (HT4/CCR5/CXCR4). Such a cell line is capable of evaluating the neutralizing activity of antibodies for both R5 and X4 tropic viruses. In a related embodiment, the assay is performed using two target cell lines. One cell line expresses CD4 plus CCR5 (U-87/CD4/CCR5) and is used to test R5 tropic viruses. Another cell line expresses CD4 plus CXCR4 (U-87/CD4/CXCR4) and is used to evaluate X4 tropic viruses. The virus entry assay is performed by infecting individual target cell cultures with recombinant virus stocks derived from packaging host cells transfected with pHIVenv and pHIVluc or pHIVlucDU3 vectors. In this embodiment, pHIVenv vectors contain patient virus derived envelope sequences and express HIV-1 envelope proteins (gp120SU, gp41TM). In this embodiment, viruses from different patient populations (see above description of patient population), and/or different virus populations (see above description for virus population) are used to construct pHIVenv vectors. Pseudotyped HIV derived from pHIVenv vectors are evaluated in the virus entry assay to determine if they are susceptible to neutralization by a panel of specific, well-characterized antibody preparations. Such antibodies represent a "standard antibody panel". Some, but not all, reasonable examples of antibodies that may

constitute a standard panel are listed in Table 4. In this embodiment virus neutralization is evaluated using target cells cultured in 96 well plates. Typically, target cells are plated at 5,000 cells per well for HT4/CCR5/CXCR4 or 10,000 cells per well for U-87/CD4/CCR5 and U-87/CD4/CXCR4 one day prior to infection. Prior to infection, each patient derived virus stock is incubated with the each of the antibody preparations (typically for 1 h) in the standard antibody panel. The sera or antibody preparations are tested undiluted and at various dilutions (typically four to five serial 10-fold dilutions). Infection of target cells with each virus stock is also performed in the absence of drug (no drug). Virus neutralization is assessed by comparing the amount of luciferase activity produced in target cells, both in the presence and absence of antibody. In this embodiment, the results of the assay are interpreted by comparing the ability of each antibody to preferentially block infection of target cells (reduce or eliminate luciferase activity). Virus neutralization activity is quantified by noting the highest antibody dilution (most dilute) that is able to block target cell infection (e.g. the highest dilution that is able to reduce the luciferase activity produced in the absence of antibody by 50%).

EXAMPLE 7

Identifying HIV-1 Envelope Amino Acid Sequences that Elicit Alter, or Prevent Neutralizing Antibody Responses

This example provides a means and method for identifying HIV-1 envelope amino acid sequences that elicit/promote, or alter, or prevent antibody mediated neutralization of HIV-1 infection (also referred to as virus neutralization in this

application).

Envelope sequences derived from patient samples, or individual clones derived from patient samples, or envelope sequences engineered by site directed mutagenesis to contain specific mutations, are tested in the entry assay to determine co-receptor tropism as described in **Example 6**.

In one embodiment, antibody mediated neutralization is evaluated in longitudinal patient samples (viruses collected from the same patient at different time points) is evaluated. For example, virus neutralization is evaluated prior to vaccination, during a course of vaccination, and at incremental time points after the course of vaccination is completed. In one embodiment, virus neutralization is evaluated for preventative vaccines. In another embodiment, virus neutralization is evaluated for therapeutic vaccines.

In another embodiment, virus neutralization is evaluated for samples collected from a large number of different patients. In a further embodiment, virus neutralization is evaluated for samples collected from a large number of patients representing different virus and patient populations. Such patient populations may include, but are not limited to, newly infected patients, chronically infected patients, patients with advanced disease, patients undergoing antiretroviral therapy or immuno-therapy, vaccinated and unvaccinated individuals. Such virus populations may include, but are not limited to, viruses with distinct genetic characteristics (clade A, B, C, D, E, F, G), viruses susceptible to antiretroviral drugs, viruses with reduced susceptibility/resistance to antiretroviral drugs, primary

isolates or isolates adapted for growth in cell culture (often referred to as lab-adapted viruses), syncytia inducing (SI) viruses or non-syncytia inducing (NSI) viruses, macrophage (M) tropic viruses, T-cell (T) tropic viruses and dual tropic (M and T) viruses.

Genotypic analysis of patient HIV samples

Envelope sequences representing patient sample pools, or clones derived from patient pools, can be analyzed by any broadly available DNA sequencing methods. In one embodiment of the invention, patient HIV sample sequences are determined using viral RNA purification, RT/PCR and dideoxynucleotide chain terminator sequencing chemistry and capillary gel electrophoresis (Applied Biosystems, Foster City, CA). Envelope sequences of patient virus pools or clones are compared to reference sequences, other patient samples, or to a sample obtained from the same patient prior to initiation of therapy, if available. The genotype is examined for sequences that are different from the reference or pre-treatment sequence and correlated to differences in entry inhibitor susceptibility.

Antibody mediated neutralization of genetically characterized viruses

Envelope amino acid sequences that correlate with virus neutralization are evaluated by constructing envelope expression vectors (pHIVenv) containing a specific mutation on a defined genetic background (e.g. NL4-3 for X4 tropism, JRCSF for R5 tropism). Mutations may be incorporated alone and/or in combination with other mutations that are thought to modulate virus neutralization. Envelope mutations are introduced into pHIVenv vectors using any of the broadly

available methods for site-directed mutagenesis. In one embodiment of this invention the mega-primer PCR method for site-directed mutagenesis is used (Sarkar, G. and Summer, S.S., 1990). A pHIVenv vector containing a specific envelope mutation or group of mutations are tested using the virus entry assay described in **Example** 6. Specific antibody preparations (i.e. well-characterized monoclonal or polyclonal antibody preparations), serum from HIV infected patients, or serum from vaccinated individuals can be selected to compare neutralizing activity. Antibody neutralization of the virus containing envelope mutations is compared to antibody neutralization of a genetically defined virus that lacks the specific mutations under evaluation. The ability of a specific mutation to confer, alter, or prevent antibody neutralization is confirmed or disproved by introducing the mutation into well-characterized reference virus and evaluating the antibody mediated neutralization of the mutant virus in the virus entry assay as described in **Example** 6. Observed changes in virus neutralization are attributed to the specific mutations introduced into the pHIVenv vector.

In one embodiment of the invention, genetic determinants of virus neutralization are identified by evaluating amino acid sequences within the V3 loop of the gp120 surface envelope protein. The amino acid sequences under evaluation are identified by comparing the amino acid sequences of large numbers of viruses that can, or cannot be neutralized by various well-characterized antibody preparations, patient sera, or sera from vaccinated individuals. Consistent differences in V3 loop amino acid sequences between viruses that can, or cannot be neutralized are selected for

evaluation. Isogenic viruses based on an well-characterized parental clone (e.g NL4-3, HXB2, JRCSF) containing specific "virus neutralization candidate" mutations in the V3 loop of the gp120 envelope protein are constructed by site directed mutagenesis and tested for antibody mediated neutralization as described in **Example 6**. Cells expressing CD4 plus CCR5 (e.g. U-87/CD4/CCR5), CD4 plus CXCR4 (U-87/CD4/CXCR4), or CD4 plus CCR5 and CXCR4 (HT4/CCR5/CXCR4) are infected. Amino acid substitutions that change that elicit, alter, or prevent antibody neutralization are deemed important to virus neutralization.

In a related embodiment of the invention, genetic determinants of virus neutralization are identified by evaluating amino acid sequences within the entire gp120 surface envelope protein.

In a related embodiment of the invention, genetic determinants of virus neutralization are identified by evaluating amino acid sequences within the gp41 transmembrane envelope protein.

EXAMPLE 8

Measuring Susceptibility to Virus Entry Inhibitors to Guide Treatment Decisions

This example provides a means and method for using virus entry inhibitor susceptibility to guide the treatment of HIV-1. This example further provides a means and method for using virus entry inhibitor susceptibility to guide the treatment of patients that have received previous antiretroviral treatment with a virus entry inhibitor. This

invention further provides the means and methods for using virus entry inhibitor susceptibility to guide the treatment of patients that have not received previous treatment with a virus entry inhibitor.

In one embodiment, the susceptibility of patient's viruses to virus entry inhibitors is used to guide the treatment of patients failing antiretroviral regimens that include one or more virus entry inhibitors. Treatment failure (also referred to as virologic failure) is generally defined as partially suppressive antiviral treatment resulting in detectable levels of virus, which is typically measured in the patient plasma). Guidance may include, but is not limited to, (a) clarification of available drug treatment options, (b) selection of more active treatment regimens, (c) clarification of the etiology of rising viral load in treated patients (i.e. poor adherence, drug resistance), and (d) reduction in the use of inactive and potentially toxic drugs. In this embodiment, resistance test vectors are derived from a patient virus samples and tested for susceptibility to various virus entry inhibitors using the phenotypic virus entry assay. Virus entry inhibitors may include, but are not limited to, fusion inhibitors (e.g. T-20, T-1249), co-receptors antagonists (AMD3100, AMD8664, TAK779, PRO542, and peperidin-1yl butane compounds) and CD4 antagonists (MAb 5A8). Appropriate treatment decisions are based on the results of the virus entry assay **(e.g. see Figure 4B)** and additional relevant laboratory test results and clinical information.

In another embodiment, the susceptibility of patient's viruses to virus entry inhibitors is used to guide the

treatment of patients that have not been previously treated with antiretroviral regimens that include one or more virus entry inhibitors. Guidance may include, but is not limited to, (a) clarification of available drug treatment options, (b) selection of more active treatment regimens, (c) clarification of the baseline susceptibility to virus entry inhibitors, and (d) reduction in the use of inactive and potentially toxic drugs. Determining baseline susceptibility of virus entry inhibitors in treatment naïve patients is important for two reasons. First, the natural susceptibility of viruses to entry inhibitors can vary widely **(e.g. see Figure 4A)**. Second, the increased use of virus entry inhibitors will undoubtedly result in the generation of drug resistant variants that can be transmitted to newly infected individuals. In this embodiment, resistance test vectors are derived from a patient virus samples and tested for susceptibility to various virus entry inhibitors using the phenotypic virus entry assay. Virus entry inhibitors may include, but are not limited to, fusion inhibitors (e.g. T-20, T-1249), co-receptors antagonists (AMD3100, AMD8664, TAK779, PRO542, and peperidin-1yl butane compounds) and CD4 antagonists (MAb 5A8). Appropriate treatment decisions are based on the results of the virus entry assay and additional relevant laboratory test results and clinical information.

EXAMPLE 9

Measuring HIV-1 Co-receptor Tropism to Guide Treatment Decisions

This example provides a means and method for using HIV-1 co-receptor (CCR5, CXCR4) tropism to guide the treatment of HIV-1. This example further provides a means and method for

using HIV-1 co-receptor tropism to guide the treatment of patients failing antiretroviral drug treatment. This invention further provides the means and methods for using HIV-1 co-receptor tropism to guide the treatment of patients newly infected with HIV-1.

This example provides a means and method for using virus HIV-1 co-receptor tropism to guide the treatment of HIV-1. This example further provides a means and method for using HIV-1 co-receptor tropism to guide the treatment of patients that have received previous antiretroviral treatment with a virus entry inhibitor. This invention further provides the means and methods for using HIV-1 co-receptor tropism to guide the treatment of patients that have not received previous treatment with a virus entry inhibitor.

In one embodiment, the co-receptor tropism of a patient's virus is used to guide the treatment of a patient failing antiretroviral regimens that include one or more co-receptor antagonists. Treatment failure (also referred to as virologic failure) is generally defined as partially suppressive antiviral treatment resulting in detectable levels of virus, which is typically measured in the patient plasma). Guidance may include, but is not limited to, (a) clarification of the etiology of rising viral load in treated patients (i.e. poor adherence, drug resistance, change in co-receptor tropism), (b) clarification of available drug treatment options, (c) selection of more active treatment regimens, and (d) reduction in the use of inactive and potentially toxic drugs. Monitoring co-receptor tropism in patients receiving treatment with CCR5 antagonists has clinical significance, since drug pressure may result in a

switch to CXCR4 co-receptor tropism. X4 viruses (CXCR4 co-receptor tropism) are associated with a poorer prognosis compared to R5 viruses (CCR5 co-receptor tropism). In this embodiment, resistance test vectors are derived from a patient virus samples and tested for susceptibility to various co-receptor antagonists using the phenotypic virus entry assay. Co-receptor antagonists may include, but are not limited to, AMD3100, AMD8664, TAK779, PRO542, and peperidin-1yl butane compounds. Appropriate treatment decisions are based on the results of the virus entry assay **(e.g. see Figure 4B)** and additional relevant laboratory test results and clinical information.

In another embodiment, co-receptor tropism of a patient's virus is used to guide the treatment of patients that have not been previously treated with antiretroviral regimens that include one or more co-receptor antagonists. Guidance may include, but is not limited to, (a) clarification of the baseline co-receptor tropism, (b) clarification of available drug treatment options, (c) selection of more active treatment regimens, (d) reduction in the use of inactive and potentially toxic drugs. Determining baseline co-receptor tropism has significant clinical significance. Treatment with the appropriate co-receptor antagonist (R5 vs. X4 tropism), or antagonists (dual tropism or mixed tropism) is likely to result in a more potent and durable response. In this embodiment, resistance test vectors are derived from a patient virus samples and tested for susceptibility to various virus entry inhibitors using the phenotypic virus entry assay. Co-receptors antagonists may include, but are not limited to, AMD3100, AMD8664, TAK779, PRO542, and peperidin-1yl butane compounds. Appropriate treatment

decisions are based on the results of the virus entry assay and additional relevant laboratory test results and clinical information.

References

1. Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Caney, A. Rabson, and M. A. Martin. 1986. Production of Acquired Immunodeficiency Syndrome-associated Retrovirus in Human and Nonhuman Cells Transfected with an Infectious Molecular Clone. J. Virol. 59:284-291.

2. Alkhatib, G., C. Combadiere, C.C. Broder, Y. Feng, P.E. Kennedy, P.M. Murphy, and E.A. Berger. 1996. CC CKR5: A Rantes, MIP-1alpha, MIP-1 Beta Receptor as a Fusion Cofactor for Macrophage-tropic Hiv-1. Science 272:1955-8.

3. Allaway G.P., Ryder A.M., Beaudry G.A., and Maddon P.J. 1993. Synergistic Inhibition of HIV-1 Envelope-Mediated Cell Fusion by CD4-based Molecules in Combination with Antibodies to Gp120 or Gp41. Aids Res. Hum. Retroviruses 9:581-7.

4. Baba, M., O. Nishimura, N. Kanzaki, M. Okamoto, H. Sawada, Y. Iizawa, M. Shiraishi, Y. Aramaki, K. Okonogi, Y. Ogawa, K. Meguro, and M. Fujino. 1999. A Small-molecule, Nonpeptide CCR5 Antagonist with Highly Potent and Selective Anti-hiv-1 Activity. Proc. Natl. Acad. Sci. USA 96:5698-703.

5. Baxter, J., D. Mayers, D. Wentworth, J. Neaton, and T. Merigan. 1999. A Pilot Study of the Short-term Effects of Antiretroviral Management Based on Plasma Genotypic Antiretroviral Resistance Testing (Gart) in Patients Failing Antiretroviral Therapy. Presented at the 6th Conference on Retroviruses and Opportunistic Infections. Chicago, Il.

6. Bernard P., Kezdy K.e., Van Melderen L., Steyaert J., Wyns L., Pato M.l., Higgins P.N., and Couturier M. 1993. The F Plasmid CcdB protein Induces Efficient ATP-dependent Dna Cleavage by Gyrase. J. Mol. Biol. 23:534-41.

7. Bernard, P. and Couturier, M. 1992. Cell Killing by the F Plasmid Ccdb protein Involves Poisoning of DNA-topoisomerase II Complexes. J. Mol. Bio. 226:735-45.

8. Bleul, C.C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, and T.A. Springer. 1996. The Lymphocyte Chemoattractant Sdf-1 Is a Ligand for Lestr/fusin and Blocks Hiv-1 Entry. Nature 382:829-33.

9. Bridger G.J, Skerlj R.T., Padmanabhan S., Martellucci S.A., Henson G.W., Struyf S., Witvrouw M., Schols D., and De Clercq E. 1999. Synthesis and Structure-activity Relationships of Phenylenebis(methylene)-linked Bis-azamacrocycles That Inhibit HIV-1 and HIV-2 Replication by Antagonism of the Chemokine Receptor CXCR4. J. Med. Chem. 42:3971-81.

10. Carpenter, C.J., Cooper D.A., Fischl, M.A., Gatell J.M., Gazzard B.G., Hammer S.M., Hirsch M.s., Jacobsen D.M., Katzenstein D.A., Montaner J.S., Richman D., Saag M.S., Schechter M., Schooley R.T., Thompson M.A., Vello S., Yeni P.G., and Volberding P.A. 2000. Antiretroviral Therapy in Adults. JAMA 283:381-89.

11. CDC (Centers for Disease Control and Prevention). HIV/AIDS Surveillance Report, 1999;11(no. 1).

12. Coffin, J.m. 1995. HIV Population Dynamics in Vivo: Implications for Genetic Variation, Pathogenesis, and Therapy. Science 267:483-489.

13. DHHS (Department of Health and Human Services). Henry Kaiser Family Foundation: Guidelines for the Use of Antiretrovirals Agents in HIV-infected Adults and Adolescents. (Jan 28, 2000).

14. Gerdes, K., L.k. Poulsen. T. Thisted, A.k. Nielson, J. MaRTInussen, and P.h. Andreasen. 1990. The Hok Killer Gene Family in Gram-negative Bacteria. The New Biologist: 2:946-956.

15. Hertogs, K., M.-p. De Béthune, V. Miller, T. Ivens, P. Schel, A. V. Cauwenberge, C. Van Den Eynde, V. Van Gerwen, H. Azijn, M. Van Houtte, F. Peeters, S. Staszewski, M. Conant, S. Bloor, S. Kemp, B. Larder, and R. Pauwels. 1998. A Rapid Method for Simultaneous Detection of Phenotypic Resistance to Inhibitors of protease and Reverse Transcriptase in Recombinant

Human Immunodeficiency Virus Type 1 Isolates from Patients Treated with Antiretroviral Drugs. Antimicrob. Agents Chemother. 42:269-276.

16. Hwang, J.-j., L. Li, W.f. Anderson. 1997. A Conditional Self-inactivating Retrovirus Vector That Uses a Tetracycline-responsive Expression System. J. Virol. 71: 7128-7131.

17. Japour, A. J., D. L. Mayers, V. A. Johnson, D. R. Kuritzkes, L. A. Beckett, J.-m. Arduino, J. Lane, B. R.j., P. S. Reichelderfer, R. T. D-aquila, C. S. Crumpacker, T. R.-s.-Group, T. A. C. T. Group, and V. C. R. W. Group. 1993. Standardized Peripheral Blood Mononuclear Cell Culture Assay for Determination of Drug Susceptibilities of Clinical Human Immunodeficiency Virus Type 1 Isolates. Antimicrob. Agents Chemother. 37:1095-1101.

18. Judice J.k., Tom J.y., Huang W., Wrin T., Vennari J., Petropoulos C.j., and Mcdowell R.s. 1997. Inhibition of HIV Type 1 Infectivity by Constrained Alpha-helical Peptides: Implications for the Viral Fusion Mechanism. *proc. Natl. Acad. Sci. U S a* 94:13426-30.

19. Kilby Jm, Hopkins S, Venetta Tm, Dimassimo B, Cloud Ga, Lee Jy, Alldredge L, Hunter E, Lambert D, Bolognesi D, Matthews T, Johnson Mr, Nowak Ma, Shaw Gm, and Saag Ms . 1998. Potent Suppression of Hiv-1 Replication in Humans by T-20, a Peptide Inhibitor of Gp41-mediated Virus Entry. *Nat Med* 4:1302-7.

20. Mascola, J.r., G. Stiegler, T.c. Vancott, H. Katinger, C.b. Carpenter, C.e. Hanson, H. Beary, D. Hayes, S.s. Frankel, D.l. Birx, and M.g. Lewis. 2000. protection of Macaques Against Vaginal Transmission of a Pathogenic Hiv-1/siv Chimeric Virus by Passive Infusion of Neutralizing Antibodies. Nature Med. 6:207-210.

21. Miyoshi, H., B. Ulrike, M. Takahashi, F.h. Gage, and I.m. Verma. 1998. Development of a Self-inactivating Lentivirus Vector. J. Virol. 72:8150-5157.

22. Naviaux, R.k., E. Costanzi, M. Haas, and I.m. Verma. 1996. The Pcl Vector System: Rapid production of Helper-free, High-titer, Recombinant Retroviruses. J. Virol. 70: 5701-5705.

23. Petropoulos, C.j., N.t. Parkin, K.l. Limoli, Y.s. Lie, T. Wrin, W. Huang, H. Tian, D. Smith, G.a. Winslow, D. Capon and J.m. Whitcomb. 2000. A Novel Phenotypic Drug Susceptibility Assay for Hiv-1. Antimicrob. Agents & Chem. 44:920-928.

24. Phrma (Pharmaceutical Research and Manufacturers of America). New Medicines in Development for Aids 1999. Http://www.phrma.org.

25. Piketty, C., E. Race, P. Castiel, L. Belec, G. Peytavin, A. Si-mohamed, G. Gonzalez-canali, L. Weiss, F. Clavel, and M. Kazatchkine. 1999. Efficacy of a Five-drug Combination Including Ritonavir, Saquinavir and Efavirenz in Patients Who Failed on a

Conventional Triple-drug Regimen: Phenotypic Resistance to protease Inhibitors predicts Outcome of Therapy. Aids: 13:f71-f77.

26. Porter, C.c., K.v. Lukacs, G. Box, Y. Takeuchi, and M.k.l. Collins. 1998. Cationic Liposomes Enhance the Rate of Transduction by a Recombinant Retroviral Vector in Vitro and in Vivo. J. Virol. 72:4832-4840.

27. Reimann K.a., Cate R.l., Wu Y., Palmer L., Olson D., Waite B.c., Letvin N.l., and Burkly L.c. 1995. In Vivo Administration of CD4-specific Monoclonal Antibody: Effect on provirus Load in Rhesus Monkeys Chronically Infected with the Simian Immunodeficiency Virus of Macaques. *Aids Res. Hum. Retroviruses* 11:517-25

28. Retroviruses. Coffin, J., S. Hughes, H. Varmus (Eds). 1997. Cold Spring Harbor Laboratory press, Cold Spring Harbor, Ny.

29. Richman, D. 1998. Nailing down Another HIV Target. Nature Med. 4:1232-1233.

30. Rimsky, L. T., D. C. Shugars, and T. J. Matthews. 1998. Determinants of Human Immunodeficiency Virus Type 1 Resistance to Gp41-derived Inhibitory Pepitides. J. Virol. 72:986-993.

31. Rodriguez-rosado, R., Briones, C. and Soriano, V. 1999. Introduction of HIV Drug-resistance Testing in Clinical practice. Aids 13:1007-1014.

32. Schinazi, R.f, Larder, B.a., and Mellors, J.w. 1999. Mutations in Retroviral Genes Associated with Drug Resistance. Intl. Antiviral News: 7:46-69

33. Shi C., and J.w. Mellors. 1997. A Recombinant Retroviral System for Rapid in Vivo Analysis of Human Immunodeficiency Virus Type 1 Susceptibility to Reverse Transcriptase Inhibitors. Antimicrob. Agents Chemother 41:2781-2785.

34. Stephenson, J. 1999. New Class of Anti-hiv Drugs. Jama 282:1994.

35. Who, Unaids/world Health Organization. Report: Aids Epidemic Update: December 1999. Http://www.unaids.org/publications/documents/epidemiology.

36. Wild, C., T. Oak, C. Mcdanal, D. Bolognesi, and T. Matthews. 1992. A Synthetic Peptide Inhibitor of HIV Replication: Correlation Between Solution Structure and Viral Inhibition. Proc. Natl. Acad. Sci. USA 89:10537-10541.

37. Zennou, V., F. Mammamo, S. Paulous, D. Mathez, and F. Clavel. 1998. Loss of Viral Fitness Associated with Multiple Gag and Gag-pol processing Defects in Human Immunodeficiency Virus Type 1 Variants Selected for Resistance to protease Inhibitors in Vivo. J. Virol: 72:3300-06.

38. Ziermann, R., K. Limoli, K. Das, E. Arnold, C.j. Petropoulos, and N.t. Parkin. 2000. A Mutation in Hiv-1 protease, N88s, That Causes in Vitro Hypersensitivity to Amprenavir. J. Virol. 74:4414-4419.

Table 1

Cells

Cell	Receptor
5.25	CXCR4, CD4, CCR5 (not expressed well) BONZO
5.25.Luc4.M7	CD4, CCR5, BONZO
HOS.CD4.CCR5	CD4, CCR5
HOS.CD4.CXCR4	CD4, CXCR4
HOS.CD4	CD4, low level expression of CCR5 and CXCR4
HOS HT4 R5 GFP wt	CD4, CXCR4, CCR5
HOS.CD4.CCR5.GFP.M7#6*	CD4, CXCR4, CCR5
P4.CCR5	CD4, CXCR4, CCR5
U87.CD4	CD4
U87.CD4 R5	CD4, CCR5
U87.CD4 X4	CD4, CXCR4
MT2	CD4, CXCR4
MT4	CD4, CXCR4
PM1	CD4, CXCR4, CCR5
CEM NKr CCR5	CD4, CXCR4, CCR5

Table 2 Representative viruses and reagents

Viruses	Envelope[a]	Source
89.6, SF2	R5-X4/SI/B	ARRRP[b]
92BR014,92US076	R5-X4/SI/B	ARRRP
JR-CSF, 91US005	R5/NSI/B	ARRRP
91US054	SI/B	ARRRP
NL43, MN, ELI	X4/B	ARRRP
92HT599	X4	ARRRP
92UG031	R5/NSI/A	ARRRP(IN-HOUSE)
92TH014, 92TH026	R5/NSI/B	ARRRP(IN-HOUSE)
92BR025, 93MW959	R5/SI/C	ARRRP(IN-HOUSE)
92UG035	R5/NSI/D	ARRRP(IN-HOUSE)
92TH022, 92TH023	R5/NSI/E	ARRRP(IN-HOUSE)
93BR020	R5-X4/SI/F	ARRRP(IN-HOUSE)
Antibodies	Epitope	SOURCE
Mabs 2F5, 1577	gp41 TM	ARRRP
Mabs IG1b12, 2G12, 17b, 48D	gp120 SU	ARRRP
Neutralization sera #2, HIV-IG	Polyclonal	ARRRP
Entry inhibitors	Target	Source
CD4-IG	gp120 SU	Genentech
CD4-IGG2	gp120 SU	Adarc
SCD4	Sigma	Progenics
T20 (DP178)	gp41 TM	Trimeris
Rantes, MIP1a/b	CCR5	SIGMA/ARRRP
SDF1a/b	CXCR4	SIGMA/ARRRP
AMD 3100	CXCR4	AnorMed
Dextran sulfate, Heparin	Non-specific	Sigma

[a]R5 (CCR5 co-receptor), X4 (CXCR4 co-receptor)
SI (syncytium inducing), NSI (non-syncytium inducing), A,B,C,D,E,F (envelope clade designation)
[b]AIDS Research and Reference Reagent Program

Table 3

Primers Tested for the Amplification of HIV Envelope	
RT PRIMERS	
RT env_N3	5'-GGA GCA TTT ACA AGC AGC AAC ACA GC-3'
RT env 9720	5' -TTC CAG TCA VAC CTC AGG TAC-3'
RT env 9740	5'-AGA CCA ATG ACT TAY AAG G-3'
5' PCR PRIMERS	
5'env	5' -GGG CTC GAG ACC GGT CAG TGG CAA TGA GAG TGA AG- 3'
5'env_Xho/Pin	5'-GGG CTC GAG ACC GGT GAG CAG AAG ACA GTG GCA ATG A-3'
5'env_START	5'-GGG CTC GAG ACC GGT GAG CAG AAG ACA GTG GCA ATG -3'
3' PCR PRIMERS	
3' env	5' -GGG TCT AGA ACG CGT TGC CAC CCA TCT TAT AGC AA- 3'
3'env_Xba/Mlu	5'-GGG TCT AGA ACG CGT CCA CTT GCC ACC CAT BTT ATA GC-3'
3'env_STOP	5'-GGG TCT AGA ACG CGT CCA CTT GCC ACC CAT BTT A-3'
3' delta CT	5' -GAT GGT CTA AGA CGC TGT TCA ATA TCC CTG CCT AAC TC- 3'
All Experiments are located in Virologic Book number 0188	

Table 4 (Panel 1)

Anti-HIV Drugs

Drug/Compound	Generic Name	Trademark	Manufacturer
RT Inhibitors (NRTIs; nucleotide analogs)			
AZT, ZDV	Zidovudine	Retrovir	Glaxo/Wellcome
3TC	Lamivudine	Epivir	Glaxo/Wellcome
AZT + 3TC		Combivir	Glaxo/Wellcome
d4T	Stavudine	Zerit	Bristol-Myers/Squibb
ddI	Didanosine	Videx	Bristol-Myers/Squibb
ddC	Zalcitabine	Hivid	Hoffman La Roche
1592U89	Abacavir	Ziagen	Glaxo/Wellcome
AZT + 3TC + 1592U89		Trizivir	Glaxo/Wellcome
(-)FTC (5-fluoro-3TC; Corviracil)	Emtricitabine		Triangle Pharmaceuticals
(-)FTC + (+)FTC (50:50)	Racivir		QuadPharma
DAPD (DXG active)	Amdoxovir		Triangle Pharmaceuticals
F-ddA (2-fluoro-ddA)	Lodenosine		MedImmune Oncology (US Bioscience)
BCH-10652, dOTC (2-deoxy-3-oxa-4-thiocytidine)			BioChem Pharma, Inc
D-d4FC			Triangle Pharmaceuticals (Schinazi)
RT Inhibitors (NRTI; nucleotide analogs)			
bis-POC PMPA (GS-4331)	Tenofovir		Gilead Sciences
bis-POM PMEA (GS-840)	Adefovir dipivoxil		Gilead Sciences
RT Inhibitors (NNRTIs; non-nucleosides)			
BI-RG-587	Nevirapine	Viramune	Boehringer/Ingleheim (Roxanne)
BHAP PNU-90152T	Delavirdine	Rescriptor	Pharmacia & Upjohn
DMP 266 (L-743,726)	Efavirenz	Sustiva	Dupont Pharmaceuticals (Avid)
MKC442 (Coactinon)	Emivirine		Triangle/Mitsubishi Kasei
AG-1549 (S1153) (on hold)	Capravirine		Agouron Pharmaceuticals
PNU-142721			Pharmacia & Upjohn
DPC-961, -963, -083, -087			DuPont Pharmaceuticals
SJ-3366	Also entry inhibitor?		Samjin Pharmaceuticals
BHAP PNU-87201	Atevirdine		Upjohn
GW420867X (quinoxaline)	(2nd gen. HBY 097)		Glaxo/Wellcome (Hoechst Bayer)
TMC 120 (R147681)			Tibotec
TMC 125 (R165335)			Tibotec
R86183	tivirapine		Janssen Pharmaceuticals
Calanolide A			Sarawak Medichem Pharmaceuticals
Protease Inhibitors (PI)			
Ro 31-8959	Saquinavir-(hgc) Saquinavir-(sgc)	Invirase Fortivase	Hoffman-La Roche
MK-639 (L-735,524)	Indinavir	Crixivan	Merck Research Laboratories
ABT-538 (A-84538)	Ritonavir	Norvir	Abbott Laboratories
AG1343	Nelfinavir	Viracept	Agouron Pharmaceuticals
141W94 (VX-478)	Amprenavir	Agenerase	Glaxo-Wellcome/Vertex
ABT-378/r	Lopinavir/ritonavir	Kaletra	Abbott Laboratories
BMS 232,632 (aza-peptide)			Bristol-Myers-Squibb
PNU-140690	Tipranavir		Pharmacia & Upjohn
DMP 450 (cyclic urea)	Mozenavir		Triangle/Avid (ph I/II)
TMC 126 (Erickson's compound)			Tibotec
GW433908 (VX-175)	amprenavir pro-drug		Glaxo/Wellcome/Vertex
L756,423 (on hold)			Merck
PD-178390 (dihydropyrone)			Parke Davis (Boehringer-Ingleheim)
? new candidate			Roche
DPG 681 and 684			DuPont Pharmaceuticals
AG-1776 (JE-2147 = KNI-764)			Agouron Pharmaceuticals
Envelope/Receptor Inhibitors			
T-20 (gp41)	Pentafuside		Trimeris Pharmaceuticals
T-1249 (gp41)			Trimeris Pharmaceuticals
D-peptide inhibitor (gp41) small mol.	SCH-C		Schering-Plough
AMD-3100 (CXCR4)	(bicyclam)		AnorMED
AMD-3664 (CXCR4)	(macrocyclam)		AnorMED
ALX40-4C (CXCR4)			U-PA
FP21399			Fuji Pharmaceuticals
PRO 542 (gp120)	CD4IgG2		Progenics Pharmaceuticals
PRO-140 (CCR5)	MAb CCR5		Progenics Pharmaceuticals
T-22 (CXCR4)	(peptide, 18-mer)		
Met-SDF-1 (CXCR4)			
TAK 779 (CCR5 antagonist)			Takeda
AOP-Rantes (CCR5)			Gryphon Sciences

Table 4 (Panel 2)

Rantes 9-68 (CCR5)			
CCR5 antagonists	4-(piperidin-1-yl) butane class		Merck
α-immunokine-NNS03 (CCR5, CXCR4)	α-cobratoxin		PhytoMed Corp.
Integrase inhibitors			
AR-177	Zintevir		Aronex Pharmaceuticals
Diketo acids			Merck Research Laboratories
Nucleocapsid inhibitors			
RB 2121	cyclic peptide p7 mimic		(see PNAS 96:4886-4891 (1999))
CI-1012			Achelion Pharmaceuticals
RNase H inhibitors			
SP1093V (BBNH Fe+3 derivative)			(Parniak)

FDA approved drugs are shown in boldface,red = discontinued development,blue = not sure about development status

Table 4 (Panel 3)

Generic Name (abbreviation)	Brand Name	Firm	FDA Approval Date
zidovudine, AZT	Retrovir	Glaxo Wellcome	March 87
didanosine, ddI	Videx	Bristol Myers-Squibb	October 91
zalcitabine, ddC	Hivid	Hoffman-La Roche	June 92
stavudine, d4T	Zerit	Bristol Myers-Squibb	June 94
lamivudine, 3TC	Epivir	Glaxo Wellcome	November 95
saquinavir, SQV, hgc	Invirase	Hoffman-La Roche	December 95
saquinavir, SQV, sgc	Fortovase	Hoffman-La Roche	November 97
ritonavir, RTV	Norvir	Abbott Laboratories	March 96
indinavir, IDV	Crixivan	Merck & Co., Inc.	March 96
nevirapine, NVP	Viramune	Boehringer Ingelheim	June 96
nelfinavir, NFV	Viracept	Agouron Pharmaceuticals	March 97
delavirdine, DLV	Rescriptor	Pharmacia & Upjohn	April 97
ZDV+3TC	Combivir	Glaxo Wellcome	September 97
efavirenz, EFV	Sustiva	DuPont Pharmaceuticals	September 98
abacavir, ABC	Ziagen	Glaxo Wellcome	February 99
amprenavir	Agenerase	Glaxo Wellcome	April 99
lopinavir/ritonavir	Kaletra	Abbott	September 2000
ZDV+3TC+ABC	Trizivir	GlaxoSmithKline	November 2000

CORECEPTOR ASSAY SCREEN TEMPLATE

active RLU limit: 100 tropism ratio limit: 5 CXCR4 CCR5 DUAL [redacted] drug 1: L83 drug 2: AMD

	1			2			3			4			5			6		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	34	26,471	0.00	14,140	55	257.09	46	6,849	0.01	21,656	38,144	0.57	30	7,336	0.00	3,984	31	128.65
L83 RLU		19,258		54				4,696		27	25,542			5,466		34		
AMD RLU		32		12,186				40		7,226	32			39		3,864		
	13			14			15			16			17			18		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	4,552	19,102	0.24	149	226	0.66	67,389	72	935.96	611	19,715	0.03	46	3,948	0.01	284	4,930	0.07
L83 RLU	26	9,956		40	153		99			30	14,985			2,953		34	2,916	
AMD RLU	836	34		41	24		42,295			76	1,022			152		25	46	
	25			26			27			28			29			30		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	67,826	68	997.47	14,982	12,020	1.25	3,788	4,384	0.86	50	37	[redacted]	658	242	2.72	231	35	6.60
L83 RLU	129			111	10,839		59	3,387					32	221		47		
AMD RLU	51,146			8,580	3,364		2,049	536					493	36		218		
	37			38			39			40			41			42		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	100	39	[redacted]	3,724	2,310	1.61	450	668	0.67	148	24	6.17	32	22	[redacted]	60	61	[redacted]
L83 RLU				38	2,656		37	483		33								
AMD RLU				1,984	272		206	72		139								
	49			50			51			52			53			54		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	35	107	0.33	83	4,709	0.02	16,099	8,451	2.14	39,257	38	1033.08	5,413	40	135.33	97,733	29	3370.10
L83 RLU		66			3,502		13,896	6,220		85			35			30		
AMD RLU		34			88		16,980	8,711		27,832			5,043			46,452		
	61			62			63			64			65			66		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	3,768	40	94.23	786	48	16.38	340	21	16.19	5,189	43	120.67	4,318	37	116.70	196	51	3.84
L83 RLU	79			39			31			34			28			23		
AMD RLU	2,773			797			282			5,356			3,461			184		
	73			74			75			76			77			78		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	391	26	15.04	98	38	[redacted]	4,449	28	158.89	4,357	9,102	0.48	6,090	105	58.00	1,886	3,635	0.52
L83 RLU	50						43			34	7,932		73	77		38	2,097	
AMD RLU	340						3,767			905	36		4,473	51		279	27	
	85			86			87			88			89			90		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	510	33	15.45	979	59	16.59	491	33	14.88	300	3,815	0.08	297	3,615	0.08	39	61,994	0.00
L83 RLU	47			55			49			29	3,661		32	2,946			56,739	
AMD RLU	504			813			376			268	36		256	30			40	

	7			8			9			10			11			12		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	30	1,217	0.02	49	1,128	0.04	42,206	70	602.94	45	1,159	0.04	44,569	97	459.66	32	29,116	0.00
L83 RLU		814			934		90				761		60				22,279	
AMD RLU		42			37		33,192				33		30,510				31	
	19			20			21			22			23			24		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	100	5,204	0.02	11,299	52	217.29	1,273	20	63.65	7,375	10,856	0.68	4,397	7,130	0.62	16,115	50	322.30
L83 RLU		3,058		22			26			40	6,455		42	3,874		66		
AMD RLU		34		8,232			1,129			3,961	2,283		1,656	1,285		11,980		
	31			32			33			34			35			36		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	53	974	0.05	177	32	5.53	24,739	33	749.67	612	45	13.60	2,997	9,696	0.31	416	31	13.42
L83 RLU		826		32			31			48			22	7,761		28		
AMD RLU		95		361			21,767			479			925	35		597		
	43			44			45			46			47			48		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	45	336	0.13	714	45	15.87	11,029	18	612.72	2,906	43	67.63	13,997	52	269.17	24,377	34	716.97
L83 RLU		207		31			18			31			73			59		
AMD RLU		29		366			6,390			2,306			12,139			16,045		
	55			56			57			58			59			60		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	9,254	73,646	0.39	5,249	20,393	0.26	470	39	12.05	19,175	126	152.18	513	31	16.55	3,264	28	116.57
L83 RLU	43	9,150		36	8,290		37			96	183		35			30		
AMD RLU	2,219	40		784	38		310			19,636	35		500			3,038		
	67			68			69			70			71			72		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	3,853	77	50.04	411	34	12.09	7,857	41	191.63	25,437	38	669.39	17,443	45	387.62	16,707	38	439.66
L83 RLU	39			22			42			35			40			104		
AMD RLU	2,106			253			4,595			14,749			13,706			12,486		
	79			80			81			82			83			84		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	263	36	6.92	3,890	9,677	0.41	2,089	1,698	1.23	475	43	11.05	8,475	38	223.03	4,107	33	124.45
L83 RLU	33			44	6,140		33	1,274		30			30			28		
AMD RLU	143			986	45		1,105	160		159			6,862			2,571		
	91			92			93			94			95			96		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	443	13,452	0.03	34	128,238	0.00	22	62	[redacted]	45	33	[redacted]	43	42	[redacted]	46	35	[redacted]
L83 RLU	33	12,051			85,018													
AMD RLU	451	51			45													

CORECEPTOR ASSAY SCREEN TEMPLATE

active RLU limit: 100 tropism ratio limit: 5 CXCR4 CCR5 DUAL [redacted] drug 1: L83 drug 2: AMD

	1			2			3			4			5			6		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	34	26,471	0.00	14,140	55	257.09	46	6,845	0.01	21,655	38,144	0.57	30	7,336	0.00	3,966	31	128.65
L83 RLU		19,256		54				4,696		27	25,542			5,468		34		
AMD RLU		32		12,186				48		7,226	32			30		3,664		
	13			14			15			16			17			18		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	4,552	19,102	0.24	149	225	0.66	67,380	72	935.96	611	19,715	0.03	40	3,946	0.01	284	4,330	0.07
L83 RLU	28	9,956		40	153		99			30	14,965			2,953		38	2,918	
AMD RLU	838	34		41	24		42,295			76	1,022			152		25	46	
	25			26			27			28			29			30		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	67,828	68	997.47	14,962	12,020	1.25	3,788	4,384	0.86	50	37	[redacted]	658	242	2.72	231	35	6.60
L83 RLU	129			111	10,839		59	3,397					32	221		47		
AMD RLU	51,146			8,580	3,384		2,049	536					493	38		219		
	37			38			39			40			41			42		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	100	39	[redacted]	3,724	2,310	1.61	450	668	0.67	148	24	6 17	32	22	[redacted]	60	61	[redacted]
L83 RLU				38	2,656		37	463		33								
AMD RLU				1,984	272		206	72		139								
	49			50			51			52			53			54		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	35	107	0.33	83	4,209	0.02	18,099	8,451	2 14	39,257	38	1033.08	5,413	40	135.33	97,733	29	3370.10
L83 RLU		68			3,502		13,896	6,220		86			38			30		
AMD RLU		34			66		16,980	8,711		27,832			5,043			48,452		
	61			62			63			64			65			66		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	3,769	40	94.23	786	46	16.36	340	21	16.19	5,169	43	120.67	4,016	37	110.70	196	51	3.84
L83 RLU	29			39			31			34			26			23		
AMD RLU	2,773			787			282			5,356			3,461			184		
	73			74			75			76			77			78		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	391	26	15.04	99	38	[redacted]	4,449	28	158.89	4,357	9,102	0.48	6,000	105	58.00	1,886	3,635	0.52
L83 RLU	50						43			34	7,932		73	77		36	2,092	
AMD RLU	340						3,752			906	36		4,473	51		279	27	
	85			86			87			88			89			90		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	510	33	15.45	979	59	16.59	491	33	14.88	300	3,615	0.08	297	3,615	0.08	39	61,594	0.00
L83 RLU	42			56			49			29	3,861		32	2,048			56,739	
AMD RLU	564			813			378			266	36		256	30			40	

	7			8			9			10			11			12		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	30	1,217	0.02	40	1,128	0.04	42,206	70	602.94	45	1,159	0.04	44,589	97	459.68	32	29,116	0 00
L83 RLU		614			934		99				761		60				22,279	
AMD RLU		42			37		33,192				33		30,510				31	
	19			20			21			22			23			24		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	100	5,204	0.02	11,299	52	217.29	1,273	20	63 65	7,375	10,856	0.68	4,397	7,130	0.62	16,115	50	322.30
L83 RLU		3,058		22			26			40	6,455		42	3,874		56		
AMD RLU		34		8,232			1,128			3,961	2,283		1,656	1,285		11,980		
	31			32			33			34			35			36		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	53	974	0.05	177	32	5.53	24,739	33	749.67	612	45	13.60	2,097	9,695	0 31	410	31	13.42
L83 RLU		828		32			31			48			22	7,261		28		
AMD RLU		95		361			21,767			470			925	35		597		
	43			44			45			46			47			48		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	45	338	0.13	714	45	15.87	11,029	18	612.72	2,908	43	67.63	13,097	52	269.17	24,377	34	716.97
L83 RLU		207		31			18			31			73			56		
AMD RLU		29		366			8,390			2,306			12,139			16,045		
	55			56			57			58			59			60		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	9,254	23,846	0.39	5,248	20,393	0.26	470	39	12.05	19,175	126	152.18	513	31	16.55	3,264	28	116.57
L83 RLU	43	9,150		38	8,200		37			90	183		35			30		
AMD RLU	2,219	40		764	38		310			19,638	35		500			3,038		
	67			68			69			70			71			72		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	3,853	77	50.04	411	34	12.09	7,857	41	191.63	25,437	38	669.39	17,443	45	387.62	16,707	38	439.66
L83 RLU	38			22			42			35			40			104		
AMD RLU	2,108			253			4,595			14,749			13,708			12,486		
	79			80			81			82			83			84		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	263	38	6.92	3,890	9,577	0.41	2,089	1,698	1.23	475	43	11 05	8,475	38	223.03	4,107	33	124 45
L83 RLU	33			44	6,140		33	1,224		30			30			26		
AMD RLU	143			986	46		1,105	180		159			6,882			2,571		
	91			92			93			94			95			96		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	443	13,452	0.03	34	126,236	0.00	22	62	[redacted]	45	33	[redacted]	45	42	[redacted]	46	35	[redacted]
L83 RLU	33	12,051			85,018													
AMD RLU	451	51			45													

CORECEPTOR ASSAY SCREEN TEMPLATE

active RLU limit: 100 tioplasm ratio limit: 8 CXCR4 CCR5 DUAL drug %inhib limit: 10 L83 resistant AMD resistant

	1			2			3			4			5			6		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	34	26,471	0 00	14,140	55	257.09	46	6,845	0 01	21,656	38,144	0 57	30	7,336	0.00	3,988	31	128 65
L83 RLU		19,258		54				4,696		27	25,542			5,468		34		
AMD RLU		32		12,186				40		7,226	32			39		3,664		
%Inhib by L83		27		100				31		100	33			25		9		
%Inhib by AMD		100		14				99		67	100			99		8		
	13			14			15			16			17			18		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	4,552	19,102	0.24	149	226	0.66	67,389	72	935 96	611	19,715	0 03	46	3,948	0.01	284	4,330	0 07
L83 RLU	28	9,936		40	153		99			30	14,985			2,953		38	2,918	
AMD RLU	838	28	6.00	41	24		42,295			76	1,022			152		25	48	
%Inhib by L83	99	48		73	32		100			95	24			25		67	30	
%Inhib by AMD	82	100		72	69		37			88	86			86		91	89	
	25			26			27			28			29			30		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	67,828	68	997 47	14,982	12,020	1.25	3,788	4,384	0 86	50	37		656	242	2 72	231	35	6 69
L83 RLU	129			111	10,838		59	3,397					32	221		47		
AMD RLU	51,146			8,580	3,384		2,049	536					493	38		218		
%Inhib by L83	100			99	10		98	23					95	9		80		
%Inhib by AMD	25			43	72		46	88					25	84		5		
	37			38			39			40			41			42		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	100	39		3,724	2,310	1 61	450	668	0 67	148	24	6 17	32	22		60	61	
L83 RLU				38	2,656		37	463		33								
AMD RLU				1,864	272		206	72		138								
%Inhib by L83				99	(15)		92	31		76								
%Inhib by AMD				47	88		34	89		6								
	49			50			51			52			53			54		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	35	107	0 33	83	4,209	0 02	18,099	8,451	2 14	39,257	38	1033 08	5,413	40	135 33	97,733	29	3370 10
L83 RLU		68			3,502		13,856	6,220		85			35			30		
AMD RLU		34			86		16,980	8,711		27,837			5,043			48,457		
%Inhib by L83		36			17		23	26		100			99			100		
%Inhib by AMD		68			96		6	(3)		29			7			50		
	61			62			63			64			65			66		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	3,769	40	94.23	786	48	16.38	340	21	16 19	5,189	43	120 67	4,318	37	116.70	190	51	3 84
L83 RLU	29			39			31			34			28			23		
AMD RLU	2,773			797			282			5,356			3,461			184		
%Inhib by L83	99			95			91			99			99			88		
%Inhib by AMD	26			(1)			17			(3)			20			6		
	73			74			75			76			77			78		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	391	26	15 04	98	38		4,449	28	158 89	4,357	9,102	0 48	6,090	105	58 00	1,886	3,635	0 52
L83 RLU	50						43			34	7,932		73	77		38	2,092	
AMD RLU	340						3,752			906	36		4,473	51		279	27	
%Inhib by L83	87						99			98	13		99	27		86	42	
%Inhib by AMD	13						16			79	100		27	51		85	99	
	85			86			87			88			89			90		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	510	33	15 45	979	59	16 59	491	33	14 88	300	3,815	0 08	297	3,615	0.08	39	61,594	0 00
L83 RLU	42			55			49			29	3,661		32	2,946			56,739	
AMD RLU	564			813			376			266	36		256	30			40	
%Inhib by L83	92			94			90			90	4		89	19			8	
%Inhib by AMD	(11)			17			23			11	99		14	99			100	

	7			8			9			10			11			12		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	30	1,217	0 02	49	1,128	0 04	42,206	70	602 94	45	1,159	0 04	44,589	97	459 68	32	29,118	0 00
L83 RLU		814			934		90				761		60				22,270	
AMD RLU		42			37		33,192				33		30,510				31	
%Inhib by L83		33			17		100				34		100				23	
%Inhib by AMD		97			97		21				97		32				100	
	19			20			21			22			23			24		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	100	6,294	0.02	11,299	52	217 29	1,273	20	63 65	7,375	10,856	0 68	4,397	7,130	0 62	16,115	50	322 30
L83 RLU		3,038		22			26			40	6,455		42	3,874		68		
AMD RLU		34		8,232			1,129			3,961	2,283		1,656	1,285		11,980		
%Inhib by L83		41		100			96			99	41		89	46		100		
%Inhib by AMD		86		27			11			46	78		62	82		20		
	31			32			33			34			35			36		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	53	974	0 05	177	32	5.53	24,739	33	749 67	612	45	13 60	2,997	9,695	0 31	416	31	13 42
L83 RLU		826		32			31			48			22	7,261		28		
AMD RLU		85		361			21,767			479			925	35		587		
%Inhib by L83		15		82			100			92			69	25		93		
%Inhib by AMD		90		(104)			12			22			69	100		(44)		
	43			44			45			46			47			48		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	45	336	0.13	714	45	15 87	11,029	18	612 72	2,906	43	67.63	13,997	52	269.17	24,377	34	716 97
L83 RLU		207		31			18			31			73			59		
AMD RLU		29		366			8,390			2,306			12,139			16,045		
%Inhib by L83		35		96			100			95			99			100		
%Inhib by AMD		91		49			24			21			13			34		
	55			56			57			58			59			60		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	5,254	23,846	0 39	5,249	20,393	0 26	470	39	12 05	19,175	126	152 18	513	31	16 55	3,264	28	116 57
L83 RLU	43	9,150		38	8,290		37			96	183		35			30		
AMD RLU	2,219	40		784	38		310			19,638	35		500			3,036		
%Inhib by L83	100	62		99	59		92			99	(45)		93			99		
%Inhib by AMD	76	100		85	100		34			(2)	72		3			7		
	67			68			69			70			71			72		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	3,853	77	50 04	411	34	12 09	7,857	41	191 63	25,437	38	669 39	17,443	45	387.62	16,707	38	439 66
L83 RLU	39			22			42			35			40			104		
AMD RLU	2,108			253			4,595			14,748			13,708			12,486		
%Inhib by L83	99			95			99			100			100			99		
%Inhib by AMD	45			38			42			42			21			25		
	79			80			81			82			83			84		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	263	38	6.92	3,890	9,577	0 41	2,088	1,698	1 23	475	43	11 05	8,475	38	223 03	4,107	33	124 45
L83 RLU	33			44	6,140		33	1,224		30			30			28		
AMD RLU	143			986	45		1,105	180		159			6,862			2,571		
%Inhib by L83	87			99	36		98	28		94			100			99		
%Inhib by AMD	46			76	100		47	91		67			19			37		
	91			92			93			94			95			96		
	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4	R5	X4	R5:X4
No Drug RLU	443	13,452	0.03	34	128,236	0 00	22	62		45	33		43	42		46	35	
L83 RLU	33	12,061			85,018													
AMD RLU	451	51			45													
%Inhib by L83	93	10			34													
%Inhib by AMD	(2)	100			100													

R5

No Drug											
34	14,140	46	21,655	30	3,988	30	49	42,206	45	44,589	32
4,552	149	67,389	611	46	284	100	11,299	1,273	7,375	4,397	16,115
67,828	14,982	3,788	50	658	231	53	177	24,739	612	2,997	416
100	3,724	450	148	32	60	45	714	11,029	2,908	13,997	24,377
35	83	18,099	39,257	5,413	97,733	9,254	5,249	470	19,175	513	3,264
3,769	786	340	5,189	4,318	196	3,853	411	7,857	25,437	17,443	16,707
391	98	4,449	4,357	6,090	1,886	263	3,890	2,089	475	8,475	4,107
510	979	491	300	297	39	443	34	22	45	43	45
L83											
30	54	29	27	32	34	27	37	90	35	60	40
28	40	99	30	21	38	32	22	26	40	42	68
129	111	59	38	32	47	28	32	31	48	22	28
29	38	37	33	30	30	32	31	18	31	73	59
31	115	13,896	85	35	30	43	38	37	96	35	30
29	39	31	34	28	23	39	22	42	35	40	104
50	31	43	34	73	38	33	44	33	30	30	28
42	55	49	29	32	25	33	35	33	36	20	27
AMD											
39	12,186	32	7,226	36	3,664	34	40	33,192	44	30,510	35
838	41	42,295	76	45	25	30	8,232	1,129	3,961	1,656	11,980
51,146	8,580	2,049	53	493	219	26	361	21,767	479	925	597
153	1,984	206	139	33	42	30	366	8,390	2,306	12,139	16,045
38	37	16,980	27,832	5,043	48,452	2,219	784	310	19,638	500	3,038
2,773	797	282	5,356	3,461	184	2,108	253	4,595	14,749	13,708	12,486
340	47	3,752	906	4,473	279	143	986	1,105	159	6,862	2,571
564	813	376	268	256	35	451	35	27	28	44	29

X4

No Drug											
26,471	55	6,849	38,144	7,336	31	1,217	1,128	70	1,159	97	29,118
19,102	226	72	19,715	3,948	4,330	5,204	52	20	10,856	7,130	50
68	12,020	4,384	37	242	35	974	32	33	45	9,695	31
39	2,310	668	24	22	61	338	45	18	43	52	34
107	4,209	8,451	38	40	29	23,846	20,393	39	125	31	28
40	48	21	43	37	51	77	34	41	38	45	38
26	38	28	9,102	105	3,635	38	9,577	1,698	43	38	33
33	59	33	3,815	3,615	61,594	13,452	128,238	62	33	42	35
L83											
19,258	32	4,696	25,542	5,468	28	814	934	32	761	46	22,279
9,956	153	38	14,985	2,953	2,918	3,058	33	33	6,455	3,874	38
43	10,839	3,397	60	221	43	828	33	36	54	7,261	33
30	2,656	463	35	28	65	207	35	23	32	39	33
68	3,502	6,220	34	33	34	9,150	8,290	33	183	33	33
24	38	42	28	28	27	87	34	47	39	43	29
35	45	40	7,932	77	2,092	58	6,140	1,224	50	44	30
27	42	55	3,661	2,946	56,739	12,051	85,018	33	39	37	25
AMD											
32	28	40	32	39	142	42	37	40	33	37	31
34	24	32	1,022	152	48	34	35	29	2,283	1,285	33
39	3,384	538	49	38	34	95	28	34	40	35	30
28	272	72	30	26	36	29	40	43	30	28	40
34	88	8,711	35	43	33	40	38	38	35	42	47
41	22	40	39	44	26	34	28	37	38	32	24
34	31	39	36	51	27	33	45	160	36	27	36
34	69	55	36	30	40	51	45	17	39	41	27

Plate	Repeat	End time	Start temp	End temp.	BarCode
1	1	6:39:36 PM	21.6	21.7	N/A

0.5 CPS (CPS)
18

26648	54	6970	37406	7158	38	1386	930	100	1184
19248	262	72	18972	3478	3946	5010	46	14	11004
106	9038	4002	32	238	26	976	34	38	48
38	2000	528	16	16	36	298	52	14	54
122	2984	7264	40	38	20	23344	15340	44	156
36	52	28	40	28	40	62	32	38	22
22	32	28	9858	68	3802	46	11470	1958	42
30	64	36	3846	3390	57858	12620	126186	68	34

Plate	Repeat	End time	Start temp	End temp.	BarCode
2	1	6:41:51 PM	21.5	21.6	N/A

0.5 CPS (CPS)
34

26294	56	6728	38882	7514	24	1048	1326	40	1134
18956	190	72	20458	4418	4714	5398	58	26	10708
30	15002	4766	42	246	44	972	30	28	42
40	2620	808	32	28	86	378	38	22	32
92	5434	9638	36	42	38	24348	25446	34	96
44	44	14	46	46	62	92	36	44	54
30	44	28	8346	142	3468	30	7684	1438	44
36	54	30	3784	3840	65330	14284	130290	56	32

Plate	Repeat	End time	Start temp	End temp.	BarCode
3	1	6:44:06 PM	21.6	21.6	N/A

0.5 CPS (CPS)
16

18590	18	4306	23902	5386	30	924	894	32	660
8698	148	34	14088	2880	3142	3160	40	46	7616
30	10252	3542	38	172	34	842	30	32	44
28	2396	370	28	26	66	172	32	22	28
64	3784	4822	32	28	24	6188	5702	34	120
16	28	62	26	28	30	38	38	38	44
38	52	40	7020	68	1798	62	7324	1076	50
32	50	70	3824	3138	53670	13088	104608	32	38

Plate	Repeat	End time	Start temp	End temp.	BarCode
4	1	6:46:20 PM	21.7	21.8	N/A

0.5 CPS (CPS)
24

19926	46	5086	27182	5550	26	704	974	32	862

11214	158	42	15882	3026	2694	2956	26	20	5294
56	11426	3252	82	270	52	814	36	40	64
32	2916	556	42	30	64	242	38	24	36
72	3220	7618	36	38	44	12112	10878	32	246
32	48	22	30	28	24	136	30	56	34
32	38	40	8844	86	2386	54	4956	1372	50
22	34	40	3498	2754	59808	11014	65428	34	40

Plate	Repeat	End time	Start temp	End temp.	BarCode
5	1	6:48:35 PM	21.9	21.9	N/A

0.5 CPS (CPS)
38

38	28	40	30	38	234	46	30	46	30
26	20	32	976	190	58	46	24	28	2298
42	3260	420	50	36	34	102	26	34	24
28	378	88	32	34	32	40	42	54	26
36	62	11690	38	44	32	42	42	26	36
46	22	44	38	58	32	38	30	32	36
22	38	52	40	42	26	34	50	200	36
36	80	52	40	36	40	52	54	22	40

Plate	Repeat	End time	Start temp	End temp.	BarCode
6	1	6:50:50 PM	21.8	21.9	N/A

0.5 CPS (CPS)
30

26	28	40	34	40	50	38	44	34	36
42	28	32	1068	114	38	22	46	30	2268
36	3508	656	48	40	34	88	30	34	56
28	166	56	28	18	40	18	38	32	34
32	114	5732	32	42	34	38	34	50	34
36	22	36	40	30	20	30	26	42	40
46	24	26	32	60	28	32	40	120	36
32	58	58	32	24	40	50	36	12	38

112	30488
7474	60
9736	32
52	38
40	32
44	28
42	24
38	36

82	27748
6786	40
9654	30
52	30
22	24
46	48
34	42
46	34

48	23382
2842	32
7616	34
36	26
34	24
62	34
42	32
50	32

44	21176

4906	44
6906	32
42	40
32	42
24	24
46	28
24	18

34	22
1290	26
40	24
30	48
46	42
38	12
34	36
42	36

40	40
1280	40
30	36
26	32
38	52
26	36
20	36
40	18

Plate	Repeat	End time	Start temp.	End temp.	BarCode
1	1	########	21.7	21.8	N/A

0.5 CPS (CPS)
32

24	14218	56	21586	28	4034	36	52	42088	40
4780	174	71276	730	48	326	28	12022	1288	7198
69452	15306	4008	44	792	180	50	182	25292	718
72	3922	546	128	44	66	30	900	11984	3194
30	88	18820	40438	4882	105946	8466	4934	470	20386
3420	934	344	6268	5012	170	3546	504	8164	22214
344	82	5344	5330	6710	1880	338	4286	2112	466
580	1018	516	318	302	40	414	32	20	52

Plate	Repeat	End time	Start temp.	End temp.	BarCode
2	1	########	21.9	21.9	N/A

0.5 CPS (CPS)
22

44	14062	36	21726	32	3942	24	46	42324	50
4324	124	63502	492	44	242	172	10576	1258	7552
66204	14658	3568	56	524	282	56	172	24186	506
128	3526	354	168	20	54	60	528	10074	2622
40	78	17378	38076	5944	89520	10042	5564	470	17964
4118	638	336	4110	3624	222	4160	318	7550	28660
438	114	3554	3384	5470	1892	188	3494	2066	484
440	940	466	282	292	38	472	36	24	38

Plate	Repeat	End time	Start temp.	End temp.	BarCode
3	1	########	21.9	22	N/A

0.5 CPS (CPS)
36

36	38	28	28	38	28	36	42	114	40
32	34	80	26	16	36	30	18	26	30
126	106	34	42	30	46	22	26	32	58
26	46	42	38	34	20	18	34	24	36
28	146	15756	76	32	26	42	52	36	106
34	50	20	24	32	18	38	18	36	38
38	38	28	36	80	50	38	44	34	38
40	30	38	30	24	22	38	36	34	36

Plate	Repeat	End time	Start temp.	End temp.	BarCode
4	1	########	21.9	22	N/A

0.5 CPS (CPS)
16

24	70	30	26	26	40	18	32	66	30

24	46	118	34	26	40	34	26	26	50
132	116	84	34	34	48	34	38	30	38
32	30	32	28	26	40	46	28	12	26
34	84	12036	94	38	34	44	24	38	86
24	28	42	44	24	28	40	26	48	32
62	24	58	32	66	26	28	44	32	22
44	80	60	28	40	28	28	34	32	36

Plate	Repeat	End time	Start temp.	End temp.	BarCode
5	1	########	22	22	N/A

0.5 CPS (CPS)
34

38	11132	26	6696	34	3960	44	40	29548	40
762	48	39888	88	40	30	36	8416	1262	4096
52058	8050	2524	60	450	168	32	340	22410	534
106	2080	172	170	38	38	30	444	7478	2458
30	46	16428	25792	4240	45094	2092	630	334	17130
2498	732	358	5044	3236	202	2124	292	4806	13736
290	30	3986	886	4584	240	110	940	1286	162
456	816	438	294	260	44	524	40	34	22

Plate	Repeat	End time	Start temp.	End temp.	BarCode
6	1	########	22	22.1	N/A

0.5 CPS (CPS)
32

40	13240	38	7756	38	3368	24	40	36836	48
914	34	44702	64	50	20	24	8048	996	3826
50234	9110	1574	46	536	270	20	382	21124	424
200	1888	240	108	28	46	30	288	9302	2154
46	28	17532	29872	5846	51810	2346	938	286	22146
3048	862	206	5668	3686	166	2092	214	4384	15762
390	64	3518	926	4362	318	176	1032	924	156
672	810	314	242	252	26	378	30	20	34

43332	38
4314	17856
3572	424
16106	23794
548	3376
15146	19592
7752	4078
48	42

45846	26
4480	14374
2422	408
11888	24960
478	3152
19740	13822
9198	4136
38	50

60	46
48	80
8	28
100	60
34	24
50	58
30	28
22	26

60	34

36	56
36	28
46	58
36	36
30	150
30	28
18	28

30628	34
1598	12372
1046	512
10024	15470
388	2730
13012	12386
6768	2204
38	20

30392	36
1714	11588
804	682
14254	16620
612	3346
14404	12586
6956	2938
50	38

	73		
	R5 cells	X4 cells	R5:X4
no drug	34	26,471	0
R5 inhibitor		19,258	
X4 inhibitor		32	
%inhib by R5 inhibitor		27	
%inhib by X4 inhibitor		100	

	21		
	R5	X4	R5:X4
no drug	67,389	72	936
R5 inhibitor	99		
X4 inhibitor	42,295		
%inhib by R5 inhibitor	100		
%inhib by X4 inhibitor	37		

0.00	257.09	0.01	0.57	0.00	128.65	0.02	0.04	602.94	0.04	459.68	0.00
0.24	0.66	935.96	0.03	0.01	0.07	0.02	217.29	63.65	0.02	0.02	322.30
997.47	1.25	0.86	[illegible]	2.72	6.60	0.05	5.53	749.67	13.60	0.31	13.42
[illegible]	1.61	0.67	6.17	[illegible]	[illegible]	0.13	15.87	612.72	67.63	269.17	716.97
0.33	0.02	2.14	1033.08	135.33	3370.10	0.35	0.26	12.05	152.18	16.55	116.57
94.23	16.38	16.19	120.67	116.70	3.84	50.04	12.09	191.63	669.39	387.62	439.66
15.04	[illegible]	158.89	0.46	58.00	0.52	6.92	0.41	1.23	11.05	223.03	124.45
15.45	16.59	14.88	0.08	0.08	0.00	0.03	0.00	[illegible]	[illegible]	[illegible]	[illegible]

CXCR4
CCR5
DUAL or MIXED
DEAD

	79		
	R5	X4	R5:X4
no drug	14,982	12,020	1
R5 inhibitor	111	10,839	
X4 inhibitor	8,580	3,384	
%inhib by R5 inhibitor	99	10	
%inhib by X4 inhibitor	43	72	

	96		
	R5	X4	R5:X4
no drug	[illegible]	[illegible]	[illegible]
R5 inhibitor	[illegible]	[illegible]	[illegible]
X4 inhibitor	[illegible]	[illegible]	[illegible]
%inhib by R5 inhibitor	[illegible]	[illegible]	[illegible]
%inhib by X4 inhibitor	[illegible]	[illegible]	[illegible]

What is claimed is:

1. A method for identifying whether a compound inhibits entry of a virus into a cell which comprises:

(a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus;

(b) co-transfecting into a first cell

(i) the nucleic acid of step (a), and

(ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal,

such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient;

(c) contacting the viral particles produced in step (b) with a second cell in the presence of the compound, wherein the second cell expresses a cell surface receptor to which the virus binds;

(d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and

(e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of

the compound, wherein a reduced amount of signal measured in the presence of the compound indicates that the compound inhibits entry of the virus into the second cell.

2. The method of claim 1, wherein the indicator nucleic acid comprises an indicator gene.

3. The method of claim 2, wherein the indicator gene is a luciferase gene.

4. The method of claim 1, wherein the cell surface receptor is CD4.

5. The method of claim 1, wherein the cell surface receptor is a chemokine receptor.

6. The method of claim 1, wherein the cell surface receptor is CXCR4 or CCR5.

7. The method of claim 1, wherein the patient is infected with the HIV-1 virus.

8. The method of claim 1, wherein the nucleic acid of step (a) comprises DNA encoding gp120 and gp41.

9. The method of claim 1, wherein the viral expression vector comprises HIV nucleic acid.

10. The method of claim 9, wherein the viral expression vector comprises an HIV gag-pol gene.

11. The method of claim 9, wherein the viral expression vector comprises DNA encoding vif, vpr, tat, rev, vpu, and nef.

12. The method of claim 1, wherein the first cell is a mammalian cell.

13. The method of claim 12, wherein the mammalian cell is a human cell.

14. The method of claim 13, wherein the human cell is a human embryonic kidney cell.

15. The method of claim 14, wherein the human embryonic kidney cell is a 293 cell.

16. The method of claim 1, wherein the second cell is a human T cell.

17. The method of claim 1, wherein the second cell is a human T cell leukemia cell line.

18. The method of claim 1, wherein the second cell is a peripheral blood mononuclear cell.

19. The method of claim 1, wherein the second cell is an astroglioma cell.

20. The method of claim 19, wherein the astroglioma cell is a U87 cell.

21. The method of claim 1, wherein the second cell is a human osteosarcoma cell.

22. The method of claim 2, wherein the human osteosarcoma cell is an HT4 cell.

23. The method of claim 1, wherein the compound binds to

the cell surface receptor.

24. The method of claim 1, wherein the compound is a ligand of the cell surface receptor.

25. The method of claim 23, wherein the compound comprises an antibody.

26. The method of claim 1, wherein the compound inhibits membrane fusion.

27. The method of claim 1, wherein the compound is a peptide, a peptidomimetic, an organic molecule, or a synthetic compound.

28. The method of claim 1, wherein the compound binds the viral envelope protein.

29. A method for making a composition which comprises admixing the compound identified by claim 1 with a carrier.

30. The method of claim 29, wherein the carrier is saline, polyethylene glycol, a buffer solution, a starch, or an organic solvent.

31. A method for identifying a cell surface receptor which is bound by a virus upon infection of a cell by the virus which comprises:

(a) obtaining viral particles which comprise (i) a viral nucleic acid and (ii) an indicator nucleic acid which produces a detectable signal;

(b) contacting a cell which expresses a cell surface receptor with the viral particles from step (a); and

(c) measuring the amount of detectable signal produced

within the cell, wherein production of the signal indicates the cell surface receptor expressed by the cell is bound by the virus, thereby identifying the cell surface receptor as being bound by the virus upon infection of the cell.

32. A method for identifying whether an antibody inhibits entry of a virus into a cell which comprises:

(a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus;

(b) co-transfecting into a first cell

(i) the nucleic acid of step (a), and

(ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal,

such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient;

(c) contacting the viral particles produced in step (b) with a second cell in the presence of the antibody, wherein the second cell expresses a cell surface receptor to which the virus binds;

(d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and

(e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of the compound, wherein a reduced amount of signal measured in the presence of the antibody indicates

that the antibody inhibits entry of the virus into the second cell.

33. A method for determining susceptibility of a virus to a compound which inhibits viral cell entry which comprises:

(a) obtaining nucleic acid encoding a viral envelope protein from a patient infected by the virus;

(b) co-transfecting into a first cell

(i) the nucleic acid of step (a), and

(ii) a viral expression vector which lacks a nucleic acid encoding an envelope protein, and which comprises an indicator nucleic acid which produces a detectable signal,

such that the first cell produces viral particles comprising the envelope protein encoded by the nucleic acid obtained from the patient;

(c) contacting the viral particles produced in step (b) with a second cell in the presence of the compound, wherein the second cell expresses a cell surface receptor to which the virus binds;

(d) measuring the amount of signal produced by the second cell in order to determine the infectivity of the viral particles; and

(e) comparing the amount of signal measured in step (d) with the amount of signal produced in the absence of the compound, wherein a reduced amount of signal measured in the presence of the compound indicates that the virus is susceptible to the compound.

34. A method for determining resistance of a virus to a compound which inhibits viral entry into a cell which comprises:

(a) determining susceptibility of a virus to a compound according to the method of claim 33, wherein a nucleic acid encoding a viral envelope protein is obtained from a patient at a first time;

(b) determining susceptibility of the virus to the compound according to the method of claim 33, wherein the nucleic acid encoding the viral envelope protein is obtained from the patient at a later second time; and

(c) comparing the susceptibilities determined in steps (a) and (b), wherein a decrease in susceptibility at the later second time indicates resistance of the virus to the compound.

35. A method for identifying a mutation in a virus that confers resistance to a compound that inhibits viral entry into a cell which comprises:

(a) determining the nucleic acid sequence or the amino acid sequence of the virus prior to any treatment of the virus with the compound;

(b) obtaining a virus resistant to the compound;

(c) determining the nucleic acid sequence or the amino acid sequence of the resistant virus from step (b); and

(d) comparing the nucleic acid sequence or the amino acid sequences of steps (a) and (c), respectively, so as to identify the mutation in the virus that confers

resistance to the compound.

36. The method of claim 35, wherein the virus obtained in step (b) is the virus of step (a) grown in the presence of the compound until resistance is developed.

37. The method of claim 35, wherein the virus obtained in step (b) is isolated from a patient which has been undergoing treatment with the compound.

SEQUENCE LISTING

<110> ViroLogic, Inc.

<120> COMPOSITIONS AND METHODS FOR EVALUATING VIRAL RECEPTOR/ CO-RECEPTOR USAGE AND INHIBITORS OF VIRUS ENTRY USING RECOMBIN ANT VIRUS ASSAYS

<130> 2793/65166APCT

<140> 09/874,475, 60/295,871

<141> 2001-06-04

<160> 16

<170> PatentIn version 3.1

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26

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35

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<213> PCR Primer

<400> 6
gggctcgaga ccggtgagca gaagacagtg gcaatg
36

<210> 7

<211> 35

<212> DNA

<213> PCR Primer

<400> 7
gggtctagaa cgcgttgcca cccatcttat agcaa
35

<210> 8

<211> 38

<212> DNA

<213> PCR Primer

<400> 8
gggtctagaa cgcgtccact tgccacccat bttatagc
38

<210> 9

<211> 34

<212> DNA

<213> PCR Primer

<400> 9
gggtctagaa cgcgtccact tgccacccat btta
34

<210> 10

<211> 38

<212> DNA

<213> PCR Primer

<400> 10
gatggtctaa gacgctgttc aatatccctg cctaactc
38

<210> 11

<211> 10

<212> PRT

<213> Viral Entry Inhibitor Resistance Mutation

<400> 11

Gln Leu Leu Ser Gly Ile Val Gln Gln Gln
1 5 10

<210> 12

<211> 10

<212> PRT

<213> Viral Entry Inhibitor Resistance Mutation

<400> 12

Gln Leu Leu Ser Ser Ile Met Gln Gln Gln
1 5 10

<210> 13

<211> 10

<212> PRT

<213> Viral Entry Inhibitor Resistance Mutation

<220>

<221> MISC_FEATURE

<222> (5)..(5)

<223> Where X= G or S

<220>

<221> MISC_FEATURE

<222> (7)..(7)

<223> Where X= V or M

<400> 13

Gln Leu Leu Ser Xaa Ile Xaa Gln Gln Gln
1 5 10

<210> 14

<211> 10

<212> PRT

<213> Viral Entry Inhibitor Resistance Mutation

<400> 14

Gln Leu Leu Ser Asp Ile Val Gln Gln Gln
1 5 10

<210> 15

<211> 10

<212> PRT

<213> Viral Entry Inhibitor Resistance Mutation

<220>

<221> MISC_FEATURE

<222> (5)..(5)

<223> Where X= G or D

<400> 15

Gln Leu Leu Ser Xaa Ile Val Gln Gln Gln
1 5 10

<210> 16

<211> 36

<212> PRT

<213> Fusion Inhibitor Peptide

<400> 16

Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gl
n
1 5 10 15

Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Le
u
20 25 30

Trp Asn Trp Phe
35