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(54) Title: DETECTION OF HUMAN IMMUNODEFICIENCY VIRUS CO-RECEPTOR TROPISM IN AVIREMIC SUBJECTS

(57) Abstract: Methods for detecting human immunodeficiency virus (HIV) co-receptor tropism or replication-competent virus in aviremic subjects, and methods of selecting optimal therapies for aviremic subjects.



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**DETECTION OF HUMAN IMMUNODEFICIENCY VIRUS CO-
RECEPTOR TROPISM IN AVIREMIC SUBJECTS**

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application Serial
5 No. 61/026,623, filed on February 6, 2008, the entire contents of which are hereby
incorporated by reference.

FIELD OF THE INVENTION

The invention relates to human immunodeficiency virus (HIV) co-receptor
tropism detection assays, and methods of selecting optimal therapies for aviremic
10 subjects.

BACKGROUND OF THE INVENTION

HIV-1 positive individuals undergoing combination antiviral therapy (i.e.,
receiving two or more anti-HIV-1 compounds) can exhibit decreased viral loads in the
peripheral blood. In some cases, after several weeks or months of therapy, HIV-1 RNA
15 cannot be detected in the peripheral blood using standard methods. Unfortunately, if such
aviremic patients stop therapy, the HIV can rebound very rapidly, indicating the presence
of a reservoir of replication-competent virus in the patient that is not detectable using
standard methods. It would be desirable to select a therapy that is specifically targeted to
the genotype of that viral reservoir, however, the limited sensitivity of standard HIV
20 tropism detection assays provides a challenge to further advances in therapy.

SUMMARY OF THE INVENTION

The invention is based on the discovery that patients having no virus detectable in the blood by known means, e.g., patients undergoing drug therapy, such as combination drug therapy, can nevertheless harbor replication-competent HIV, and that this viral reservoir is indicated by the presence of 2-LTR circles in peripheral blood mononuclear cells (PBMCs). The presence of the 2-LTR circles indicates the persistence of viral spread in those patients, in spite of the fact that these patients have no detectable virus in the blood. Since the 2-LTR circles are related to the replication-competent virus, the genotype of the circles can be used to determine the tropism of the virus. This information can be used to guide the selection of antiviral medications that inhibit the entry of virus into cells ("entry inhibitors") by blocking the virus/cell surface receptor interaction, for subjects who are aviremic (i.e., have no detectable levels of cell-free HIV viral RNA in their blood).

Thus, the invention provides methods for determining co-receptor tropism of replication competent virus in an HIV-positive subject, e.g., a mammal, e.g., a primate, e.g., a human, who has less than 50 cell-free viral RNA molecules/ml of serum. In general, the methods include providing a sample comprising a cell (e.g., a peripheral blood mononuclear cell (PBMC)) from the subject; detecting an HIV 2-LTR circle DNA molecule in the sample; and determining the co-receptor tropism of the 2-LTR circle DNA. As the 2-LTR circles are derived from infectious virus, the co-receptor tropism of the 2-LTR circle DNA indicates the co-receptor tropism of the replication competent virus in the subject.

In some embodiments, determining the co-receptor tropism of the 2-LTR circle comprises determining the genotype of (e.g., sequencing) the DNA. In some embodiments, the DNA molecule is amplified before determining the genotype of the DNA, e.g., using polymerase chain reaction (PCR) with primers specific for an envelope protein or a portion thereof, e.g., the V3 loop. In some embodiments, the envelope protein is gp120 or gp41.

In some embodiments, determining the co-receptor tropism of the 2-LTR circle comprises performing a phenotypic tropism assay, e.g., a cell-based assay as described herein.

In some embodiments, the subject is being treated with highly active antiretroviral therapy (HAART). In some embodiments, cell-free HIV viral RNA cannot be detected in the blood of the mammal.

In some embodiments, the co-receptor tropism of the 2-LTR circle DNA indicates that the replication competent virus is primarily M-tropic, primarily T-tropic, primarily dual-tropic; or of mixed tropism.

5 In some embodiments, the methods further include selecting an entry inhibitor based on the co-receptor tropism of the replication competent virus. For example, the methods can include selecting a CCR5-specific entry inhibitor based on the presence of co-receptor tropism for CCR5 or of dual tropism, or selecting a CXCR4-specific entry inhibitor based on the presence of co-receptor tropism for CXCR4.

Also provided herein are methods for treating an HIV-infected subject, e.g., a
10 mammal, e.g., a primate, e.g., a human, who has less than 50 cell-free viral RNA molecules/ml of serum. The methods generally include providing a sample comprising a cell from the subject; detecting an HIV 2-LTR circle DNA molecule in the sample; determining the co-receptor tropism of the 2-LTR circle DNA; determining co-receptor tropism of replication-competent virus in the subject based on the co-receptor tropism of
15 the 2-LTR circle DNA; selecting an entry inhibitor suitable for the HIV co-receptor tropism of the virus in the subject; and administering an effective amount of the selected entry inhibitor to the subject, thereby treating the subject.

In some embodiments, the methods include selecting a CCR5-specific entry inhibitor for a subject in whom the co-receptor tropism is for a receptor other than
20 CXCR4. In some embodiments, the methods include selecting a CXCR4-specific entry inhibitor for a subject in whom the co-receptor tropism is for CXCR4.

In some embodiments, the methods described herein include a step of confirming that the subject has less than about 50 cell-free viral RNA molecules/ml of serum.

An HIV-positive individual is one who produces antibodies that specifically bind
25 to an HIV viral protein.

Cell-free HIV viral RNA is RNA that is not associated with a cell (e.g., RNA in plasma).

Analysis of 2-LTR circles can be applied to determine HIV genotype(s) in infected individuals. Genotype analysis is useful for determining therapy. For example,
30 therapy with CCR5 inhibitors is contraindicated in patients infected with a CXCR4-tropic virus because CCR5 inhibitors are ineffective against CXCR4-tropic viruses, and may promote outgrowth of pathogenic strains. (CXCR4 refers to Chemokine, CXC motif, Receptor 4; CCR5 refers to Chemokine, CC motif, Receptor 5). Accordingly, the invention includes methods in which the genotype of 2-LTR circle DNA from a subject
35 (e.g., an aviremic subject) is determined. In some embodiments, the genotyping includes

determining the co-receptor tropism of the HIV virus (e.g., CCR5 or CXCR4 tropism, e.g., by evaluating the envelope gene). In some embodiments, therapy with a CCR5 inhibitor is indicated for a subject infected with a virus that is not CXCR4-tropic. In some embodiments, therapy with a CCR5 inhibitor is not indicated for a subject infected with a CXCR4-tropic virus. Likewise, in some embodiments, therapy with a CXCR4 inhibitor is indicated for a subject infected with a CXCR4-tropic virus. In some embodiments, therapy with a CXCR4 inhibitor is contraindicated for a subject infected with a CCR5-tropic virus.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although suitable methods and materials for the practice or testing of the present invention are described below, other methods and materials similar or equivalent to those described herein, which are well known in the art, can also be used. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B are graphs of viral DNA (copies/ 10^5 cells) versus time post RT inhibition, showing data for HIV-1_{LAI} and HIV-1_{ADA}, respectively.

Figs. 2A-2D are graphs of HIV-1 RNA or genomes versus time in weeks, showing the data for patients Gu, Sm, Za, and Ha, respectively.

Fig. 3 is a data point plot of number of virus-positive and virus-negative cultures for patient designations.

DETAILED DESCRIPTION

The invention relates to a methods of determining the co-receptor tropism of replication-competent HIV in aviremic HIV-positive patients, for example, patients undergoing antiviral drug therapy, by detecting and genotyping HIV 2-LTR circles in the patients' cells. Present methods of determining co-receptor tropism rely on the presence of plasma viral RNA (e.g., at least 100 to several thousand molecules of viral RNA/ml plasma), however, as discussed herein, patients without detectable plasma viral RNA may still retain replication-competent virus. The present methods can be used to determine co-receptor tropism of that replication-competent virus in patients who are aviremic, i.e., who test negative for plasma viral RNA (below 40 or 50 molecules of viral RNA/ml plasma). This allows the selection and administration of entry inhibitors that are specific for the co-receptors used by the virus present in the subject.

Viral Tropism

HIV entry into cells is mediated through sequential interactions between HIV envelope glycoproteins (Env) and two cellular molecules: CD4 and a co-receptor, typically either CCR5 or CXCR4 (Coakley et al., Curr Opin Infect Dis. 2005 Feb;18(1):9-15). The envelope glycoprotein of HIV-1 consists of a complex of gp120 and gp41. gp120 determines viral tropism (the specificity of a virus for a particular cell or tissue) by binding to a specific co-receptor, while gp41 mediates fusion between viral and cellular membranes. See, e.g., Moore and Doms, Proc Natl Acad Sci U S A. 2003;100:10598-10602.

Macrophage (M-tropic, or R5) strains of HIV-1 use the β -chemokine receptor CCR5 for entry. The CCR5 co-receptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype. T-tropic (or X4) isolates use the α -chemokine receptor, CXCR4, for entry. Dual-tropic (D) HIV-1 strains are able to use both CCR5 and CXCR4 as co-receptors for viral entry, and may be transitional strains of the HIV-1 virus. Mixed-tropic (M) virus populations may contain various combinations of R5 virus, X4 virus, and/or dual-tropic viruses. See, e.g., (Coakley et al., Curr Opin Infect Dis. 2005 Feb;18(1):9-15; Deng et al., Nature 1996;381(6584):661-6; Feng et al., Science 1996;272(5263):872-7).

Present methods of determining viral tropism use plasma viral RNA, and thus cannot be used on subjects who are aviremic.

Sample Preparation

A variety of biological samples can be analyzed by the methods of the invention, including blood and solid-tissue biopsies (e.g., a lymph node biopsy). For example, blood can be collected from an HIV-positive individual undergoing combination therapy.

5 PBMC can be isolated by standard ficoll-based isolation procedures. The PBMC are then lysed and the total or extrachromosomal DNA isolated.

Total cellular DNA can be extracted by lysing the PBMC in detergent, digesting the cellular protein, and precipitating the DNA (Pauza et al., *Virology*, 205:470-478, 1984; and Panther et al., *J. Acquir. Immune. Defic. Syndr. Hum. Retro.*, 17:303-313, 10 1998). Extrachromosomal DNA can be isolated by any method known in the art, including standard alkaline lysis, Hirt extraction, or guanidinium thiocyanate precipitation (Jurriana et al., *J. Gen. Virol.*, 73:1537-1541, 1992; Stevenson et al., *J. Virol.*, 64:2421-2425, 1990; and Sambrook et al., eds., *Molecular Cloning: A Laboratory Manual*, 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring 15 Harbor, NY, 1989).

Although the standard alkaline lysis technique is best known for isolating plasmid DNA from bacteria, this technique can also be used according to the invention to isolate 2-LTR circles from mammalian cells. The Spin Miniprep Kit available from Qiagen (Cat. No. 27104) is especially useful for this purpose. The methods of the invention 20 include the use of this technique to isolate and purify 2-LTR circle DNA.

When possible, extrachromosomal DNA, instead of total DNA, should be isolated since the number of target 2-LTR circles per microgram of extrachromosomal DNA is expected to be far greater than the number of 2-LTR circles per microgram of total cellular DNA.

25 2-LTR Circles and Methods of Detection

In vitro studies of retroviruses have shown that the first evidence of reverse transcription is unintegrated viral DNA appearing in the cytoplasm, which is transported to the nucleus within hours after infection of a cell (Shank et al., *J. Virol.* 25:104-114, 1978; Clayman et al., *Science*, 206:582-584, 1979; and Stevenson et al., *EMBO J.*, 30 9:1551-1560, 1990). In the case of HIV-1, this unintegrated DNA exists in several forms, including incompletely or completely reverse-transcribed linear DNA, circular DNA containing one LTR, and circular DNA containing two LTRs (2-LTR circles). 2-LTR circles are identical to integrated proviruses, except that the ends of the LTR are joined in a head-to-tail fashion via a covalent linkage.

PCR can be used to specifically amplify a small segment (a few hundred base pairs) spanning the 2-LTR junction. The PCR is specific for 2-LTR circles, since no proviruses, single LTR circles, or other incomplete viral reverse transcription products will be amplified. Methods of detecting and/or quantifying 2-LTR circles are described herein and in the art. See, e.g., U.S. Pat. Nos. 7,232,657 and 6,797,464; and U.S. Pat. Pub. No. 2005-0064393.

For example, 2-LTR circles can be detected using known techniques, including those that do not require nucleic acid amplification, such as Southern blotting. The DNA sample obtained as described herein can be hybridized with 2-LTR circle-specific probes that are directly or indirectly labeled with chromogenic, radioactive, fluorescent, or luminescent labels.

Where amplification of the 2-LTR circles is desired, e.g., before a detection step, the 2-LTR circles can be amplified by any method well known in the art. These methods include polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,195 and 4,683,202) and variants thereof. Another suitable nucleic acid amplification method is ligation chain reaction (LCR) or variants thereof (Landegran et al., *Science*, 241:1077-1080, 1988; and Nakazawa et al., *Proc. Natl. Acad. Sci. USA*, 91:360-364, 1994). Methods for performing quantitative PCR using 2 LTR-specific primers is described in Stevenson et al., *J. Virol.* 64:2421-2425 (1990).

Other amplification methods include: self-sustained sequence replication (Guatelli et al., *Proc. Natl. Acad. Sci. USA*, 87:1874-1878, 1990), transcriptional amplification system (Kwoh, et al., *Proc. Natl. Acad. Sci. USA*, 86:1173-1177, 1989), and Q-Beta Replicase (Lizardi et al., *Bio/Technology*, 6:1197, 1988).

However the 2-LTR circles are detected, a threshold level of 2-LTR circles per million cells is useful to define meaningful numbers of the circles. If the assay is capable of single-molecule sensitivity, a base threshold can be established at one circle per million PBMC. This threshold is appropriate when determining whether eradication of HIV has been achieved in a patient. Whenever a patient tests above this threshold, the patient is said to exhibit active viral infection. Whenever a patient tests below the threshold, the patient is said to have undetectable levels of infection and may be a candidate for removal from antiviral therapy. In other contexts, such as when the level of 2-LTR circles is used to determine the efficacy of any antiviral regime, thresholds above one per million PBMC can be appropriate (e.g., 10, 50, 100, or 250 circles/ 10^6 PBMC).

Any of the above methods can be combined in a method of the invention to achieve suitable 2-LTR detection efficiencies. The amplification products can then be

genotyped using methods known in the art to determine the predominant tropism of the replication-competent virus.

Detection of HIV viral RNA

Various assays have been developed to detect HIV viral RNA in plasma. A
5 common HIV-1 detection assay utilizes quantitative polymerase chain reaction (PCR) as
a means to amplify and detect viral RNA present in patient plasma. For example, plasma
viral RNA in a sample can be measured using the AMPLICOR® HIV Monitor Test kit
(Roche Molecular Systems, Inc., Branchburg, NJ), employing HIV-1-specific quantitative
PCR, following manufacturer's directions. The threshold of detection for this standard
10 HIV-1 RNA detection assay is about 40-50 viral RNA molecules per milliliter of plasma.
In some embodiments, the assay is an "ultrasensitive" assay, which allows input of RNA
from 10-fold more plasma per amplification reaction, and has a detection threshold of
about 4-5 molecules/ml plasma. See, e.g., Sun et al., *J Clin Microbiol.* 1998 October;
36(10): 2964-2969.

Determining Co-Receptor Tropism Based on 2-LTR Circles

Viral tropism plays a role in HIV pathogenesis. Knowledge of the HIV tropism in
an individual is useful for monitoring the course of infection and determining appropriate
therapy (e.g., by selecting therapy with antagonists directed against the receptor tropism
of the HIV strain in the infected individual, and by avoiding therapy with antagonists that
20 would be ineffective and/or promote outgrowth of pathogenic strains in the individual).

Methods of determining co-receptor tropism are known in the art and include
phenotypic assays (e.g., cell-based assays) and genotyping (e.g., using nucleic acid
sequencing or heteroduplex tracking assays). Some methods are described, e.g., in
Poveda et al., *J. Med. Virol.*, 79(8):1040-1046, 2007; Foeglein and Walter, *Eur. J. Med.*
25 *Res.*, 12(9):473-482, 2007; and Van Baelaen et al., *J. Virol. Meth.*, 146:61-73, 2007.

Genotyping generally includes predicting viral tropism based on the sequence of a
viral protein, e.g., an envelope protein, i.e., gp120 or gp41. Most of the genetic
determinants of coreceptor usage reside in the HIV envelope, in particular, the V3 loop
(Fouchier et al., 1992; *J. Virol.* 66:3183-3187; Hoffman et al., 2002; *J. Virol.* 76:3852-
30 3864; Jensen et al., *J Virol.* 2003 Dec;77(24):13376-88). Thus, this region can be used
for genotypic predictors of coreceptor usage. One approach, known as the "11/25 rule"
classifies a virus as X4 if positively charged amino acids (lysine or arginine) are present
at positions 11 and/or 25 of the V3 loop (DeJong et al., 1992; *J. Virol.* 66:6777-6780;

Fouchier et al., 1995; *J. Clin. Microbiol.* 33:906-911; Fouchier et al., 1992; *J. Virol.* 66:3183-3187). Bioinformatics-based genotypic predictors, such as neural networks (Resch et al., 2001; *Virology* 288:51-62), position-specific scoring matrices (Jensen et al., 2003; *AIDS Rev.* 5:104-112), and support vector machines (SVMs), indicate that the
 5 coreceptor phenotypes may be accurately determined with genotypic predictors (Sing et al., abstr. 30. Abstr. 1st Int. Workshop Targeting HIV Entry, Bethesda, MD) when tested on clonally derived sequence data. See, e.g., Garrido et al., *J Clin Microbiol.* 2008 Mar;46(3):887-91. Epub 2008 Jan 16; Skrabal et al., *J Clin Microbiol.* 2007 Feb;45(2):279-84. Epub 2006 Nov 22; and Raymond et al., *AIDS.* 2008 Sep
 10 12;22(14):F11-6; Low et al., *AIDS Rev.* 2008 Jul-Sep;10(3):143-51; and Sierra et al., *Genotypic Coreceptor Analysis.* 2007; *Eur J Med Res* 12:453-462.

In addition, there are several commercially available tropism assays: cell-based phenotypic recombinant virus assays (RVAs) (e.g., single-cycle assays as described in Whitecomb et al., *Antimicrob Agents Chemother.* 2007;51:566-575; in Poveda et al.,
 15 *AIDS: Volume* 2007;21(11):1487-1490; and in Dam et al., 5th International Workshop on HIV Drug Resistance and Treatment Strategies, June 4-8, 2001; Scottsdale, Arizona; and embodied in the TRT assay, Monogram Diagnostics' TROFILE™ assay, and PHENOSCRIP™ assay from VIRalliance); and a heteroduplex tracking assay HTA (see, e.g., WO2007084568, and Pathway Diagnostics' SENSITROP™ and SENSITROP
 20 IT™(available through Quest Diagnostics, Inc.)). Exemplary HTA assay methods include amplification of the V3 env region from the 2-LTR circles; hybridization of the patient HIV V3-DNA with standard HIV V3-DNA probes from CCR5-tropic HIV strains; and electrophoretic separation and detection of CXCR4/CCR5 HIV V3-DNA heteroduplexes in non-denaturing polyacrylamide gels. Combinations of phenotypic and genotypic
 25 assays, e.g., the XTRACKC/PHENX-R™ assay (from inPheno) and an assay from Virco. For additional information on assays for determining co-receptor tropism, see, e.g., Braun and Wiesman, *Eur J Med Res.* 2007 Oct 15;12(9):463-72; Skrabal et al., *J Clin Microbiol.* 2007 Feb;45(2):279-84. Epub 2006 Nov 22; WO9967429; WO2007088201; WO0179540; WO0157245; and US Pat. Nos. 6057102 and 6727060. One of skill in the
 30 art would readily be able to adapt the art-known methods for use with 2-LTR DNA in place of.

Antiviral Drugs

A number of HAART regimens are presently used; exemplary regimes can include combinations of Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Protease

Inhibitors (PI), and/or non-nucleoside reverse transcriptase inhibitors (NNRTIs). In some embodiments, the subject is being treated with a regimen that includes 2 NRTIs plus an NNRTI and/or a PI. In some embodiments, the subject's treatment includes an additional drug or drugs that targets viral integration into genomic DNA (i.e., an integration inhibitor, e.g., raltegravir). The present methods can include the intensification of the subject's treatment by the addition of an additional drug or drugs that inhibits entry of the virus into cells (entry inhibitors).

NRTIs

NRTIs are the nucleoside and nucleotide analogs, which replace the normal endogenous nucleotides/nucleosides, preventing the reverse transcriptase from transcribing viral RNA. Exemplary NRTIs are listed in Table A.

Table A: Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

Brand Name	Generic Name	Pharmaceutical Company
COMBIVIR	zidovudine + lamivudine	GlaxoSmithKline
EMTRIVA	emtricitabine	Gilead Sciences
EPIVIR	lamivudine	GlaxoSmithKline
EPZICOM	abacavir + lamivudine	GlaxoSmithKline
RETROVIR	zidovudine	GlaxoSmithKline
TRIZIVIR	abacavir + zidovudine + lamivudine	GlaxoSmithKline
TRUVADA	tenofovir DF + emtricitabine	Gilead Sciences
VIDEX & VIDEX EC	didanosine	Bristol-Myers Squibb
VIREAD	tenofovir disoproxil fumarate (DF)	Gilead Sciences
ZERIT	stavudine	Bristol-Myers Squibb
ZIAGEN	abacavir	GlaxoSmithKline
RACIVIR		Pharmasset
	amoxovir	RFS Pharma
	apricitabine	Avexa Limited
	elvucitabine	Achillion Pharmaceuticals

PIs

PIs inhibit the activity of the HIV protease, preventing the production of functional viral particles. Exemplary PIs are listed in Table B.

Table B: Protease Inhibitors (PIs)

Brand Name	Generic Name	Pharmaceutical Company
AGENERASE	amprenavir	GlaxoSmithKline and Vertex
APTIVUS	tipranavir	Boehringer Ingelheim
CRIVAN	indinavir	Merck & Co
INVIRASE	saquinavir	Hoffmann-La Roche
KALETRA	lopinavir + ritonavir	Abbott Laboratories
LEXIVA	fosamprenavir	GlaxoSmithKline
NORVIR	ritonavir	Abbott Laboratories
PREZISTA	darunavir	Tibotec
REYATAZ	atazanavir	Bristol-Myers Squibb
VIRACEPT	nelfinavir	Pfizer

NNRTIs

NNRTIs bind to reverse transcriptases and prevent the transcription of viral RNA.

5 Exemplary NNRTIs are listed in Table C.

Table C: Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Brand Name	Generic Name	Pharmaceutical Company
INTELENCE	etravirine	Tibotec
RESCRIPTOR	delavirdine	Pfizer
SUSTIVA	efavirenz	Bristol-Myers Squibb
VIRAMUNE	nevirapine	Boehringer Ingelheim
	rilpivirine	Tibotec

Entry Inhibitors

Entry inhibitors (also known as fusion inhibitors) target the gp120 or gp41 HIV
 10 envelope proteins, or the CD4 protein, or CCR5 or CXCR4 receptors on a CD4 cell's
 surface. A number of entry inhibitors are known in the art, e.g., enfuvirtide (Trimeris and
 Hoffmann-La Roche, targets gp41); maraviroc (SELZENTRY™, Pfizer, targets CCR5;
 see, e.g., Mayer et al., AIDS 2006 – XVI International AIDS Conference; August 13–18,
 2006; Toronto, Canada. Abstract THLB0215); vicriviroc (Schering-Plough Corporation,
 15 targets CCR5); PRO 140 (Progenics Pharmaceuticals, targets the CD4 protein);
 Pentafuside (T-20, Hoffman-LaRoche/Trimeris, targets gp41, see Kilby et al., Nat. Med.
 1302-1307, 1998); T-1249 (Hoffman-LaRoche/Trimeris, targets gp41); and NBD-556 and

analogs thereof, e.g., as described in Madani et al., *Structure* 2008 Nov;16:1689-1701.

Additional entry inhibitors include the CXCR4 antagonists AMD 3100 (Johnson
Matthey/Anormed Pharmaceuticals), T22 (Seikagaku Pharmaceuticals), and ALX40C-4C
(Allelix Pharmaceuticals), and the natural CCR5 ligands (e.g. MIP-1 α , MIL-1 β , and
5 RANTES), and analogs thereof (e.g., TAK-779 and AOP-RANTES). See, e.g., Eron,
PRN Notebook, 5(3):10-14 (2000).

Inhibitory antibodies targeting the various proteins, e.g., antibodies that
specifically bind gp120, gp41, CD4, CCR5, or CXCR4, can also be used, e.g., TNX-355
(Tanox, Inc., targets the CD4 protein).

10 Dosages, specific formulations, and routes of administration of HIV antiviral
drugs are known in the art. See, e.g., *Physicians' Desk Reference*, 63rd edition (Medical
Economics Company, Montvale, NJ, 2009); Panel on Antiretroviral Guidelines for Adult
and Adolescents, "Guidelines for the use of antiretroviral agents in HIV-1-infected adults
and adolescents." Department of Health and Human Services. November 3, 2008; pp 1-
15 139 (available at aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf); and
Kuritzkes et al. (1999, *AIDS* 13:685-694).

EXAMPLES

The invention will be further described in the following example, which does
not limit the scope of the invention described in the claims.

20 Example 1

Blood samples were obtained using standard techniques from 20 HIV-1-
infected individuals who began and continued to receive combination anti-HIV drug
therapy. All of these patients exhibited a period of time in which, after commencement of
combination therapy, no plasma viral RNA could be detected by quantitative PCR.

25 PBMC were isolated from each blood sample, and the extrachromosomal DNA was
purified using the Spin Miniprep Kit available from Qiagen as Cat. No. 27104, generally
following the manufacturer's directions.

HIV-1 2-LTR circles were detected by quantitative PCR using the 2-LTR-
specific primers described in Stevenson et al., *J. Virol.*, 64:2421-2425 (1990). The (-)
30 strand primer spanned nucleotides 9591 to 9610 (or 507-526) of the HXB2 strain of
HIV-1, while the (+) strand primer spanned nucleotides 9650-9669 (or 566-585) of the
HXB2 strain of HIV-1 (Ratner et al., *Nature*, 313:277-284, 1985). Plasma viral RNA in
each sample was also measured using the Amplicor HIV Monitor $\square$ kit (Roche Molecular

Systems, Inc., Branchburg, NJ), employing HIV-1-specific quantitative PCR, following manufacturer's directions. The threshold of detection for this standard HIV-1 RNA detection assay was about 50 viral RNA molecules per milliliter of plasma. On the other hand, the threshold for the method of the invention at which the number of 2-LTR circles was conservatively estimated to give a positive result was set at 1 molecule or circle per million PBMC (roughly about 0.1 to 1 ml whole blood). Higher thresholds could be set, but such thresholds may lead to more false negatives. Considering the consequences of false negatives, the lowest practical threshold should be used. The results are summarized in Table 1.

Table 1

Patient	# Viral RNA/ ml Plasma	# 2-LTR Circles/ 10 ⁶ cells	Months without Detectable Viral RNA
1	<50	20	N/A
2	155	25	8
3	<50	47	12
4	<50	872	9
5	N/A	13	N/A
6	<50	<1	9
7	<50	6200	7
8	<50	<1	17
9	N/A	1	N/A
10	<50	1	N/A
11	<50	240	19
12	121	36	19
13	N/A	3	15
14	<50	48	10
15	<50	<1	24
16	<50	1	9
17	<50	3	15
18	<50	<1	16
19	<50	271	15
20	69	117	4

Table 1 illustrates the unexpectedly superior sensitivity of 2-LTR circle detection as compared to the standard plasma viral load assay. Some HIV-positive individuals, who do not have detectable plasma virus, nevertheless harbor newly HIV-infected blood cells, as indicated by the presence of 2-LTR circles (i.e., patients 1-5, 7, 9-14, 16, 17, 19, and 20). These individuals should not cease antiviral therapy, since infectious virus is still present in the body.

On the other hand, in some patients with undetectable plasma virus, no 2-LTR circles were detected in their PBMC (i.e., patients 6, 8, 15, and 18). These individuals may have completely eradicated HIV from their bodies, and are candidates for removal from antiviral therapy.

Example 2

The stability of 2-LTR circle forms of viral DNA were initially examined in acutely infected cells *in vitro*. CD4⁺ MT-4 T cells and Jurkat-CCR5 cells were infected with the X4 variant HIV-1_{LAI} and the R5 variant HIV-1_{ADA}, respectively. Synthesis of viral cDNA was allowed to proceed for 24 hours, and further rounds of virus infection and cDNA synthesis were then restricted by the addition of reverse transcriptase inhibitors ZDV (5 μ M) or Nevirapine (1 μ M) to HIV-1_{LAI} and HIV-1_{ADA} infected cells, respectively. Cells were then maintained in the presence of the RT inhibitors.

The experimental procedures used in Examples 2-4 are briefly described.

The relationship between 2-LTR circle frequency and either the duration of undetectable plasma viral RNA or the frequency of positive virus co-cultures was examined using Spearman's correlation coefficient. Mean frequency of positive co-cultures in 2-LTR circle positive individuals and 2-LTR circle negative individuals as shown in Fig. 3 was further compared by a paired t-test.

Ficoll-purified PBMC ($2-40 \times 10^6$) were collected by centrifugation at $1300 \times g$ for 2 minutes. Cell pellets were resuspended in buffer P1 and extrachromosomal DNA was purified by a QIAprepTM spin miniprep kit (Qiagen, Valencia, CA) using the modification for the isolation of low copy number plasmids as recommended by the manufacturer. Chromosomal DNA was recovered from the sodium acetate -SDS precipitate using DNAzolTM reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Total cellular DNA was purified using an IsoquickTM nucleic acid extraction kit (ORCA Research, Bothell, WA).

2-LTR circle junctions were amplified from 10-30 μ l of extrachromosomal DNA in a 50 μ l reaction containing 1 x HotStarTaqTM buffer, 200 nM dNTPs, 400 nM primers, and 1.5 units HotStarTaqTM (Qiagen, Valencia, CA). The reverse primer was 5'-cagatctggcttaaccagaga-3' (SEQ ID NO:1), and the forward primer was 5'-gtaactagagatccctcagac-3' (SEQ ID NO:2), which annealed to nucleotides 9157-9137 (HIV-1 LTR R region) and nucleotides 130-150 (HIV-1 LTR U5 region) of HIV-1_{LAI}, respectively (see GenBank Accession No. K02013 for numbering). After an initial denaturation step (95°C, 10 minutes), PCR amplification proceeded for 45 cycles (95°C, 30 seconds; 60°C, 30 seconds; 72°C, 60 seconds) followed by a final extension (72°C, 5 minutes).

To control for the effect of sequence polymorphisms at primer binding sites, amplification was performed with internal primers which were reversed in orientation to those listed above. Amplification with the internal LTR primers proceeded for 35 cycles

using conditions outlined above. Polymorphisms in the region of the LTR that is recognized by the fluorogenic probe can affect annealing of the probe and potentially result in "false negatives." Consequently, Taqman reaction products were subsequently analyzed on agarose-TBE gels and stained with ethidium bromide to ensure that those reactions did not contain episome-specific PCR products. For quantitation of 2-LTR circle frequency in patient PBMC, PCR reactions were performed using an ABI prism 7700 sequence detection system with the addition of 200 nM fluorogenic probe (5'-agtggcgagccctcagatgctgc-3'; SEQ ID NO:3) to the reaction. The probe anneals to nucleotides 9081-9103 of HIV-1_{LAI} and was modified with 6-FAM (6-carboxyfluorescein) reporter dye on the 5' end and 6-TAMRA (6-carboxytetramethylrhodamine) quencher dye on the 3' end. Copy number estimates of 2-LTR circles were determined by extrapolation from a plot of standards versus band intensity or by using the ABI prism 7700 quantitation software. For sequencing, 2-LTR circle junctions were cloned into a TA cloning vector (Invitrogen, San Diego, CA) and analyzed on an ABI 377 DNA sequencer according to the manufacturer's protocol.

Patient PBMC were separated by Ficoll-Paque (Amersham-Pharmacia) and depleted of CD8⁺ T lymphocytes using antibody-coated beads (Dyna). Cells were seeded in flasks in aliquots of 1×10^7 cells in RPMI 1640 medium supplemented with 10% fetal calf serum and activated by PHA (5 µg/ml) for 12 hours. CD8⁺-depleted PBMC from HIV-1 seronegative individuals were activated for 12 hours with PHA and added in equal numbers to flasks of patient PBMC together with 20 IU/ml of interleukin-2 (Genzyme). At weekly intervals, half of the culture supernatant was replaced with fresh medium containing 20 IU/ml IL-2 and 10^7 freshly isolated, CD8⁺-depleted, PHA activated donor PBMC from HIV-1 seronegative individuals. HIV-1 Gag p24 antigen in culture supernatants was evaluated by ELISA (Beckman Coulter) after 4 weeks.

Within 24-48 hours following addition of the RT inhibitors, 2-LTR circle number fell by over ten fold in both HIV-1_{LAI} and HIV-1_{ADA} infected cells (Figs. 1A and 1B). The copy number of other viral DNA forms identified by the internal LTR primers (predominantly linear and integrated viral genomes) remained relatively constant over the same interval. Thus, 2-LTR circles appeared to be labile intermediates in the virus lifecycle.

Example 3

Whether 2-LTR circles were labile *in vivo* was next evaluated. PBMC samples were obtained from four HIV-1 infected individuals (Gu, Sm, Za, Ha) who, following adjustment of their antiretroviral regimens to more potent combinations, exhibited steady declines in plasma viral RNA levels. Patient Gu, who had been maintained on a two-drug RT inhibitor combination, was subsequently changed (week 0) to a three-drug regimen (ZDV/3TC/NFV). Patient Sm, who had been on a two-drug regimen (ZDV/3TC) was changed at week 68 to ddI/EFV/NFV. Patient Za, who had been on a four-drug regimen (3TC/D4T/ddI/NFV) was adjusted (week 1) to ZDV/ddC/NFV/RTV. Patient Ha, previously on a three-drug regimen (ZDV/ddI/NVP) was subsequently adjusted (week 0) to D4T/3TC/NVP. Marked declines in 2-LTR circle copy number were observed over the interval in which there was a rapid drop in levels of plasma viral RNA (Figs. 2A-2D). In contrast, when samples were analyzed in parallel with internal LTR primers, HIV-1 viral genome levels (detected via cDNA) fluctuated by no more than three fold (Figs. 2A-2C). Collectively, the results suggest that 2-LTR circles are labile, both *in vitro* and *in vivo*, relative to integrated viral genomes.

Example 4

A larger patient population than that of Example 1 was then examined. The 2-LTR HIV-1 episomes were examined in 63 patients (four of whom were included in the study described in Example 1) who, through treatment with high activity antiretroviral therapy (HAART), had undetectable levels of plasma viral RNA for sustained periods of time (Table 2). Fifty of these patients (80%) had undetectable levels of plasma viral RNA (assay limit of sensitivity was 400 copies/ml) for 12 months or longer (Table 2). Of these 50 patients, 24 (48%) exhibited undetectable levels of plasma viral RNA for 12 months or more using an assay with a sensitivity of 50 copies/ml. In 48 of the 63 patients (76%), 2-LTR circles were detected in their PBMC (Table 2). 2-LTR circle copy numbers ranged from less than 1 copy/ 10^6 PBMC to 620 copies/ 10^6 PBMC. There did not appear to be any significant relationship between the frequency of 2-LTR circles in patient PBMC and the time during which plasma viral RNA was undetectable. This data indicated that labile replication intermediates are present in a substantial proportion of HIV-1 infected individuals who exhibit sustained suppression of plasma viral RNA while on HAART. 2-LTR circles were not detectable in PBMC from 15 (24%) patients (Table 2).

Table 2 below lists AIDS patients on HAART and the level of 2-LTR circles and viral RNA in the blood. The abbreviations for Table 2 are as follows. Anti-retroviral

therapy: ZDV, Zidovudine; 3TC, Lamivudine; D4T, Stavudine; ddl, Didanosine; NVP, Nevirapine; RTV, Ritonavir; EFV, Efavirenz; SQV, Saquinavir; IDV, Indinavir; NFV, Nelfinavir; ddC, Zalcitabine; and ABV, Abacavir. CD4⁺ T cell measurements were determined at or just prior to time PBMC were collected for PCR analysis of viral cDNA intermediates.

For the column labeled "Period of Undetectable Viral RNA," plasma viral RNA was detected using an assay with a sensitivity of about 400 copies/ml. Numbers in parentheses indicated the period for which viral RNA was below the level of detection using a second assay with a sensitivity of 50 copies/ml. Plasma viral RNA measurements were determined approximately every three months.

The 2-LTR circle copy number in most cases were determined in duplicate on independent PBMC samples. Values less than 1 indicated that more than 1 million PBMC were required for detection of 2-LTR circles.

The total number of PBMC from which extrachromosomal DNA was isolated and analyzed for the presence of 2-LTR circles was determined as follows. In all patients, 2-LTR circles were quantitated by fluorescence-based PCR using Taqman software (ABI Prism 7700 Software). Similar 2-LTR circle numbers were obtained when samples were quantitated by comparison of PCR band intensity to a standard dilution of synthetic 2-LTR circles.

Table 2

Patient Number	Drug Regimens	CD4 ⁺ T Cells (cells/ml)	Period of Undetectable Viral RNA (months)	2-LTR circles (Copies/10 ⁶ PBMC)	#PBMC Analyzed (millions)
W1	RTV,ZDV,3TC	475	23 (14)	3	1.0
W2	NFV,ZDV,3TC	827	13 (13)	<1	5.5
W3	IDV,D4T,3TC	436	23 (14)	27	1.0
W4	IDV,D4T,3TC	505	22 (12)	37	1.0
W6	IDV,D4T,3TC	248	19 (11)	15	1.0
W7	SQV,D4T,3TC	443	19 (13)	8	1.0
W8	ddl,D4T	870	18 (15)	<1	4.0
W9	NFV,D4T,3TC	641	22 (11)	59	1.0
W10	IDV,ZDV,3TC	656	22 (15)	<1	4.0
W11	IDV,ZDV,3TC	344	22 (15)	65	1.0
W12	ZDV,3TC,DLV	626	26 (16)	<1	5.5
W13	NFV,ZDV,3TC	699	13 (13)	<1	5.5
W14	NFV,D4T,3TC	685	21 (15)	47	1.0
W15	NFV,3TC,NVP	866	25 (12)	17	1.0
W16	RTV,D4T,3TC	572	22 (14)	2	5.5
W17	IDV,ZDV,3TC	364	26 (15)	31	1.0
W18	IDV,ZDV,3TC	119	21 (16)	<1	2.0
W19	SQV,ZDV,3TC	153	16 (10)	4	4.0
W20	IDV,ZDV,3TC	360	27 (15)	<1	4.0
W21	NFV,D4T,3TC	208	13 (13)	<1	2.0
W22	D4T,3TC	495	23 (15)	<1	4.0

Patient Number	Drug Regimens	CD4 ⁺ T Cells (cells/ml)	Period of Undetectable Viral RNA (months)	2-LTR circles (Copies/10 ⁶ PBMC)	#PBMC Analyzed (millions)
W28	NFV,ddI,D4T	527	22 (8)	9	1.0
W30	D4T,3TC	575	22 (17)	<1	4.0
M1	NFV,D4T,NVP	287	14 (9)	31	1.0
M3	IDV,ddI,NVP	440	16 (7)	22	1.0
M4	IDV,ZDV,3TC	586	13 (ND)	264	1.0
M6	NFV,ZDV,3TC	317	24 (7)	63	1.0
M7	NFV,3TC,NVP	175	11 (ND)	4	5.5
M8	IDV,ZDV,3TC,NVP	357	13 (2)	15	1.0
M12	NFV,D4T,3TC	749	12 (7)	35	1.0
M13	ZDV,3TC,EFV	670	10 (0)	67	1.0
M14	IDV,ZDV,3TC	728	14 (14)	41	1.0
M15	IDV,ZDV,3TC	565	10 (10)	82	1.0
M16	NFV,3TC,NVP	403	12 (8)	3	4.0
L2	3TC,D4T,RTV	852	8 (8)	5	1.0
L3	ZDV,3TC,IDV	448	12 (8)	10	1.0
L4	ZDV,3TC,RTV	978	21 (12)	180	1.0
L6	D4T,RTV,SQV	577	10 (7)	<1	4.0
L7	D4T,ddI,NVP	394	11 (7)	610	1.0
L8	ZDV,3TC,NFV	173	17 (8)	<1	1.0
L9	3TC,D4T,EFV	482	8 (5)	<1	2.2
L11	ZDV,3TC,RTV	615	19 (12)	84	1.0
L12	3TC,D4T,RTV	389	19 (6)	7	1.0
L13	D4T,SQV,NFV	312	15 (3)	<1	7.8
L14	3TC,D4T,IDV	375	14 (7)	116	1.0
L15	3TC,RTV,SQV,ABV	91	30 (17)	<1	1.5
L16	3TC,D4T,SQV,RTV	575	12 (12)	4	8.1
L17	3TC,D4T,SQV	198	15 (15)	14	1.0
L18	ZDV,3TC,IDV	175	16 (13)	<1	10.2
L19	3TC,D4T,RTV,SQV	499	15 (6)	620	1.0
L22	ZDV,D4T,IDV	223	14 (12)	6	1.0
L23	3TC,ddC,IDV	534	14 (12)	<1	4.8
L26	3TC,D4T,SQV,NFV	911	17 (6)	36	1.0
L27	ZDV,3TC,IDV	185	17 (17)	<1	3.2
L28	D4T,ABV,EFV	80	8 (8)	275	1.0
L29	ZDV,ddC,SQV,NFV	121	21 (1)	3	2.0
L32	3TC,D4T,EFV	219	7 (1)	<1	10.0
L33	3TC,D4T,IDV	610	16 (1)	<1	14.4
L36	ddI,D4T,NFV	172	14 (4)	2	5.6
L37	ZDV,ddC,3TC,IDV	279	13 (7)	<1	5.6
L41	ZDV,3TC,RTV	990	22 (1)	100	1.0
L42	3TC,D4T,SQV	117	18 (1)	<1	2.0
L46	3TC,D4T,NFV	180	7 (1)	4	20.0

It was suspected that in 2-LTR circle positive patients, there would also be cells harboring replication competent virus. To investigate this, high-input viral co-culture assays were performed on PBMC from nine 2-LTR circle positive and four 2-LTR circle negative patients. The results are shown in Fig. 3. Replication competent virus could readily be isolated from eight of the nine patients who were 2-LTR circle positive. Virus could not be isolated from patient W1 who had a very low circle copy number. Intriguingly, infectious virus could not be isolated from three patients who were 2-LTR

circle negative even though co-culture was conducted on between 40 and 60 million CD8+-depleted patient PBMC. In patient L8 who was also 2-LTR circle negative, only one of three cultures yielded infectious virus (Fig. 3). Collectively, these results suggested a correlation between the presence of 2-LTR circles and cells harboring replication competent virus. Plasma based viral RNA assays therefore, unlike the 2-LTR circle assay, failed to reveal the full extent of viral activity in infected individuals who are being treated with HAART.

This study has important implications for the development of strategies to eradicate virus replication in HIV-1 infected individuals. Although complete elimination of HIV-1 replication may be difficult with current antiretroviral regimens, this study suggests instances in which even the most sensitive assays fail to reveal ongoing replication in some well suppressed patients. It is also likely that, as more potent antiretrovirals enter the clinic, ongoing or "covert" virus replication may be arrested in a higher percentage of patients. A better understanding of the nature of the reservoir which sustains virus replication in aviremic patients on HAART may lead to the development of more effective strategies for arrest of virus replication. Monitoring of the 2-LTR, as a superior surrogate marker for viral replication, can be integral to the understanding of viral reservoirs.

Example 5

2-LTR circle DNA is obtained from a subject who is well-suppressed on HAART, and the sequence of the viral DNA encoding the V3 loop of the viral envelope glycoprotein, which is contained within 2LTR amplicons generated with specific primers, is determined. Tropism is predicted based on the deduced sequence of amino acids within the V3 loop of the viral envelope glycoprotein. The overall charge of amino acids within the V3 loop strongly predicts the X4 versus R5 tropism of the envelope. A computer algorithm is used to predict tropism from the net charge of amino acids within the V3 loop. This is then optionally corroborated by inserting amplified V3 envelope sequences into an infectious molecular clone of HIV-1. Tropism of this recombinant virus is then assessed on cell lines that express either the CXCR4 co-receptor or the CCR5 co-receptor.

Based on the tropism determined by this method, an appropriate treatment is selected.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the
5 claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of determining co-receptor tropism of replication competent virus in an HIV-positive subject who has less than 50 cell-free viral RNA molecules/ml of serum, the method comprising:

- providing a sample comprising a cell from the subject;
- detecting an HIV 2-LTR circle DNA molecule in the sample; and
- determining the co-receptor tropism of the 2-LTR circle DNA;

wherein the co-receptor tropism of the 2-LTR circle DNA indicates the co-receptor tropism of the replication competent virus in the subject.

2. The method of claim 1, wherein determining the co-receptor tropism of the 2-LTR circle comprises determining the genotype of the DNA.

3. The method of claim 2, wherein the DNA molecule is amplified before determining the genotype of the DNA using polymerase chain reaction (PCR) with primers specific for an envelope protein.

4. The method of claim 3, wherein the envelope protein is gp120 or gp41.

5. The method of claim 1, wherein determining the co-receptor tropism of the 2-LTR circle comprises performing a phenotypic tropism assay.

6. The method of claim 1, wherein the subject is being treated with highly active antiretroviral therapy (HAART).

7. The method of claim 1, wherein the subject is a human.

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

9. The method of claim 1, wherein cell-free HIV viral RNA cannot be detected in the blood of the mammal.

10. The method of claim 1, wherein the co-receptor tropism of the 2-LTR circle DNA indicates that the replication competent virus is primarily M-tropic.

11. The method of claim 1, wherein the co-receptor tropism of the 2-LTR circle DNA indicates that the replication competent virus is primarily T-tropic.

12. The method of claim 1, wherein the co-receptor tropism of the 2-LTR circle DNA indicates that the replication competent virus is primarily dual-tropic.
13. The method of claim 1, further comprising selecting an entry inhibitor based on the co-receptor tropism of the replication competent virus.
14. The method of claim 13, comprising selecting a CCR5-specific entry inhibitor based on the presence of co-receptor tropism for CCR5 or of dual tropism.
15. The method of claim 13, comprising selecting a CXCR4-specific entry inhibitor based on the presence of co-receptor tropism for CXCR4.
16. A method of treating an HIV-infected subject who has less than 50 cell-free viral RNA molecules/ml of serum, the method comprising:
 - providing a sample comprising a cell from the subject;
 - detecting an HIV 2-LTR circle DNA molecule in the sample;
 - determining the co-receptor tropism of the 2-LTR circle DNA;
 - determining co-receptor tropism of replication-competent virus in the subject based on the co-receptor tropism of the 2-LTR circle DNA;
 - selecting an entry inhibitor suitable for the HIV co-receptor tropism of the virus in the subject; and
 - administering an effective amount of the selected entry inhibitor to the subject, thereby treating the subject.
17. The method of claim 16, comprising selecting a CCR5-specific entry inhibitor for a subject in whom the co-receptor tropism is for a receptor other than CXCR4.
18. The method of claim 16, comprising selecting a CXCR4-specific entry inhibitor for a subject in whom the co-receptor tropism is for CXCR4.
19. The method of any of claims 1-18, further comprising confirming that the subject has less than about 50 cell-free viral RNA molecules/ml of serum.

FIG. 1A

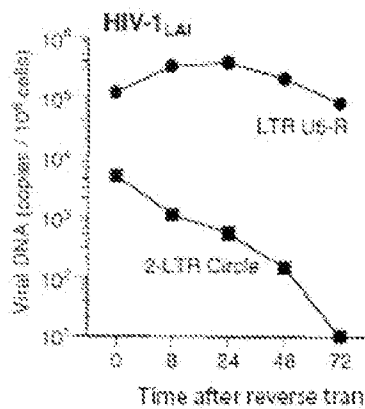


FIG. 1B

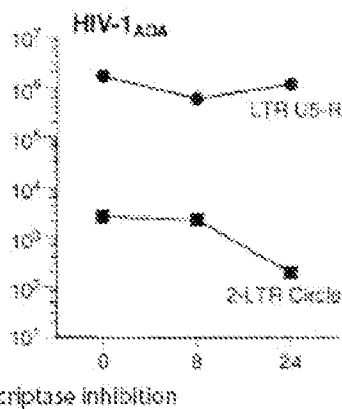


FIG. 2A

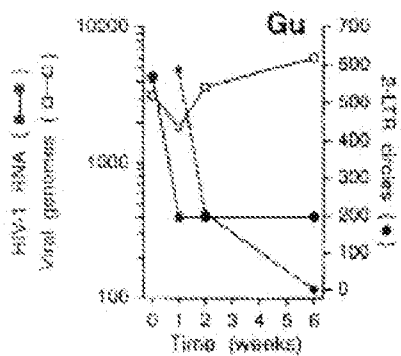


FIG. 2B

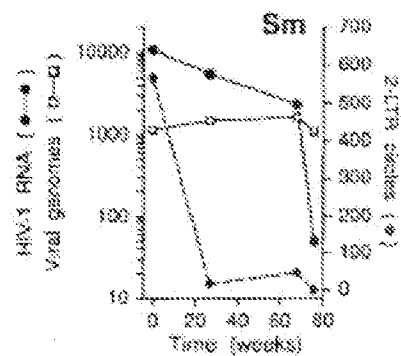


FIG. 2C

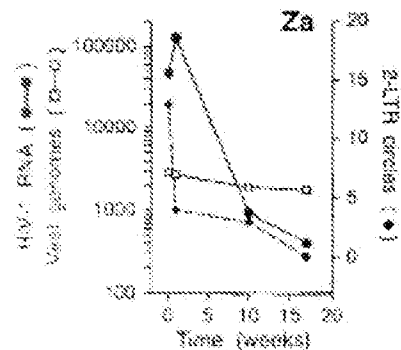


FIG. 2D

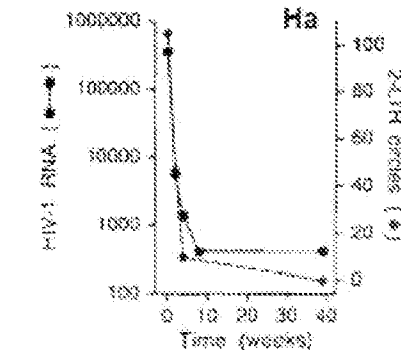


FIG. 3

