INHIBITION OF HIV-1 REPLICATION BY DISRUPTION OF THE PROCESSING OF THE VIRAL CAPSID-SPACER PEPTIDE 1 PROTEIN

Statement Regarding Federally-Sponsored Research and Development

[0001] The U.S. Government has a paid-up license in this invention and may have the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. 2R44AI051047-02 awarded by NIH/NIAID.

Background of the Invention

Field of the Invention

[0002] The invention includes methods of inhibiting, inhibitors and methods of discovery of inhibitors of HIV infection.

Background

[0003] Human Immunodeficiency Virus (HIV) is a member of the lentiviruses, a subfamily of retroviruses. The viral genome contains many regulatory elements which allow the virus to control its rate of replication in both resting and dividing cells. Most importantly, HIV infects and invades cells of the immune system; it breaks down the body's immune system and renders the patient susceptible to opportunistic infections and neoplasms. The immune defect appears to be progressive and irreversible, with a high mortality rate that approaches 100% over several years.

[0004] HIV-1 is trophic and cytopathic for T4 lymphocytes, cells of the immune system which express the cell surface differentiation antigen CD4, also known as OKT4, T4 and leu3. The viral tropism is due to the interactions between the viral envelope glycoprotein, gpl20, and the cell-surface CD4 molecules (Dalgleish *et al., Nature 312*:763-767 (1984)). These interactions not only mediate the infection of susceptible cells by HIV, but are also

responsible for the virus-induced fusion of infected and uninfected T cells. This cell fusion results in the formation of giant multinucleated syncytia, cell death, and progressive depletion of CD4 cells in HIV-infected patients. These events result in HIV-induced immunosuppression and its subsequent sequelae, opportunistic infections and neoplasms.

[0005] In addition to $CD4^+$ T cells, the host range of HIV includes cells of the mononuclear phagocytic lineage (Dalgleish *et al., supra*), including blood monocytes, tissue macrophages, Langerhans cells of the skin and dendritic reticulum cells within lymph nodes. HIV is also neurotropic, capable of infecting monocytes and macrophages in the central nervous system causing severe neurologic damage. Macrophage and monocytes are major reservoirs of HIV. They can interact and fuse with CD4-bearing T cells, causing T cell depletion and thus contributing to the pathogenesis of AIDS.

[0006] Considerable progress has been made in the development of drugs for HIV-1 therapy. Therapeutic agents for HIV can include, but not are not limited to, at least one of AZT, 3TC, ddC, d4T, ddI, tenofovir, abacavir, nevirapine, delavirdine, emtricitabine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, lopinavir, amprenavir, atazanavir and fosamprenavir, or any other antiretroviral drugs or antibodies in combination with each other, or associated with a biologically based therapeutic, such as, for example, gp41-derived peptides enfuvirtide (Fuzeon; Timeris-Roche) and T-1249 (Trimeris), or soluble CD4, antibodies to CD4, and conjugates of CD4 or anti-CD4, or as additionally presented herein. Combinations of these drugs are particularly effective and can reduce levels of viral RNA to undetectable levels in the plasma and slow the development of viral resistance, with resulting improvements in patient health and life span.

[0007] Despite these advances, there are still problems with the currently available drug regimens. Many of the drugs exhibit severe toxicities, have other side-effects (e.g., fat redistribution) or require complicated dosing schedules that reduce compliance and thereby limit efficacy. Resistant strains of HIV often appear over extended periods of time even on combination therapy. The high cost of these drugs is also a limitation to their widespread use, especially outside of developed countries.

[0008] There is still a major need for the development of additional drugs to circumvent these issues. Ideally these would target different stages in the viral life cycle, adding to the armamentarium for combination therapy, and exhibit minimal toxicity, yet have lower manufacturing costs.

[0009] HIV virion assembly takes place at the surface membrane of the infected cell where the viral Gag polyprotein accumulates, leading to the assembly of immature virions that bud from the cell surface. Within the virion, Gag is cleaved by the viral proteinase (PR) into the matrix (MA), capsid (CA), nucleocapsid (NC), and C-terminal p6 structural proteins (Wiegers K. *et al., J. Virol. 72*:2846-2854 (1998)). Gag processing induces a reorganization of the internal virion structure, a process termed "maturation." In mature HIV particles, MA lines the inner surface of the membrane, while CA forms the conical core which encases the genomic RNA that is complexed with NC. Cleavage and maturation are not required for particle formation but are essential for infectivity (Kohl, N. *et al., Proc. Natl. Acad. Sci. USA 85*:4686-4690, (1998)).

[0010] CA and NC as well as NC and p6 are separated on the Gag polyprotein by short spacer peptides of 14 and 10 amino acids (p2), respectively (spacer peptide 1 (SP1) and SP2, respectively) (Wiegers K. *et al., J. Virol. 72*:2846-2854 (1998), Pettit, S.C. *et al., J. Virol. 68*:8017-8027 (1994), Liang et al. *J. Virol.* 76:11729-11737 (2002)). These spacer peptides are released by PR-mediated cleavages at their N and C termini during particle maturation. The individual cleavage sites on the HIV Gag and Gag-Pol polyproteins are processed at different rates and this sequential processing results in Gag intermediates appearing transiently before the final products. Such intermediates may be important for virion morphogenesis or maturation but do not contribute to the structure of the mature viral particle (Weigers *et al.* and Pettit, *et al., supra*). The initial Gag cleavage event occurs at the C terminus of SP1 and separates an N-terminal MA-CA-SP1 intermediate from a C-terminal NC-SP2-p6 intermediate. Subsequent cleavages separating MA from CA-SP1 and NC-SP2 from p6 occur at an approximately 10-fold-lower rate. Cleavage of SP1 from the C terminus of CA is a late event and occurs at a 400-fold-lower rate than cleavage at the SP1-NC site (Weigers *et al.* and Pettit,

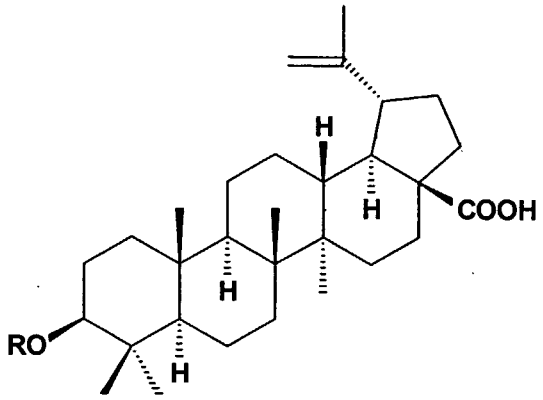
et al., supra). The uncleaved CA-SP1 intermediate protein is alternatively termed "p25," whereas the cleaved CA protein is alternatively termed "p24" and the cleaved SP1 peptide is alternatively termed "p2".

[0011] Cleavage of SP1 from the C terminus of CA appears to be one of the last events in the Gag processing cascade and is required for final capsid condensation and formation of mature, infectious viral particles. Electron micrographs of mature virions reveal particles having electron dense conical cores. On the other hand, electron microscopy studies of viral particles defective for CA-SP1 cleavage show particles having a spherical electron-dense ribonucleoprotein core and a crescent-shaped, electron-dense layer located just inside the viral membrane (Weigers *et al., supra*). Mutations at or near the CA-SP1 cleavage site have been shown to inhibit Gag processing and disrupt the normal maturation process, thereby resulting in the production of non-infectious viral particles (Weigers *et al., supra*). Phenotypically, these particles exhibit a defect in Gag processing (which manifests itself in the presence of a p25 (CA-SP1) band in Western blot analysis) and the aberrant particle morphology described above which results from defective capsid condensation.

[0012] Previously, betulinic acid and platanic acid were isolated from *Syzigium claviflorum* and were determined to have anti-HIV activity. Betulinic acid and platanic acid exhibited inhibitory activity against HIV-1 replication in H9 lymphocyte cells with EC_{50} values of 1.4 μM and 6.5 μM, respectively, and therapeutic index (T.I.) values of 9.3 and 14, respectively. Hydrogenation of betulinic acid yielded dihydrobetulinic acid, which showed slightly more potent anti- HIV activity with an EC_{50} value of 0.9 and a T.I. value of 14 (Fujioka, T., *et al., J. Nat. Prod. 57*:243-247 (1994)). Esterification of betulinic acid with certain substituted acyl groups, such as 3',3'-dimethylglutaryl and 3',3'-dimethylsuccinyl groups produced derivatives having enhanced activity (Kashiwada, Y., *et al., J. Med. Chem. 39*:1016-1017 (1996)). Acylated betulinic acid and dihydrobetulinic acid derivatives that are potent anti-HIV agents are also described in U.S. Patent No. 5,679,828. Anti-HIV assays indicated that 3-*O*-(3',3'-dimethylsuccinyl)-betulinic acid (DSB) and the dihydrobetulinic acid analog both demonstrated extremely potent anti-

HIV activity in acutely infected H9 lymphocytes with EC_{50} values of less than 1.7×10^{-5} μM, respectively. These compounds exhibited remarkable T.I. values of more than 970,000 and more than 400,000, respectively.

[0013] U.S. Patent No. 5,468,888 discloses 28-amido derivatives of lupanes that are described as having a cytoprotecting effect for HIV-infected cells.

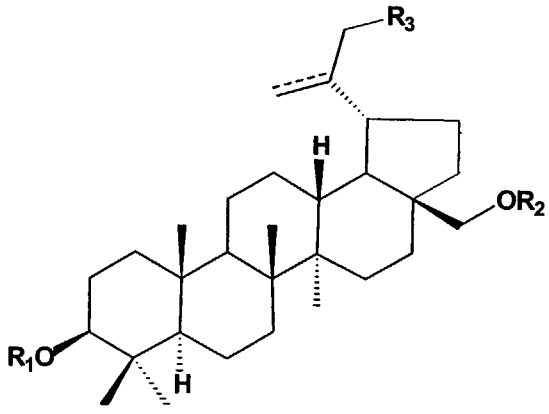


R = H (Betulinic acid)

[0015] Japanese Patent Application No. JP 01 143,832 discloses that betulin and 3,28-diesters thereof are useful in the anti-cancer field.

[0016] U.S. Patent No. 6,172,110 discloses betulinic acid and dihydrobetulin derivatives which have the following formulae or pharmaceutically acceptable salts thereof,

Betulin and Dihydrobetulin Derivatives



wherein R_1 is a C_2-C_{20} substituted or unsubstituted carboxyacyl, R_2 is a C_2-C_{20} substituted or unsubstituted carboxyacyl; and R_3 is hydrogen, halogen, amino, optionally substituted mono- or di-alkylamino, or --OR_4, where R_4 is hydrogen, C_{1-4} alkanoyl, benzoyl, or C_2-C_{20} substituted or unsubstituted

carboxyacyl; wherein the dashed line represents an optional double bond between C20 and C29.

[0017] U.S. Patent Application No. 60/413,451 discloses 3,3-dimethylsuccinyl betulin and is herein incorporated by reference. Zhu, Y-M. *et al., Bioorg. Chem Lett. 11*:3115-3118 (2001); Kashiwada Y. *et al., J. Nat. Prod. 61*:1090-1095 (1998); Kashiwada Y. *et al., J. Nat. Prod. 63*:1619-1622 (2000); and Kashiwada Y. *et al., Chem. Pharm. Bull. 48*:1387-1390 (2000) disclose dimethylsuccinyl betulinic acid and dimethylsuccinyl oleanolic acid. Esterification of the 3' carbon of betulin with succinic acid produced a compound capable of inhibiting HIV-1 activity (Pokrovskii, A.G. *et al., Gos. Nauchnyi Tsentr Virusol. Biotekhnol. "Vector," 9*:485-491 (2001)).

[0018] Published International Application No. WO 02/26761 discloses the use of betulin and analogs thereof for treating fungal infections.

[0019] There exists a need for new HIV inhibition methods that are effective against drug resistant strains of the virus. The strategy of this invention is to provide therapeutic methods and compounds that inhibit the virus in different ways from approved therapies.

[0020] The compound and methods of the present invention have a novel mechanism of action and therefore are active against HIV strains that are resistant to current reverse transcriptase and protease inhibitors. As such, this invention offers a completely new approach for treating HIV/AIDS.

Brief Summary of the Invention

[0021] Generally, the invention provides methods of inhibiting, inhibitory compounds and methods of identifying inhibitory compounds that target proteolytic processing of the HIV-1 Gag protein. In one embodiment, such compounds may directly or indirectly inhibit the interaction of a protease enzyme with HIV-1 Gag protein. In another embodiment, such inhibition of interaction occurs via the binding of a compound to Gag. The inhibition of protease cleavage of the CA-SP1 protein of HIV-1 Gag by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB) is one example, but other proteolytic cleavage sites can be targeted by a similar approach using inhibitory

compounds that interact with the substrate in a manner similar to that in which DSB interacts with Gag.

[0022] Another aspect of the invention is directed to a method of inhibiting the processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but having no effect on other Gag processing steps.

[0023] A further aspect of the invention is directed to a method for identifying compounds that inhibit processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but have no effect on other Gag processing steps.

[0024] In one aspect, the invention is drawn to a compound or pharmaceutical composition identified by the method for identifying compounds that inhibit HIV-1 replication disclosed herein.

[0025] In another aspect, the present invention is directed to a polynucleotide comprising a sequence which encodes an amino acid sequence containing a mutation in the Gag p25 protein, said mutation resulting in a decrease in the inhibition of processing of p25 to p24 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. This aspect of the invention is also directed to a vector, virus and host cell comprising said polynucleotide, and a method of making said protein.

[0026] A further aspect of the present invention is directed to an amino acid sequence containing a mutation in the Gag p25 protein, said mutation resulting in a decrease in the inhibition of processing of p25 to p24 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid.

[0027] An additional aspect of the invention is directed to an antibody which selectively binds an amino acid sequence containing a mutation in the Gag p25 protein, said mutation resulting in a decrease in the inhibition of processing of p25 to p24 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. Also included in this aspect of the invention are a method of making said antibody, a hybridoma producing said antibody and a method of making said hybridoma.

[0028] In a further embodiment, the invention is directed to a kit comprising a polynucleotide, polypeptide or antibody disclosed herein.

[0029] The invention further relates to a method of inhibiting HIV-1 infection in cells of an animal by contacting said cells with a compound that blocks the maturation of virus particles released from treated infected cells. In one

embodiment, the released virus particles exhibit non-condensed cores and a distinctive thin electron-dense layer near the viral membrane and have reduced infectivity. A method is included of contacting animal cells with a compound that both inhibits processing of the viral Gag p25 protein and that disrupts the maturation of virus particles. Also, included is a method of treating HIV-infected cells, wherein the HIV infecting said cells does not respond to other HIV therapies.

[0030] This invention further includes a method for identifying compounds that inhibit processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but have no significant effect on other Gag processing steps. The method involves contacting HIV-1 infected cells with a test compound, and thereafter analyzing virus particles that are released to detect the presence of p25. Methods to detect p25 include western blotting of viral proteins and detecting using an antibody to p25, gel electrophoresis, and imaging of metabolically labeled proteins. Methods to detect p25 also include immunoassays using an antibody to p25 or SP1 (p2) or to an epitope tag inserted into the SP1 sequence.

[0031] The invention is further directed to a method for identifying compounds involving contacting HIV-1 infected cells with a compound, and thereafter analyzing virus particles released by the contacted cells, by thin-sectioning and transmission electron microscopy, and determining whether viral particles with non-condensed cores and a distinctive thin electron-dense layer near the viral membrane are present.

[0032] The invention is also directed to compounds identified by the aforementioned screening methods. In additional embodiments, the invention is drawn to a method of treating HIV-1 infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps. In related embodiments, such inhibition may be accompanied by a different observable phenotypes. For example, inhibition may not necessarily significantly reduce the quantity of virions released from treated infected cells; and/or said inhibition may have little or no significant effect on the amount of RNA incorporation into the released virions; and/or said inhibition disrupts the maturation of virions released from infected cells treated with said compound.

In related embodiments, the virion structure may be affected, and a majority of virions released from treated infected cells exhibit spherical, electron-dense cores that are acentric with respect to the viral particle; and/or possess crescent-shaped electron-dense layers lying just inside the viral membrane; and/or and have reduced or no infectivity.

[0033] In additional embodiments, the invention is drawn to a method of treating HIV-1 infection in a patient by administering a compound that inhibits the interaction of HIV protease with CA-SP1, which results in the inhibition of the processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but has no significant effect on other Gag processing steps. Such inhibition may be direct or, alternatively, indirect; and/or may involve said compound binding to the viral Gag protein such that interaction of HIV protease with CA-SP1 is inhibited. The invention is also drawn to a method of treating HIV in a patient with a compound that binds at or near the site of cleavage of the viral Gag p25 protein (CA-SP1) to p24 (CA), thereby inhibiting the interaction of HIV protease with the CA-SP1 cleavage site and resulting in the inhibition of processing of p25 to p24.

[0034] In other embodiments, the invention is drawn to a method of treating HIV-1-infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide with an amino acid sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, or which is identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTAC QGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTAC QGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26);

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119).

[0035] In other embodiments, the invention is drawn to a method of treating HIV-1-infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide encoded by a polynucleotide sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, or which is identical to a polynucleotide selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

[0036] In another aspect, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) by administration of a compound. In related embodiments, such a compound binds to a polypeptide with an amino acid sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, or which is identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTAC QGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTAC QGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26);

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119).

[0037] In related embodiments; the invention is drawns to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) by administration of a compound wherein said compound binds to a polypeptide encoded by a polynucleotide sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, or which is identical to a polynucleotide selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

[0038] The invention may be useful in the treatment of HIV in patients who are not adequately treated by other HIV-1 therapies. Accordingly, the invention is also drawn to a method of treating a patient in need of therapy, wherein the HIV-1 infecting said cells does not respond to other HIV-1 therapies. In another embodiment, methods of the invention are practiced on a subject infected with an HIV that is resistant to a drug used to treat HIV infection. In one application, the HIV is resistant to a protease inhibitor, a polymerase inhibitor, a nucleoside analog, a vaccine, a binding inhibitor, an immunomodulator, or any other inhibitor. In another embodiment, methods of the invention are practiced on a subject infected with an HIV that is resistant to a drug used to treat HIV infection is selected from the group consisting of zidovudine, lamivudine, didanosine, zalcitabine, stavudine, abacavir, nevirapine, delavirdine, emtricitabine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, tenofovir, amprenavir, adefovir, atazanavir, fosamprenavir, hydroxyurea, AL-721, ampligen, butylated hydroxytoluene; polymannoacetate, castanospermine; contracan; creme pharmatex, CS-87, penciclovir, famciclovir, acyclovir, cytofovir, ganciclovir, dextran sulfate, D-penicillamine trisodium phosphonoformate, fusidic acid, HPA-23, eflornithine, nonoxynol, pentamidine isethionate, peptide T, phenytoin, isoniazid,. ribavirin, rifabutin, ansamycin, trimetrexate, SK-818, suramin, UA001, and combinations thereof.

[0039] Compounds of the invention are also useful as part of combination of therapies. Accordingly, in one aspect the invention is drawn to a method of treating HIV in a patient, wherein said patient is administered said compound in combination with at least one anti-viral agent. Anti-viral agents suitable include, but are not limited to: zidovudine, lamivudine, didanosine, zalcitabine, stavudine, abacavir, nevirapine, delavirdine, emtricitabine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, tenofovir, adefovir, atazanavir, fosamprenavir, hydroxyurea, AL-721, ampligen,

butylated hydroxytoluene; polymannoacetate, castanospermine; contracan; creme pharmatex, CS-87, penciclovir, famciclovir, acyclovir, cytofovir, ganciclovir, dextran sulfate, D-penicillamine trisodium phosphonoformate, fusidic acid, HPA-23, eflornithine, nonoxynol, pentamidine isethionate, peptide T, phenytoin, isoniazid, ribavirin, rifabutin, ansamycin, trimetrexate, SK-818, suramin, UA001, enfuvirtide, gp41-derived peptides, antibodies to CD4, soluble CD4, CD4-containing molecules, CD4-IgG2, and combinations thereof. In another embodiment, the patient is administered said compound in combination with an immunomodulating agent, anticancer agent, antibacterial agent, antifungal agent, or a combination thereof.

[0040] The invention is also directed to compounds. Such compounds are useful in a method of treating patients infected with HIV; in a method for inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), or in a method for treating human blood and human blood products. Such compounds useful in the present invention include, but are not limited to derivatives of dimethylsuccinyl betulinic acid or dimethylsuccinyl betulin, or is selected from the group consisting of 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, 3-*O*-(3',3'-dimethylsuccinyl) betulin, 3-*O*-(3',3'-dimethylglutaryl) betulin, 3-*O*-(3',3'-dimethylsuccinyl) dihydrobetulinic acid, 3-*O*-(3',3'-dimethylglutaryl) betulinic acid, (3',3'-dimethylglutaryl) dihydrobetulinic acid, 3-*O*-diglycolyl-betulinic acid, 3-*O*-diglycolyl-dihydrobetulinic acid and combinations thereof.

[0041] Compounds of the invention may be used alone, or administered with additional compounds, including zidovudine, lamivudine, didanosine, zalcitabine, stavudine, abacavir, nevirapine, delavirdine, emtricitabine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, tenofovir, adefovir, atazanavir, fosamprenavir, hydroxyurea, AL-721, ampligen, butylated hydroxytoluene; polymannoacetate, castanospermine; contracan; creme pharmatex, CS-87, penciclovir, famciclovir, acyclovir, cytofovir, ganciclovir, dextran sulfate, D-penicillamine trisodium phosphonoformate, fusidic acid, HPA-23, eflornithine, nonoxynol, pentamidine isethionate, peptide T, phenytoin, isoniazid, ribavirin, rifabutin, ansamycin, trimetrexate, SK-818, suramin, UA001, enfuvirtide, gp41-derived peptides, antibodies to

CD4, soluble CD4, CD4-containing molecules, CD4-IgG2, and combinations thereof; an antiviral, an immunomodulating agent, anti-cancer agent, antibacterial agent, an anti-fungal agent, or combinations thereof.

[0042] In further embodiments, the invention is directed to a method of treating human blood products comprising contacting said blood products with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA). In one aspect, said compound does not significantly affect other Gag processing steps. In related embodiments of this method, said inhibition does not significantly reduce the quantity of virions released from treated infected cell; and/or has little or no significant effect on the amount of RNA incorporation into the released virions; and/or inhibits the maturation of virions released from infected cells treated with said compound; and/or affects viral morphology. Such effects on viral morphology include, but are not limited to: the virions released from treated infected cells to exhibit spherical, electron-dense cores that are acentric with respect to the viral particle; and/or possess crescent-shaped electron-dense layers lying just inside the viral membrane; and/or and have reduced or no infectivity. In related embodiments, the method involves the administration of the compound which inhibits the interaction of HIV protease with CA-SP1, which results in the inhibition of the processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) but has no significant effect on other Gag processing steps. This may be via direct, or indirect inhibition of the interaction of HIV protease with CA-SP1; and/or may involve said compound binds to the viral Gag protein such that interaction of HIV protease with CA-SP1 is inhibited; and/or said compound binds at or near the site of cleavage of the viral Gag p25 protein (CA-SP1) to p24 (CA), thereby inhibiting the interaction of HIV protease with the CA-SP1 cleavage site and resulting in the inhibition of processing of p25 to p24.

[0043] In a further embodiment, the invention is drawn to a method of treating human blood products comprising contacting said blood products with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide with an amino acid sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99%

identity, or which is identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTAC QGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTAC QGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26);

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119).

[0044] In a related embodiment, the invention is drawn to a method of treating human blood products comprising contacting said blood products with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide encoded by a polynucleotide sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, or which is identical a polynucleotide selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

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(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

[0045] The invention also embodies methods for identifying compounds that inhibit HIV-1 replication. Accordingly, the invention also includes a method of identifying compounds that inhibit HIV-1 replication in cells of an animal, comprising: contacting a Gag protein comprising a CA-SP1 cleavage site with a test compound; adding a labeled substance that selectively binds near the CA-SP1 cleavage site; and measuring competition between the binding of the test compound and the labeled substance to the CA-SP1 cleavage site. In further embodiments of this method, the compounds inhibits the interaction of HIV-1 protease with a target site by binding to said target site.

[0046] These methods also include embodiments wherein the CA-SP1 cleavage site region is contained within a polypeptide fragment or recombinant peptide; and/or wherein the labeled substance is a labeled antibody specific for CA-SP1, and measuring the change in the amount of labeled antibody bound to the protein in the presence of test compound compared with a control. Labels include, but are not limited to, an enzyme, fluorescent substance, chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, electron dense substance, radioisotope and a combination thereof.

[0047] The method of identifying compounds that inhibit HIV-1 replication in cells of an animal also comprises, in one embodiment, measuring the change in the amount of labeled 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid bound to the protein in the presence of test compound, compared with a control, and wherein the labeled substance is 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid.

[0048] In an alternative embodiment, the invention comprises a method for identifying compounds that inhibit HIV-1 replication in the cells of an animal

which comprises: contacting a polypeptide comprising a CA-SP1 cleavage site, with a protease in the presence of a test compound. Preferably the protease is related to HIV-1 protease, or is HIV protease. In one embodiment, the method comprises ; contacting a polypeptide comprising a wild type CA-SP1 cleavage site, with a protease in the presence of a test compound and also contacting a polypeptide comprising a mutant CA-SP1 cleavage site or a protein comprising an alternative protease cleavage site with HIV-1 protease in the presence of the test compound, detecting the cleavage, and comparing the amount of cleavage of the native wild-type polypeptide to the amount of cleavage of the mutant polypeptide or to amount of cleavage of the protein comprising an alternative protease cleavage site. In a related aspect of this method, the wild-type CA-SP1 or mutant CA-SP1 or alternative protease cleavage site region is contained within a polypeptide fragment or recombinant peptide. In a further related aspect, the polypeptide is labeled with a fluorescent moiety and a fluorescence quenching moiety, each bound to opposite sides of the CA-SP1 cleavage site, and wherein said detecting comprises measuring the signal from the fluorescent moiety. In another related embodiment, the polypeptide is labeled with two fluorescent moieties, each bound to opposite sides of the CA-SP1 cleavage site, and wherein said detecting comprises measuring the transfer of fluorescent energy from one moiety to the other in the presence of the test compound. In a further embodiment, the effect of the test compound on cleavage of the polypeptide is detected by measuring the amount of a labeled antibody that is bound to SP1 or p24 (CA). In a related aspect, the labeled antibody that binds CA, or the antibody that binds SP1 is labeled with a molecule selected from the group consisting of enzyme, fluorescent substance, chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, electron dense substance, radioisotope, and combinations thereof.

[0049] The invention is also directed to a method for identifying compounds that inhibit HIV-1 replication in cells of an animal. In one embodiment, the method comprises: contacting a test compound with cells infected with wild-type virus isolates and with cells infected with virus isolates having significantly reduced sensitivity to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid;

and selecting test compounds that are more active against the wild-type virus isolate compared with virus isolates that have reduced sensitivity to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. In another embodiment, the method comprises contacting HIV-1 infected cells with a test compound; lysing the infected cells or the released viral particles to form a lysate, and analyzing the lysate to determine whether cleavage of the CA-SP1 protein has occurred. In this latter embodiment, said analyzing may comprise measuring the presence or absence of p25; and or performing a western blot of viral proteins and detecting p25 using an antibody to p25; and/or performing a gel electrophoresis of viral proteins and imaging of metabolically labeled proteins; and/or performing an immunoassay. Such an immunoassay may be performed by any methods known in the art, including, but not limited to:

(a) capturing p25 and p24 on a substrate using an antibody that selectively binds p24; and

(b) detecting the presence or absence of p25 on the substrate by using an antibody that selectively binds p25. The invention also includes such modifications of the above assay as would be obvious to one of ordinary skill in the art.

[0050] In a further embodiment, the method of identifying a compound according to the invention comprises the use of an epitope tag sequence inserted into SP1 and the selective detection of p25 is performed using an antibody to the epitope tag.

[0051] The invention is also directed to a method for identifying compounds that inhibit HIV-1 replication in the cells of an animal comprising: contacting HIV-1 infected cells with a test compound and thereafter analyzing the virus particles using transmission electron microscopy. Such analysis includes for example, looking for the presence of spherical cores that are acentric with respect to the viral particle; and/or having crescent-shaped, electron-dense layers lying just inside the viral membrane.

[0052] In additional aspects, the invention is drawn to an isolated polynucleotide comprising a sequence which encodes an amino acid sequence

containing a mutation in an HIV Gag p25 protein (CA SP1), said mutation resulting in a decrease in inhibition of processing of p25 (CA-SP1) to p24 (CA) by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB). This inhibition of processing of p25 may be due to a decrease in inhibition of the interaction of HIV-1 protease with Gag; and/or a decrease in the binding of 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid to Gag; and/or a decrease in the binding of DSB at or near the CA-SP1 cleavage site of Gag. Suitable polynucleotides also include those encoding a mutation at or near the CA-SP1 cleavage site or in the SP1 domain of CA-SP1; and/or those encoding a mutation at or near the amino acid sequence G/SHKARV/ILAEAMSQV (SEQ ID NO: 1); and/or those encoding the amino acid sequences GHKARVLVEAMSQV (SEQ ID NO: 2) or SHKARILAEVMSQV (SEQ ID NO: 3); and/or isolated polynucleotide which is selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9; and/or having at least about 95% identity to a polynucleotide selected from the group consisting of SEQ ID NO: 4, and SEQ ID NO: 6; and/or having at least about 80% identity to a polynucleotide selected from the group consisting of SEQ ID NO: 8 and SEQ ID NO: 9; and/or having at least about 95% identity to a polynucleotide selected from the group consisting of SEQ NO: 5 and SEQ ID NO: 7; and/or having at least about 80% identity to a polynucleotide of SEQ ID NO: 10. In additional embodiments, the polynucleotide having more than about 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99% identity or which is identical to the polynucleotide sequences listed above.

[0053] The invention is also drawn to vectors comprising such polynucleotides as described above; to a host cell comprising such a vector; and to a method of producing a polypeptide comprising incubating the host cell containing such a vector in a medium and recovering the polypeptide from said medium.

[0054] In one embodiment, the invention is directed to an antibody. Such an antibody may bind to a polypeptide with an amino acid sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, or which is identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTAC QGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTAC QGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26);

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119).

[0055] In a further related embodiment, the invention is drawn to an antibody which binds to a polypeptide encoded by a polynucleotide sequence having at least about 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% identity, or which is identical to a polynucleotide with a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(f) about nucleotides 1370-1413 of SEQ ID NO: 18;

(g) about nucleotides 1857-1899 of SEQ ID NO: 19

(h) about nucleotides 1372-1419 of SEQ ID NO: 18;

(i) about nucleotides 1858-1905 of SEQ ID NO: 19;

(j) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(k) about nucleotides 1858-1920 of SEQ ID NO: 19.

[0056] In one embodiment, the antibody binds to amino acids of the CA-SP1 region of the HIV-1 Gag polypeptide, wherein said amino acids comprise: SHKARILAEAMSQV (SEQ ID NO: 25) or GHKARVLAEAMSQV (SEQ ID NO: 26).

[0057] In one embodiment, the invention is drawn to an antibody that inhibits the binding of 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid to the CA-SP1 region of the Gag polypeptide.

[0058] The invention is also drawn to mutant HIV-1 viruses. In one such embodiment, the invention is an isolated mutant recombinant HIV-1 virus, wherein the processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in said virus is not significantly inhibited by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. In related embodiments, this virus is not inhibited by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. In another embodiment, 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid does not inhibit the interaction of protease with the Gag polypeptide in this virus. In another, the virus does not bind to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. In further embodiments the invention is drawn to viruses wherein the amino acids of the CA-SP1 region are replaced with alternative amino acids, or amino acids are added to the CA-SP1 region, or where amino acids are deleted. In one embodiment, ; one or more amino acids are deleted from the AEAMSQV (amino acid no. 8-14 of SEQ ID NO:26) amino acid sequence in the CA-SP1 region.

[0059] A mutant viruses may be used in the methods of the invention described elsewhere herein. For example, such viruses are useful in a method of identifying a compound which inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), the method comprising comparing the ability of said compound to inhibit HIV-1 replication compared with the replication of a the mutant virus outlined above. Such inhibition may be examined in a cell, or in an animal, or *in vitro*.

[0060] The invention is also drawn to non-HIV-1 retroviruses that are sensitive to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. In some embodiment, said retrovirus encodes a CA-SP1 polypeptide with an amino acid sequence comprising the sequence AEAMSQV (amino acid no. 8-14 of SEQ ID NO: 26) at or near the CA-SP1 cleavage site. In another embodiment, the retrovirus encodes a CA-SP1 polypeptide with an amino acid sequence comprising the sequence VLAEAMSQV (amino acid no. 6-14 of SEQ ID NO: 26) at or near the CA-SP1 cleavage site. In another embodiment, the retrovirus encodes a CA-SP1 polypeptide with an amino acid sequence comprising the sequence GHKARVLAEAMSQV (SEQ ID NO: 26) at or near the CA-SP1 cleavage site; in another the retrovirus comprises the amino acid sequence having at least 60%, 70%, 80%, 90% identity or which is identical to the sequence enocoded by the polynucleotide of SEQ ID NO:26, SEQ ID NO: 90; SEQ ID NO: 92; SEQ ID NO: 94; SEQ ID NO: 96; or SEQ ID NO: 98; in another embodiment the retrovirus comprises the amino acid sequence having at least 60%, 70%, 80%, 90% identity or which is identical to the sequence of SEQ ID NO: 91; SEQ ID NO: 93; SEQ ID NO: 95; SEQ ID NO: 97; or SEQ ID NO: 99. In another embodiment, the retrovirus comprises the nucleic acid sequence having at least 70%, 80%, 90% or which is identical to the sequence of SEQ ID NO: 90; SEQ ID NO: 92; SEQ ID NO: 94; SEQ ID NO: 96; or SEQ ID NO: 98.

[0061] Retroviruses of this embodiment of the invention include, but are not limited to HIV-2, HTLV-I, HTLV-II, SIV, avian leukosis virus (ALV), endogenous avian retrovirus (EAV), mouse mammary tumor virus (MMTV), feline immunodeficiency virus (FIV), Bovine immunodeficiency virus (BIV), caprine arthritis encephalitis virus (CAEV), Visna-maedi virus, or feline leukemia virus (FeLV).

[0062] In a related embodiment, the invention is drawn to a method of making a recombinant non-HIV-1 lentivirus sensitive to DSB. This method comprises: deleting from the genome of said lentivirus the nucleotides which correspond to nucleotides 1370-1413 from SEQ ID NO: 18, in HIV-1; and inserting nucleotides 1370-1413 from SEQ ID NO: 18 or nucleotides 1857-1899 of SEQ ID NO: 19 into said region of said non-HIV-1 lentivirus.

[0063] Examples of chimeric lentiviruses that were, are or may be constructed by this method are described in Figure 10.

[0064] Such viruses may be used in the methods of the invention described elsewhere herein. For example, such recombinant non-HIV-1 lentiviruses may be used in a method of identifying a compound which inhibit processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), the method consisting of comparing of the ability of said compound to inhibit replication of a wild-type non-HIV-1 lentivirus with the DSB-sensitive recombinant variant thereof . Such inhibition may occur in a cell; in an animal; or *in vitro*.

[0065] The invention is also drawn to an animal model of lentivirus infection comprising a suitable non-human animal host infected with a lentivirus sensitive to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. In such an embodiment, the lentivirus may include, but is not limited to SIV; FIV; EIAV; BIV; CAEV; and Visna-Maedi virus.

[0066] The invention is also drawn to isolated polypeptides. In one embodiment, the invention is drawn to a polypeptide containing a mutation in an HIV CA-SP1 protein, said mutation which results in a decrease in inhibition of processing of p25 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. In a related embodiment, this polypeptide is encoded by a polynucleotide that contains a mutation located at or near the CA-SP1 cleavage site or in the SP1 domain encoded by SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 10 and/or is encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9; and/or comprises a sequence that is selected from the group consisting of GHKARVLVEAMSQV (SEQ ID NO: 2) or SHKARILAEVMSQV (SEQ ID NO: 3); and/or is encoded by an isolated polynucleotide which hybridizes under stringent conditions to a polynucleotide selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, and 10; and/or is part of a chimeric or fusion protein.

[0067] The invention is also drawn to antibodies which selectively bind to an amino acid sequence containing a mutation in an HIV CA-SP1 protein which results in a decrease in the inhibition of processing of p25 (CA-SP1) to p24 (CA) by 3-*O*-(3'3'-dimethylsuccinyl) betulinic acid. In one such embodiment, the antibody selectively binds to a mutation located at or near the CA-SP1

cleavage site or in the SP1 domain of CA-SP1; in another, the antibody selectively binds to a mutation comprising a sequence that is selected from the group consisting of GHKARVLVEAMSQV (SEQ ID NO: 2) or SHKARILAEVMSQV (SEQ ID NO: 3); in another embodiment, the antibody selectively binds an amino acid sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 3.

[0068] In another embodiment, the invention is drawn to an antibody that selectively binds SP1 but not CA-SP1; another that selectively binds CA-SP1 but not CA; another that selectively binds CA but not CA-SP1; and a further antibody that selectively binds at or near the CA-SP1 cleavage site.

[0069] The invention is also directed to a compound identified by any of the methods elucidated herein. In one embodiment, the compounds is not a compound selected from the group consisting of 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, 3-*O*-(3',3'-dimethylsuccinyl) betulin, 3-*O*-(3',3'-dimethylglutaryl) betulin, 3-*O*-(3',3'-dimethylsuccinyl) dihydrobetulinic acid, 3-*O*-(3',3'-dimethylglutaryl) betulinic acid, (3',3'-dimethylglutaryl) dihydrobetulinic acid, 3-*O*-diglycolyl-betulinic acid, 3-*O*-diglycolyl-dihydrobetulinic acid, and combinations thereof.

[0070] The invention is also drawn to a pharmaceutical composition. In one embodiment, the pharmaceutical composition comprises derivatives of dimethylsuccinyl betulinic acid or dimethylsuccinyl betulin; in another, the pharmaceutical composition comprises a compound selected from the group consisting of 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, 3-*O*-(3',3'-dimethylsuccinyl) betulin, 3-*O*-(3',3'-dimethylglutaryl) betulin, 3-*O*-(3',3'-dimethylsuccinyl) dihydrobetulinic acid, 3-*O*-(3',3'-dimethylglutaryl) betulinic acid, (3',3'-dimethylglutaryl) dihydrobetulinic acid, 3-*O*-diglycolyl-betulinic acid, 3-*O*-diglycolyl-dihydrobetulinic acid, and combinations thereof. In another embodiment, the pharmaceutical composition comprises one or more compounds identified according to the methods of the invention which are not otherwise listed; or any pharmaceutically acceptable salt, ester or prodrug thereof, and a pharmaceutically acceptable carrier. In another embodiment, the pharmaceutical composition further comprising an anti-viral agent which may include any one of zidovudine, lamivudine, didanosine, zalcitabine,

stavudine, abacavir, nevirapine, delavirdine, emtricitabine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, tenofovir, amprenavir, adefovir, atazanavir, fosamprenavir, hydroxyurea, AL-721, ampligen, butylated hydroxytoluene; polymannoacetate, castanospermine; contracan; creme pharmatex, CS-87, penciclovir, famciclovir, acyclovir, cytofovir, ganciclovir, dextran sulfate, D-penicillamine trisodium phosphonoformate, fusidic acid, HPA-23, eflornithine, nonoxynol, pentamidine isethionate, peptide T, phenytoin, isoniazid,. ribavirin, rifabutin, ansamycin, trimetrexate, SK-818, suramin, UA001, combinations thereof, any other antiviral, immunomodulating agent, anti-cancer agent, anti-fungal agent, anti-bacterial agent, or combinations thereof.

[0071] The invention is also drawn to a method of determining if an individual is infected with HIV-1 that is susceptible to treatment by a compound that inhibits p25 processing. In one embodiment, the method involves taking blood from the patient, genotyping the viral RNA and determining whether the viral RNA contains mutations in the sequence encoding the region of the CA-SP1 cleavage site.

[0072] The invention is also drawn to a method of treating a disease in a patient in need thereof comprising:

identifying a compound which inhibits the processing of viral Gag p25 protein (CA-SP1) to p24 (CA), but has no significant effect on other Gag processing steps;

obtaining regulatory approval for the sale and use of said compound;

packaging the compound for sale and treatment of a disease in a patient in need thereof.

Brief Description of the Drawings

[0073] Figure 1. DSB does not disrupt the activity of HIV-1 protease at a concentration of 50 μg/mL. In DSB-containing samples recombinant Gag is processed correctly. In contrast, indinavir blocks protease activity at 5 μg/mL as evidenced by the absence of bands corresponding to p24 and the MA-CA precursor.

[0074] Figure 2. Western blots of virion-associated Gag derived from chronically infected H9/HIV-1_{IIIB}, H9/HIV-2_{ROD}, and H9/SIVmac251 in the presence of DSB (1 μg/mL), indinavir (1 μg/mL) or control (DMSO). Gag proteins were visualized using HIV-Ig (HIV-1) or monkey anti-SIVmac251 serum (HIV-2 and SIV; NIH AIDS Research and Reference Reagent Program).

[0075] Figure 3. EM analysis of DSB-treated HIV-1 infected cells. The EM data show two primary differences between DSB-treated and untreated samples. Virions generated in the presence of DSB are characterized by an absence of conical, mature cores. In these samples the cores are uniformly spherical and often acentric. Secondly, many virions display an electron dense layer inside the lipid bilayer but outside the core (indicated with arrows in the DSB-treated sample panels). In the DSB-treated samples no mature viral particles were observed.

[0076] Figure 4 depicts amino acid sequences in the region of the CA-SP1 cleavage site from DSB-sensitive HIV-1 isolates NL4-3 and RF (#1; SEQ ID NO: 1) and DSB-resistant HIV-1 isolates (#2; SEQ ID NO: 2 (NL4-3), and #3; SEQ ID NO: 3 (RF)). The differences between the native and DSB-resistant sequences involve an alanine to valine change at the first downstream residue (#2) and an alanine to valine change in the third downstream residue (#3) from the CA-SP1 cleavage site (-|-). These residues are underlined and bolded for ease of identification.

[0077] Figure 5 depicts the + sense consensus sequence for the A364V DSB-resistant NL4-3 mutant (SEQ ID NO: 4) beginning with the start of *gag* and continuing into *pol*, including the entire protease coding region. Missense mutations not found in the wild-type NL4-3 GENBANK M19921 sequence are in bold and gray shadowing. The coding sequence for the consensus CA-SP1 cleavage site region is underlined. The shaded area including the cleavage site denotes the SP1 sequence. The first mutation is the A364V mutation.

[0078] The second amino acid change (in protease) was also found in the parental clone and has been confirmed to correspond to a sequencing error in

the original GENBANK entry. Therefore, no mutations actually occurred in protease.

[0079] Figure 6 depicts the + sense consensus sequence for the DSB-sensitive NL4-3 parental isolate (SEQ ID NO: 5) that was passaged in the absence of drug in parallel with the A364V mutant isolate.

[0080] Figure 7 depicts the + sense consensus sequence for the A366V DSB-resistant HIV-1 RF mutant (SEQ ID NO: 6) beginning with the start of the *gag* and continuing into *pol*, including the entire protease coding region. Missense mutations not found in the wild-type HIV-1 RF GENBANK M17451 sequence are shadowed in gray. The region of the CA-SP1 cleavage site is underlined. The only missense mutation not also found in the identically passaged DSB-sensitive isolate is the A366V mutation in the CA-SP1 cleavage site.

[0081] Figure 8 depicts the + sense consensus sequence for the DSB-sensitive HIV-1 RF parental isolate (SEQ ID NO: 7), that was passaged in the absence of drug in parallel with the A366V mutant isolate.

[0082] Figure 9 depicts the polynucleotide sequences, SEQ ID NO: 8 and SEQ ID NO: 9, which encode the polypeptides designated herein as SEQ ID NO: 2 and SEQ ID NO: 3, respectively. SEQ ID NO: 10 and 12 depict the nucleotide sequences that encode the parental polypeptide sequences designated as SEQ ID NO: 1. SEQ ID NO: 1 is a consensus sequence based on the sequences of the region from NL4-3 and RF

[0083] Figure 10:

10A. Amino acid sequences in the CA-SP1 region of lentiviruses. (SEQ ID NO: 13; SEQ ID NO: 11; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 20; SEQ ID NO: 27; SEQ ID NO: 28; SEQ ID NO: 29; SEQ ID NO: 30; SEQ ID NO: 30; respectively)

10B: Amino acid sequences of the CA-SP1 region in HIV-1 strains RF (SEQ ID NO: 11) and NL4-3 (SEQ ID NO: 13).

10C-10D: Nucleotide sequences of *gag* gene chimeric SIVs. The 42 nucleotide sequence encoding the seven amino acids upstream and seven amino acids downstream of the CA-SP1 cleavage site is underlined and in bold.

10E-H Nucleotide sequences of *gag* gene of chimeric FIV, EIAV and BIV to be made according to the invention. The 42 nucleotide sequences encoding the seven amino acids upstream and seven amino acids downstream of the CA-SP1 cleavage sites are underlined and in bold (nucleotide sequence: SEQ ID NO: 16; amino acid sequence SEQ ID NO: 17).

10F. Nucleotide sequence of GAG gene of Chimeric Feline Immunodeficiency Virus (FIV) containing the HIV CA-SP1 region: Chimeric FIV-GAG gene nucleotides 1-1353 corresponds to nucleotides 628-1980 in Chimeric FIV genome. Nucleotide sequence SEQ ID NO: 94 encoding amino acids SEQ ID NO: 95.

10G. Nucleotide Sequence of GAG gene of Chimeric Equine Infectious Anemia Virus (EIAV) containing the HIV1 CA-SP1 region: Chimeric EIAV-GAG gene nucleotide 1-1587 corresponds to nucleotides 450-1910 in Chimeric EIAV genome. Nucleotide SEQ ID NO: 96 encoding amino acids SEQ ID NO: 97

10H. Nucleotide Sequence of GAG gene of Chimeric Bovine Immunodeficiency Virus (BIV) containing the HIV1 CA-SP1 region: Chimeric BIV-GAG gene nucleotides 1-1471 corresponds to nucleotides 316-1746 in Chimeric BIV genome. Nucleotide SEQ ID NO: 98 encoding amino acids SEQ ID NO: 99

[0084] Figure 11: Replication kinetics of PA-457 (DSB)-resistant mutants

[0085] Figure 12: Sequential SP1 point deletions in the context of NL4-3 used to identify residues necessary for DSB activity. The amino acid sequence of SP1 domain in NL4-3 is shown. "Δ" indicates the deletion and "–" means identical residues between point deletion mutants and NL4-3 (SEQ ID NO: 13; SEQ ID NO: 33; SEQ ID NO: 34; SEQ ID NO: 35; SEQ ID NO: 36; SEQ ID NO: 85; SEQ ID NO: 86; SEQ ID NO: 87; SEQ ID NO: 88; SEQ ID NO: 89; SEQ ID NO: 100; SEQ ID NO: 101; SEQ ID NO: 102; SEQ ID NO: 103; respectively).

[0086] Figure 13. Summary of particle production and infectivity of point deletions mutants.

[0087] Figure 14. Western blots for viruses containing point deletions in SP1, in the presence (+) and absence (-) of DSB.

[0088] Figure 15. Substitution of HIV-1 CA-SP1 residues VL-AEAMSQV (SEQ ID NO:32) into SIVmac239 backbone renders SIVmac239 sensitive to DSB (SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 20; SEQ ID NO: 27; SEQ ID NO: 28; SEQ ID NO: 13; respectively).

(Top panel) Amino acid sequences near the CA-SP1 cleavage site (including entire SP1 region) are shown for SIVmac239, HIV-1 NL4-3 and a series of SIV mutants into which various NL4-3 residues (underlined) were inserted. Dashes ("–") indicates the residues are the same as those in SIVmac239.

(Bottom panel) Western blots showing the CA and CA-SP1 proteins for this series of viruses in the presence (+) or absence (-) of DSB.

[0089] Figure 16: Sequence conservation in the CA-SP1 region of Lentiviruses. Cloning Strategy: Substituting HIV-1 specific CA-SP1 residues into the corresponding Gag region of FIV, EIAV or BIV.

[0090] Figure 17. HIV-1 NL4-3 SP1 tagged with an epitope. Sequences of SP1 peptides with peptide tags inserted are shown. "Δ" indicates deleted residue and "–" indicates that the residue is identical to that in NL4-3 SP1. (Fig 17 (1); SEQ ID NO: 15; SEQ ID NO: 104; SEQ ID NO: 105; SEQ ID NO: 106; SEQ ID NO: 107; respectively); (Fig 17 (2); SEQ ID NO: 15; SEQ ID NO: 108; SEQ ID NO: 109; respectively); (Fig 17 (3); SEQ ID NO: 15; SEQ ID NO: 110; SEQ ID NO: 111; respectively); (Fig 17 (4); SEQ ID NO: 15; SEQ ID NO: 112; SEQ ID NO: 113; SEQ ID NO: 114; respectively); (Fig 17 (5); SEQ ID NO: 15; SEQ ID NO: 115; respectively).

[0091] Figure 18A-C: HIV-1 strain RF polynucleotide sequence. The nucleotide sequence of the Gag polyprotein is underlined and in bold. The 42 nucleotide sequence encoding the seven amino acids upstream and seven amino acids downstream of the CA-SP1 cleavage site is highlighted in green. An additional 129 nucleotides (43 amino acid residues) upstream of the cleavage site in CA and the remaining 21 nucleotides (seven amino acids residues) in SP1 are highlighted.

[0092] Figure 19A-E: HIV-1 strain NL4-3 polynucleotide sequence. The nucleotide sequence of the Gag polyprotein is underlined and in bold. The 42 nucleotide sequence encoding the seven amino acids upstream and seven

amino acids downstream of the CA-SP1 cleavage site is highlighted in green. An additional 129 nucleotides (43 amino acid residues) upstream of the cleavage site in CA and the remaining 21 nucleotides (seven amino acids residues) in SP1 are highlighted.

[0093] Figure 20: A schematic representation of the Gag protein and the CA-SP1 sequences of the SHIVs used in this study. The sequences flanking the CA-SP1 cleavage site for SIV Mac239 and HIV-1 NL4-3 are shown at the top and bottom of the list of sequences, respectively. A dashed line (-) represents residues that are identical to the parent SIV MAC239, a delta (Δ) represents SIV residues that are deleted in the SHIVs.

[0094] Figure 21: Western blot analysis of the Gag processing profiles for panel 1-3 SHIVs. Figure 21A shows Gag processing from cell-associated virus while Fig. 21B shows the Gag processing profile for cell-free virions. Normal Gag processing is indicated by a plus sign (+), while a defective processing profile is indicated by a minus sign (-).

[0095] Figure 22: Western blot analysis of the effect of DSB at 1μg/ml on the conversion of the capsid precursor, CA-SP1 to mature capsid protein. In panel A, the virus for Western blotting was obtained using a constant volume of cell culture supernatant. This resulted in variability in the intensity of the viral protein bands due to differences among the SHIVs in the level of virus production. Panel B shows the Gag processing profiles obtained when increased amounts of viral protein are used for Western blot analysis. Only SHIVs that exhibit normal Gag processing are included here. The asterix(*) in panel A indicates the faint CA-SP1 band for SHIV GI observed in the autoradiograph cannot be seen, however, the DSB sensitivity for this virus is scored a +/- based on results observed when the analysis is performed using increased amounts of protein (panel B).

[0096] Figure 23A-H. Alignment of the CA-SP1 region in HIV-1 clinical isolates, obtained from *"HIV Sequence Compendium 2002,"* Kuiken *et al.* eds. Los Alamos National Laboratory, Los Alamos, NM. (www.hiv.lanl.gov).

Detailed Description of the Invention

[0097] The present invention is directed to methods of inhibiting HIV-1 replication in the cells of an animal. More specifically, the invention involves methods of inhibiting HIV-1 replication in the cells of a mammal by contacting infected cells with a compound that inhibits the processing of the viral Gag p25 protein (CA-SP1) to the p24 protein (CA). More specifically, such compounds inhibit the processing of the viral Gag p25 protein (CA-SP1) to the p24 protein (CA) without significantly affecting other Gag processing steps.

[0098] "A compound that does not significantly affect other Gag processing steps" means that the compound in question predominantly inhibits processing of p25 to p24, but does not necessarily preclude the possibility of having additional minor effects on other Gag processing steps.

[0099] "Significant" or "Significantly," where not otherwise defined herein, means an observable or measurable change compared to the process in the absence of a compound. However, not all observable or measureable changes may necessarily be significant.

[0100] A number of viral phenotypes may also be observed in practicing the method of the invention. One result of contacting an infected cell with the compounds of the invention may be the formation of noninfectious viral particles. Alternatively, or in addition, contacting infected cells with a compound that inhibits p25 to p24 processing, results in the formation of non-infectious viral particles, but where there is no significant effect on other Gag processing steps. This may not significantly reduce the quantity of virus released from treated cells and/or has no little or no significant effect on the amount of RNA incorporation into the released virions.

[0101] Accordingly, the invention is also drawn to a method of inhibiting HIV infection in cells of an animal comprising contacting said cells with a compound that inhibits p25 processing and also affects other viral phenotypes, discribed above.

[0102] Mutant viruses defective in CA-SP1 cleavage have been shown to be non-infectious (Wiegers K. *et al., J. Virol. 72*:2846-2854 (1998)). 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB) is an example of a compound that

disrupts p25 to p24 processing and potently inhibits HIV-1 replication. This compound's activity is specific for the p25 to p24 processing step, not other steps in Gag processing. Furthermore, DSB treatment results in the aberrant HIV particle morphology as described in Figure 3.

1. Identification of HIV-1 determinants associated with sensitivity to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid

(a) Generation and selection of HIV-1 viruses resistant to DSB.

[0103] Mutant forms of HIV-1 have been generated in which the amino acid sequence in the region of the CA-SP1 cleavage site is modified, decreasing the sensitivity of these strains to compounds that disrupt CA-SP1 processing. Data on these mutant viruses have been used to identify the amino acid residues in wild-type Gag that are implicated in the antiviral activity of these compounds. In one embodiment, compounds that disrupt CA-SP1 processing directly or indirectly inhibit the interaction of HIV-1 protease with the region of the Gag protein containing these amino acid residues. In another embodiment, compounds that disrupt CA-SP1 processing bind to the region containing these amino acid residues. As used herein, the terms "bind," "bound" or "binding" refers to binding or attachment including, *e.g.*, ionic interactions, electrostatic hydrophobic interactions, hydrogen bonds, *etc*; and also includes associations that may be covalent, *e.g.*, by chemically coupling. Covalent bonds can be, for example, ester, ether, phosphoester, thioester, thioether, urethane, amide, amine, peptide, imide, hydrazone, hydrazide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term "bound" is broader than and includes terms such as "coupled," "conjugated" and "attached."

[0104] In another embodiment, compounds that disrupt CA-SP1 processing bind to another region of Gag and thereby inhibit the interaction of HIV-1 protease with the region of the CA-SP1 cleavage site. In another embodiment, viruses or recombinant proteins that contain mutations in the region of the CA-SP1 cleavage site can be used in screening assays to identify compounds that disrupt CA-SP1 processing.

[0105] In one set of experiments, amino acid residues in HIV-1 Gag that are involved in the disruption of CA-SP1 processing by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB) were identified by sequencing the *gag-pol* gene of virus isolates that had been selected for resistance to DSB. The amino acid sequences from these resistant viruses were compared with the *gag-pol* gene sequences from DSB-sensitive HIV-1 isolates. Two single amino acid changes were identified in the DSB-resistant viruses, an alanine (Ala) to valine (Val) substitution at residue 364 (SEQ ID NO: 4) and in a second isolate, at residue 366 (SEQ ID NO: 6), in the Gag polyprotein (see Figure 4). These residues are located immediately downstream of the CA-SP1 cleavage site (at the N-terminus of SP1). Alanine is highly conserved at these positions throughout all HIV-1 subtypes listed in the Los Alamos National Laboratory database. The five amino acid residues upstream and downstream of the CA-SP1 cleavage site are also highly conserved among the various subtypes. However, isoleucine replaces valine at the position two residues upstream of the cleavage site in a number of clades (c.f., Figure 4, SEQ ID NO. 1). (*"HIV Sequence Compendium 2002,"* Kuiken *et al.* eds. Los Alamos National Laboratory, Los Alamos, NM.)

[0106] In order to more extensively map the viral genetic determinants for DSB resistance, additional experiments were performed to select for viruses *in vitro* that are drug resistant. Multiple parallel cultures of Jurkat T cells (5 x 10^5 each) were transfected with the proviral DNA clone pNL4-3 in the presence or absence of 10-50 ng/ml DSB. The cells were passaged every two days, and fresh drug was added at each passage. Virus replication was monitored by measuring reverse transcriptase activity in culture supernatants. Virus was isolated from culture supernatants harvested at selected timepoints, and genomic DNA was amplified by RT-PCR using primers that spanned the coding region between the N-terminus of CA and the N-terminus of RT. The amplified product was then sequenced using the same set of primers.

[0107] In one experiment, an A366V mutation was identified in the SP1 region of NL4-3 virus cultured in the presence of DSB (note: numbering is relative to the Gag polyprotein). Upon further passaging, a double mutant was identified that contained a G357S mutation in CA as well as the A366V

mutation in SP1. The A366V mutation was identified previously in experiments selecting for resistant variants of the RF isolate. Interestingly, the wild-type RF sequence also contains a serine residue at position 357 in CA (Figure 4). Since serine is present at this position in isolates (such as RF) that are sensitive to DSB, the CA G357S mutation alone is not sufficient to confer resistance to DSB. To determine the contribution of each of these mutations to drug resistance, the A366V mutation and the A366V/G357S double mutation were re-engineered into the wild-type NL4-3 backbone by site-directed mutagenesis. The resulting constructs were transfected into Jurkat T cells and characterized in a virus replication assay as described above for the selection of resistance. SDS-PAGE analysis of transfected cell lysates and virus released into the media demonstrated that the A366V mutant Gag was processed and released from cells inefficiently (data not shown) and thus replicated very poorly even in the absence of drug (Figure 11) However, the A366V/G357S double mutant replicated efficiently in the absence or presence of DSB. There data indicate that the resistant mutant, A366V, requires a serine at the 357 position in the CA region of Gag to compensate for a deleterious effect on virus replication (Figure 11).

[0108] In a further experiment, ten different resistant isolates were generated. Sequencing of these isolates identified four additional mutations not previously seen in resistance selection experiments. These were H358Y, L363F and L363M in CA, and A402T in the NC region of Gag. None of these mutations are present in the consensus sequences for HIV-1 clades A-O, reflecting the breadth of activity of DSB against genetically diverse clades of HIV-1. The L363M substitution in CA was found in the consensus sequence for HIV-2, which may, in part, explain the specificity of DSB for HIV-1.

[0109] These results demonstrate the presence of specific genetic determinants for DSB activity in HIV-1, and that these determinants are centered around the CA-SP1 cleavage site.

(a) HIV-1 NL4-3 deletion and SIV insertion studies used to identify viral genetic determinants of DSB sensitivity

[0110] Results from *in vitro* resistance selection experiments indicated that the determinants of DSB HIV-1 inhibitory activity map to the region of Gag

flanking the CA-SP1 cleavage site. In order to better define the viral genetic determinant for DSB, HIV-1 point-deletion mutagenesis and SIV insertion studies were undertaken to identify the specific amino acid residues associated with compound activity. The study was carried out as follows. Single residue deletions starting with residue E365 and continuing through residue M377 were engineered into the SP1 domain of the infectious HIV-1 molecular clone NL4-3 (Figure 12). The effect of these point deletions on viral particle production, infectivity, Gag processing and sensitivity to DSB was determined. The results of these experiments were used to identify the Gag residues in the region of the CA-SP1 cleavage site that are associated with DSB activity. The residues associated with activity were inserted into the CA-SP1 cleavage site region of the DSB-resistant virus SIV (Mac 239 isolate) to generate a HIV-1, SIV chimeric virus (SHIV). Point substitution of HIV-1 residues from the N-terminus of the CA protein were made into this chimeric virus until the minimal sequence necessary to rescue DSB activity was identified. This minimal sequence necessary to gain DSB activity is considered a primary viral genetic determinant of DSB activity. It may suggest the molecular determinant of DSB activity.

1. Methods:

(a) Construction of NL4-3 single point-deletion mutants.

[0111] Single point-deletion constructs were generated using the PCR-ligation-PCR (PLP) strategy as previously described. HIV-1 NL4-3 plasmid DNA was used as the template to perform all PCR reactions for generating point deletions spanning the complete Gag SP1 domain with the exception of the first residue of SP1.

[0112] ΔE365 was generated using NL4-3 as the template with Vent DNA polymerase (NEB) by using deletion-specific downstream primer (Primer 1) with universal upstream primer (Primer 2) (Table 1). The fragment derived from this was termed as a first flanking PCR fragment. A second flanking fragment was amplified using deletion-specific upstream primer (Primer. 3) and universal downstream primer (Primer 4) (Table 1). To generate other deletion constructs (ΔA366, ΔM367, ΔS368, ΔQ369, ΔV370, ΔT371, ΔN372,

ΔP373, ΔA374, ΔT375, ΔI376, and ΔM377). PCR procedures were similarly performed by varying deletion-specific downstream and upstream primers corresponding to each specific point deletion (Table 1).

[0113] Each of these parallel two adjacent PCR fragments was gel purified, phosphorylated using T4 polynucelotide kinase (NEB), and ligated by using T4 DNA ligase (NEB). After inactivation at 65°C for 15 minutes, the ligation reaction was used for a subsequent amplification with universal upstream primer (Primer. 2) and downstream primer (Primer. 4). This product was gel purified, digested with SpeI and ApaI, and then ligated into the SpeI and ApaI sites of NL4-3 proviral DNA clone.

[0114] Standard PCR conditions were used for the above-described reactions. These included, one cycle of denaturation at 95°C for 1 minutes 30 seconds, followed by 30 cycles of denaturation at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The PCR reactions were set up using the following components:

5 μL 10 x NEB Thermophilic buffer

2 μL 10 mM dNTPs

1 μL 100nM $MgSO_2$

1 μL 50 pmol upstream primer

1 μL 50 pmol downstream primer

1 μL 50 ng/μL template DNA

0.5 μL Vent DNA polymerase

38.5 μL ddH_2O

[0115] A 10 μL aliquot was run on a 1.0% agarose gel to make sure the correct size product was amplified. The PCR products were then gel isolated and purified with a Qiaex II gel extraction Kit (Qiagen). The gel-purified two adjacent PCR fragments were individually phosphorylated in the following reaction by using T4 polynucleotide kinase (NEB) prior to ligation. The phosphorylation reaction was set up as follows:

2 μL 10X T4 polynucleotide kinase buffer

2 μL 10mM ATP

1 μL T4 polynucleotide kinase

[0116] 15 μL gel purified DNA of each of these two adjacent PCR fragments The reaction was incubated at 37°C for 1 hour. Following the inactivation at 65°C for 10 minutes, the adjacent phosphorylated PCR fragments were then ligated together by using T4 DNA ligase (NEB) under following conditions:

3 μL 10X T4 DNA ligase buffer

13 μL of each of two adjacent PCR fragments

1 μL T4 DNA ligase

[0117] After overnight incubation at 16°C the ligation reaction product was used in a second round PCR reaction to amplify the full-length PCR fragment spanning these two adjacent PCR products. The second round PCR reaction was performed as described above with the exception that only universal upstream primer (Primer. 2) and downstream primer (Primer. 4) were used. Again, a 10 μL aliquot was run on a agarose gel to make sure the correct product was amplified. The full-length PCR fragments were then gel isolated and purified using a Qiaex II kit. The purified full-length PCR fragment, together with NL4-3, were then cut with SpeI and ApaI under the following conditions:

2 μL 10X NE buffer 4 (NEB)

1 μL ApaI (NEB)

1 μL SpeI (NEB)

16 μL full length PCR product (1 μg) or NL4-3 (500 ng)

[0118] The above restriction enzyme digestion mixture was incubated at 37°C for 2 hours. Digested DNA fragments for the full-length PCR product and the NL4-3 plasmid were individually gel isolated and purified using a Qiaex II kit. The digested vector NL4-3 and full length PCR fragment were ligated using T4 DNA ligase under the following procedure:

1 μL 10X T4 DNA ligase buffer

1 μL (25-50 ng) digested NL4-3 vector

7 μL digested (200 ng-400 ng) digested PCR fragment (700 bp)

1 μL T4 DNA ligase

[0119] The ligation reaction was incubated at 16°C overnight and the ligated products were transformed into *Escherichila coli* Max Efficiency Stbl2

(Invitrogen) by heat shock according to instruction (Invitrogen). The proviral DNA clones were then screened by automatically sequencing using a Taq Dye Deoxy Terminator cycle Sequencer Kit (Applied Biosystems) individually using internal primers (Primer 29 and 30) Following the verification the mutations the proviral DNA clones were used for various future studies.

1. Construction of SIV chimeric mutants

[0120] A panel of SIV chimeric constructs harboring various residues of NL4-3 CA-SP1 boundary region was generated using the SIVmac239 molecular clone by employing PCR and cloning procedures described above. These constructs and their amino acid sequences in the CA-SP1 boundary region are shown in Figure 15. SIV mac239 was used to generate the SIV DD and DE constructs. The SIV DD construct was used to generate SIV DM. Different SIV chimeric constructs were produced in the PCR by varying respective mutagenic upstream and downstream primers corresponding to each chimera (Table 1). Each of these parallel two adjacent PCR fragments was gel purified and directly used without phospohorylation treatment for a subsequent amplification with universal upstream primer (Primer. 31) and downstream primer (Primer 32). This product was gel purified, digested with BamHI and SbfI, and then ligated into the BamHI and SbfI sites of SIVmac239 proviral DNA clone. The proviral DNA clones were then screened by automatically sequencing using a Taq Dye Deoxy Terminator cycle Sequencer Kit (Applied Biosystems) individually using an internal primer (Primer. 39). Following the verification the mutations the proviral DNA clones were used for various future studies.

2. Cell culture and DNA transfection

[0121] HeLa cells were maintained in DMEM (Invitrogen) (10% FBS, 100 U/ml penicillin, and 100 μg/ml Streptomycin) and passaged upon confluence. Jurkat cells were cultured in RPMI 1640 (Invitrogen) (10% FBS, 100 U/ml penicillin, and 100 μg/ml Streptomycin) and passaged every two or three days.

[0122] To characterize the effect of deletion or substitution on viral particle production and Gag polyprotein processing, wild-type HIV-1 NL4-3 or

SIVmac239 and respective mutant proviral DNAs were transfected into HeLa cells by employing FuGENE 6 transfection reagent (Roche). Briefly, cells were seeded into a 6-well plate (Corning) at a concentration of 0.5 x 105 per well the day before transfection to reach 60 to 80% confluence on the day of transfection. For each transfection, 3 μl of FuGENE 6 was diluted into 100 μl of serum-free DMEM followed by the addition of 1 μg of DNA. After gently mixing, the mixture of DNA-lipid complexes was gently added drop wise into the cells containing 2 ml of complete DMEM medium. Twenty-four hours post-transfection, medium containing DNA-FuGENE 6 complexes was removed, 2 ml of fresh DMEM was added into the transfected cells. At 48 h post-transfection, medium containing viral particles was collected and clarified by centrifugation at 2,000 rpm at 4°C for 20 min in a Sorvall RT 6000B centrifuge. Virus particle-containing supernatants were then concentrated through a 20% sucrose cushion in a microcentrifuge at 13,000 rpm at 4°C for 120 min and pellets were resuspended in a lysis buffer (150 mM Tris-HCl, 5% Triton X-100, 1% deoxycholate, pH 8.0). The level of viral particle production for wild type NL4-3 and point deletion mutants was determined by p24 antigen capture ELISA (ZeptoMetrix, Buffalo, NY).

[0123] To examine the effect of deletion or substitution on Gag polyprotein processing (in the absence of DSB), SDS-PAGE and Western-Blot was performed. In brief, viral proteins were separated on a 12% NuPAGE Bis-Tris Gel (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen) followed by blocking in a PBS buffer containing 0.5% Tween and 5% dry milk. The membrane was incubated with immunoglobulin from HIV-1-infected patients (HIV-Ig) (NIH AIDS research and reference reagent program) and hybridized with goat anti-human horseradish peroxidase (Sigma). For the membrane containing SIV proteins, the membrane was incubated with a reference polyclonal immune serum from a SIV-infected monkey (NIH AIDS Research and Reference Reagent Program) and hybridized with goat-anti-monkey horseradish peroxidase (Sigma). The immune complex was visualized with an ECL system (Amersham Pharmacis Biotech) according to the instructions provided by the manufacturer.

[0124] To address the effect of deletion or substitution on the ability of DSB to inhibit CA-SP1 processing, HeLa cells were transfected with wild-type HIV-1 NL4-3 or SIVmac239 and respective mutant proviral DNAs by employing the procedure described above. DSB at a concentration of 1 μg/ml and DMSO control were maintained throughout the entire culture and SDS-PAGE/Western-Blot for analyzing viral proteins derived from these transfections were performed as described in the previous paragraph.

[0125] The 50% -Tissue Culture Infectious Dose ($TCID_{50}$) per ml was used as a measure of the infectivity of each deletion mutant. Mutant viruses derived from transfections in HeLa cells were used to infect U87 CD4.CXCR4 cells. Each virus stock was tested in triplicate at a starting dilution of 1:10, followed by four-fold serial dilutions. Cells were plated the day before infection at a density of $3x10^3$ cells/well. On the day of infection, culture media was removed from the cell plate and 90μl of diluted virus was added. On days 1, 3, and 6 post infection, virus was removed from plate and 200μl of culture media was added. On days 6 and 8 post infection, supernatant was collected for p24 ELISA analysis. The virus dilution that caused 50% of the culture to be infected ($TCID_{50}$) was determined according to the method of Reed and Muench (Aldovini A. and B. Walker 1990; Dulbecco R. 1988).

3. Results

[0126] Viruses containing sequential point deletions within the Gag SP1 domain (Figure 12) were characterized for particle production, infectivity, Gag processing and sensitivity to DSB. The results from these experiments were used to identify SP1 residues associated with DSB activity.

[0127] As expected, the effect of point deletions on viral particle production varied as a function of the proximity of the change from the proteolytic cleavage site. The results from these experiments are summarized in Figure 13. Viruses with deletions at residues E366, A367 and M368 were most affected, generating <25% the number of particles normally observed in wild-type virus infection. *In vitro* infectivity assays were used to characterize the ability of the deletion mutants to support virus replication. These experiments indicated that deletion of single residues at any of the five positions E365

through Q369 resulted in a virus that was either non-infectious or significantly impaired for replication (Figure 13). In contrast, starting with residue V370 and extending away from the CA-SP1 cleavage site, none of the characterized point deletions resulted in a decrease in virus infectivity (Figure 13). With the exception of viruses with deletions at positions I376 and M377 all mutant viruses exhibited a normal or near normal Gag processing phenotype (Figure 13). The results from these three sets of experiments permitted the design and interpretation of experiments to identify the genetic determinants of DSB activity.

[0128] Sensitivity to DSB was determined in experiments that characterized the effect of DSB on a late step in Gag processing, CA-SP1 cleavage. Specifically, these assays measured the ability of DSB to disrupt CA-SP1 processing. As seen, e.g. Example 8, the DSB-induced defect in Gag processing correlates with the ability of the compound to inhibit virus replication. Results from these experiments indicate that deletion of a single residue at any of the six positions E365 through V370 significantly reduces the affect of DSB on CA-SP1 processing (Figure 14). In contrast, starting with residue T372 and extending away from the CA-SP1 cleavage site, all of the characterized point deletions are fully sensitive to DSB-induced disruption of CA-SP1 processing (Figure 14).

[0129] The SP1 residues associated with DSB activity consist of the contiguous residues E365 through V370.

[0130] Residues A364 through V370 were inserted into the analogous position of the Gag SP1 domain in the DSB-resistant retrovirus SIV (Mac 239 isolate). Additionally, the N-terminus of the CA protein of this chimeric virus was modified by cumulative substitution of residues found in SIV with HIV-1-specific residues. This approach is summarized in Figure 15. Next, the effect of DSB on the Gag processing phenotype of each of the chimeric viruses was determined. As shown in Figure 15, the SIV.DM virus displays a Gag processing phenotype indicative of sensitivity to DSB. Thus, the minimum sequence of HIV-1 CA-SP1-specific residues that needs to be inserted to rescue DSB activity in the SHIVs extends from V362 to V370

Table 1. PCR Mutagenesis Primers

Primer No.	Sequence (5' to 3')	Construct	PCR application
1	agccaaaactcttgctttatggcc (SEQ ID NO: 37)	ΔE365	First PCR fragment (with No. 2 primer)
2	agtcagtgtggaaaatctctagcagtgg (SEQ ID NO: 38)	All deletion constructs of NL4-3	All first PCR fragments
3	gcaatgagccaagtaacaaatcca (SEQ ID NO: 39)	ΔE365	Second PCR fragment (with No. 4 primer)
4	aggtatggtaaatgcagtatacttcctgaag (SEQ ID NO: 40)	All deletion constructs	All second PCR fragments
5	ttcagccaaaactcttgctttatggcc (SEQ ID NO: 41)	ΔA366	First PCR fragment (with No. 2 primer)
6	atgagccaagtaacaaatccagc (SEQ ID NO: 42)	ΔA366	Second PCR fragment (with No. 4 primer)
7	tgcttcagccaaaactcttgc (SEQ ID NO: 43)	ΔM367	First PCR fragment (with No. 2 primer)
8	agccaagtaacaaatccagct (SEQ ID NO: 44)	ΔM367	Second PCR fragment (with No. 4 primer)
9	cattgcttcagccaaaactcttgc (SEQ ID NO: 45)	ΔS368	First PCR fragment (with No. 2 primer)
10	caagtaacaaatccagctacca (SEQ ID NO: 46)	ΔS368	Second PCR fragment (with No. 4 primer)
11	gctcattgcttcagccaaaactctt (SEQ ID NO: 47)	ΔQ369	First PCR fragment (with No. 2 primer)

12	gtaacaaatccagctaccataa (SEQ ID NO: 48)	ΔQ369	Second PCR fragment (with No. 4 primer)
13	acaaatccagctaccataatgatac (SEQ ID NO: 49)	ΔV370	First PCR fragment (with No. 2 primer)
14	ttggctcattgcttcagccaaaactc (SEQ ID NO: 50)	ΔV370	Second PCR fragment (with No. 4 primer)
15	tacttggctcattgcttcagccaa (SEQ ID NO: 51)	ΔT371	First PCR fragment (with No. 2 primer)
16	aatccagctaccataatgatacag (SEQ ID NO: 52)	ΔT371	Second PCR fragment (with No. 4 primer)
17	tgttacttggctcattgcttc (SEQ ID NO: 53)	ΔN372	First PCR fragment (with No. 2 primer)
18	ccagctaccataatgatacagaaa (SEQ ID NO: 54)	ΔN372	Second PCR fragment (with No. 4 primer)
19	atttgttacttggctcattgcttc (SEQ ID NO: 55)	ΔP373	First PCR fragment (with No. 2 primer)
20	gctaccataatgatacagaaaggcaa (SEQ ID NO: 56)	ΔP373	Second PCR fragment (with No. 4 primer)
21	tggatttgttacttggctcattgc (SEQ ID NO: 57)	ΔA374	First PCR fragment (with No. 2 primer)
22	accataatgatacagaaaggc (SEQ ID NO: 58)	ΔA374	Second PCR fragment (with No. 4 primer)
23	agctggatttgttacttggctc (SEQ ID NO: 59)	ΔT375	First PCR fragment (with No. 2 primer)

24	ataatgatacagaaaggcaattttagg (SEQ ID NO: 60)	ΔT375	Second PCR fragment (with No. 4 primer)
25	ggtagctggatttgttacttg (SEQ ID NO: 61)	ΔI376	First PCR fragment (with No. 2 primer)
26	atgatacagaaaggcaattttaggaacc (SEQ ID NO: 62)	ΔI376	Second PCR fragment (with No. 4 primer)
27	tatggtagctggatttgttac (SEQ ID NO: 63)	ΔM377	First PCR fragment (with No. 2 primer)
28	atacagaaaggcaattttagg (SEQ ID NO: 64)	ΔM377	Second PCR fragment (with No. 4 primer)
29	ccacctatcccagtaggag (SEQ ID NO: 65)	Sequencing primer	For NL4-3 mutants
30	ggcacagcaagcagcagctg (SEQ ID NO: 66)	Sequencing primer	For NL4-3 mutants
31	gtagaccaacagcaccatctagcggcaga (SEQ ID NO: 67)	All substitution constructs of SIV	For SIV mutants
32	ggtaaagtaaaggcagtgtactgcctaa (SEQ ID NO: 68)	All substitution constructs of SIV	For SIV mutants
33	cactggtgcgaggacctgactcatggcttctgccatt (SEQ ID NO: 69)	SIV DD	First PCR fragment (with No.31 primer)
34	aatggcagaagccatgagtcaggtcctcgcaccagtg (SEQ ID NO: 70)	SIV DD	Second PCR fragment (with No.32primer)
35	ggcttctgccagtactctagccttctgt (SEQ ID NO: 71)	SIV DE	First PCR fragment (with No.31 primer)
36	acagaaggctagagtactggcagaagcc (SEQ ID NO: 72)	SIV DE	Second PCR fragment (with No.32primer)

37	ggcttctgccagtactctagccttctgt (SEQ ID NO: 73)	SIV DM	First PCR fragment (with No.31 primer)
38	acagaaggctagagtactggcagaagcc (SEQ ID NO: 74)	SIV DM	Second PCR fragment (with No.32primer)
39	atccaactggggttgcaaaaatgtg (SEQ ID NO: 75)	Sequencing primer	For SIV mutant

[0131] The resistance and mutagenesis data presented above suggest that the GHKARVL-AEAMSQV amino acid sequence in the region of the HIV-1 Gag CA-SP1 cleavage site serves as a genetic determinant of viral sensitivity to DSB.

Extending The Determinants Of Dsb Sensitivity To Other Lentiviruses: Ca-Sp1 Chimeras As Animal Efficacy Models For Development Of Maturation Inhibitors.

[0132] The development of anti-HIV therapeutics has been hindered by the lack of an animal efficacy model. This lack of an animal model is primarily due to the inability of most HIV-1 strains to replicate and cause disease in non-human primates. In some instances this problem has been overcome through the use of chimeric viruses that incorporate the region(s) of interest from the HIV-1 viral target into an SIV viral backbone that will support replication in a non-human primate. The most notable example of this approach involves the HIV-1/SIV (SHIV) chimeric viruses in which the proteins making up the infectious virus are exclusively SIV in origin with the exception of Env (gp120/gp41) which is derived form HIV-1. These SHIV envelope chimeras have been used extensively in HIV-1 vaccine development.

[0133] HIV-1 maturation inhibitors disrupt Gag CA-SP1 processing, which results in the formation and release of non-infectious viral particles exhibiting aberrant core morphology. See e.g. Li *et al. Proc Natl Acad Sci U S A.* 100:13555-60 (2003). The betulinic acid derivative DSB is an example of this class of inhibitors. The viral genetic determinants critical that are associated with the activity of maturation inhibitors map to amino acid residues flanking

the HIV-1 CA-SP1 cleavage site. When this determinant is introduced into the CA-SP1 cleavage sites of DSB-resistant non-HIV-1 viruses, maturation inhibitor sensitive chimeras result. These CA-SP1 chimeric viruses serve as the basis for an animal efficacy model for HIV-1 maturation inhibitors.

[0134] The region of HIV-1 CA-SP1 necessary for maturation inhibitor sensitivity is introduced into selected lentiviruses. Amino acid residues from HIV-1 CA-SP1 junction that are determinants of DSB sensitivity were used to replace the corresponding CA-SP1 amino acids in the genome of Simian Immunodeficiency (SIV). Similarly, the amino acid residues from HIV-1 CA-SP1 junction that are determinants of DSB can be replaced in Feline Immunodeficiency virus (FIV), Bovine Immunodeficiency Virus (BIV), Equine Infectious Anemia Virus (EIAV), Visna-Maedi, and Caprine Arthritis Encephalitis virus (CAEV). Table 2 depicts the Gag polypeptide sequence for HIV-1, SIV, FIV, EIAV and BIV in the region of the CA-SP1 cleavage site.

Table 2. Sequence comparison in the region of the CA-SP1 cleavage site region of HIV-1 with SIV, FIV, EIAV and BIV

	CA	SP1	NC
HIV-1	GHKARVL	AEAMSQVTNPATIM	IQKG
(SEQ ID NO: 76)			
FIV	GYKMQLL	AEALTKVQ	VVQS
(SEQ ID NO: 77)			
EIAV	KQKMMLL	AKALQ	TGLA
(SEQ ID NO: 78)			
BIV	KSKMQFL	VAAMKEMGIQSPIPAVLPHTPEAYA	SQTS
(SEQ ID NO: 79)			

[0135] The HIV-1 CA-SP1 sequence used for replacement is as follows:

CA	SP1
GHKARVL	AEAMSQV (SEQ ID NO: 80)

[0136] The method described above for generating the SHIV CA-SP1 chimeric provirus DNA clone is used to generate FIV, EIAV and BIV provirus clones containing selected residues or extended region from CA-SP1 region of HIV-1 replacing the corresponding wild-type sequence (Figure 16).

[0137] The SHIV CA-SP1 chimeric-provirus DNA clone was generated by site-directed mutagenesis employing standard molecular biology techniques. Briefly, the unique restriction enzyme sites in the SIV Gag that surrounding the CA-SP1 region were identified i.e., BamHI (in matrix) and Sbf-I (in NC). Starting from the CA-SP1 region where the mutagenisis is intended two overlapping primers, a forward and a reverse primer incorporating the mutated sequence i.e., HIV CA-SP1 at their 5' ends were synthesized. Using the wild-type SIV provirus DNA as a template, two separate PCR reactions were set up to amplify SIV-Gag fragments in either direction from the site of mutagenisis (CA-SP1 region), i.e., yield two amplified fragments that overlapped in the mutated CA-SP1 region, a Bam HI-CA-SP1 fragment and a CA-SP1-Sbf-I fragment. In a third PCR reaction, the fragments, Bam HI-CA-SP1 and CA-SP1-Sbf-I were annealed at their common HIV-CA-SP1 sequence and amplified with a forward SIV Bam HI primer and a reverse SIV Sbf-I primer to generate a full-length chimeric SHIV CA-SP1 gag fragment. The chimeric SHIV CA-SP1 PCR fragment was cloned into BamHI - Sbf-I window of SIV provirus clone replacing the SIV-Gag wild-type sequence to yield the SHIV CA-SP1 provirus cDNA clone.

[0138] Similarly, unique restriction enzyme cloning sites surrounding the CA-SP1 regions in FIV (Genbank Accession # NC_001482), EIAV (GA# AF016316), and BIV (GA# M32690) genome have been identified (Fig 16). Specific FIV/EIAV/BIV-HIV1CA-SP1 chimeric primers along with genome specific primers of FIV, EIAV or BIV incorporating the specific cloning site sequence are synthesized. These primers along with corresponding provirus DNA clone as template (FIV, EIAV or BIAV) in PCR reactions to generate the Chimeric HIV-1 CA-SP1 fragment. The chimeric HIV-1 CA-SP1 fragment is digested with the appropriate restriction enzyme and cloned into SacI-EcoRI window of FIV provirus; or (ii) KasI-EcoRV window of EIAV provirus; or (iii) BsrGI-ApaI window of BIV provirus replacing the corresponding wild type sequence (Figure 16). The chimeric FIV/EIAV/BIV-HIV-1CA-SP1 provirus DNA clones are sequenced to confirm the presence of intended mutations. Based on observed results that indicate the transfer of DSB

sensitivity, additional constructs are generated employing the above strategy in order to optimize the results.

[0139] In summary, a chimeric virus was generated in which the CA-SP1 determinant of HIV-1 maturation inhibitor sensitivity has replaced the analogous region of Gag in the maturation inhibitor-resistant simian immunodeficiency virus (SIV). Transfer of this region of HIV-1 into the genome of SIV results in a maturation inhibitor-sensitive phenotype. Infection of a non-human primate with this HIV-1/SIV chimeric virus should result in an animal efficacy model for therapeutic development of maturation inhibitors.

[0140] Analogous approaches are used to prepare and characterize HIV-1 CA-SP1 chimeras with FIV, BIV and EIAV. These additional DSB-sensitive chimeric viruses should enable the development of additional animal efficacy models for the study of HIV-1 maturation inhibitors.

Uses Of Mutant And Chimeric Viruses

[0141] The mutant and chimeric viruses of the present invention, as described above, are useful in a variety of cell based as well as animal based assays.

[0142] By comparing the phenotypes associated with a virus that is resistant to DSB, with a virus that is sensitive DSB, one may identify compounds that act by a mechanism similar to that of DSB. Thus the invention includes a method of identifying a compound that inhibits cleavage of p25 to p24 in wild type HIV-1, but does not inhibit CA-SP1 processing in HIV-1 containing a deletion in the CA-SP1 region. Compounds obtained by such a method are also included in the present invention.

[0143] Chimeras of SIV and other lentiviruses that do not readily infect humans have additional advantages. Firstly, these viruses pose a lesser safety hazard to laboratory workers. As a result, cell based assays to identify novel compounds that inhibit CA-SP1 processing, for example, can be conducted with less risk. The lower risk may allow assays to be performed that cannot be performed readily or safely with HIV, and may also lower the cost of such assays.

[0144] Furthermore, such chimeric viruses are useful in animal models. For example chimeric SIV that is sensitive to DSB may be used to identify novel compounds that inhibit CA-SP1 processing, for example; to identify pharmaceutical compositions, routes of administration and dosage regimes for treatment of disease; and for studying the effect of combination therapies, such as DSB with protease inhibitors.

[0145] As SIV is generally limited to infection of monkeys, the generation of additional lentiviral chimeras allows animal studies to be performed in animals that are less expensive, easier to handle, have a faster disease progression or otherwise more appropriate for a particular aspect of human disease, for example.

[0146] Furthermore, animal models may be used to identify appropriate pharmaceutical compositions for the treatment of animal diseases, of interest in the treatment of companion animals and other high value animals, such as agricultural breeding stock and race horses.

[0147] Chimeric viruses may be derived from any retrovirus. For example, derived HIV-2, HTLV-I, HTLV-II, SIV, avian leukosis virus (ALV), endogenous avian retrovirus (EAV), mouse mammary tumor virus (MMTV), feline immunodeficiency virus (FIV), Bovine immunodeficiency virus (BIV), caprine arthritis encephalitis virus (CAEV), Equine infectious anemia virus (EIAV), Visna-maedi virus, or feline leukemia virus (FeLV).

[0148] Such chimeric viruses may be used in the methods of the invention described elsewhere herein. For example, such recombinant non-HIV-1 lentiviruses may be used in a method of identifying a compound which inhibit processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), the method consisting of comparing of the ability of said compound to inhibit replication of a wild-type non-HIV-1 lentivirus with the DSB-sensitive recombinant variant thereof. Such inhibition may occur in a cell; in an animal; or *in vitro*.

Construction And Use Of Viruses Or Polypeptides With Epitope Tags

[0149] The present invention is also drawn to recombinant retroviruses with epitope tags in the CA-SP1 region of Gag. Epitope tags may be inserted in the

CA domain and/or in the SP1 domain. Suitable tags are well known to those of ordinary skill in the art, and include haemagglutinin epitope HA (YPYDVPDYA) (SEQ ID NO: 81), bluetongue virus epitope VP7 (QYPALT) (SEQ ID NO: 82), α-tubulin epitope (EEF), Flag (DYKDDDDK) (SEQ ID NO: 83), and VSV-G (YTDIEMNRLGK) (SEQ ID NO: 84). Examples of SP1 containing epitope tags are illustrated in Figure 17.

[0150] Such epitope tagged viruses and fragments thereof are useful in identifying novel compounds that inhibit CA-SP1 processing *in vitro*, in cell based assays, and *in vivo*, including in animal models. Additional uses of such epitope tagged viruses and fragments thereof are described elsewhere herein.

Polynucleotides, Polypeptides and Antibodies of the Invention

[0151] The invention also includes isolated polypeptides and polynucleotides. In one embodiment, the invention includes polypeptides at least about 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% identical to an amino acid sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTACQ GVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTACQ GVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26);

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119).

[0152] In another embodiment the invention includes polynucleotides encoding the above polypeptides. Polynucleotides of the invention include degenerate variants, such as those that differ in the third base of the codon but nevertheless encodes the same amino acid due to coding "degeneracy".

[0153] The term "about" as used herein refers to a value that is 10% more or less than the stated value, and preferably is 5% more or less.

[0154] The polypeptides and polynucleotides of the invention are useful in the methods of the invention. In one aspect, they may be used in an *in vitro* assay to identify compounds that bind to the CA-SP1 region of Gag. In another, they may be used in the production of antibodies useful in other methods described elsewhere herein. In another, a polynucleotide may be inserted into a vector and thereupon into a host cell for production of polypeptide. The above embodiments are exemplary and are not intended to be limiting.

[0155] The present invention comprises a polynucleotide comprising a sequence which encodes an amino acid sequence containing a mutation in the HIV Gag p25 protein (CA-SP1), said mutation resulting in a decrease in the inhibition of processing of p25 (CA-SP1) to p24 (CA) by DSB. The polynucleotide of the invention includes a mutation which is optionally located at or near the CA-SP1 cleavage site or located in the SP1domain of CA-SP1. Said mutation can be present in an amino acid sequence that is selected from the group consisting of GHKARVLVEAMSQV (SEQ ID NO: 2) and SHKARILAEVMSQV (SEQ ID NO: 3). The polynucleotide of this invention is also drawn to sequences designated as SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9. The invention also includes a vector comprising said polynucleotide, a host cell comprising said vector and a method of producing said polypeptides comprising incubating said host cell in a medium and recovering the polypeptide from the medium.

[0156] The invention further includes a polynucleotide that hybridizes under stringent conditions to a polynucleotide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9. The invention also includes a polynucleotide which hybridizes to SEQ NO: 5, SEQ ID NO: 7 or SEQ ID NO: 10 or 12, which contains a mutation which results in

the decrease in the inhibition of processing of p25 to p24 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, and also wherein said mutation is optionally located at or near the CA-SP1 cleavage site or in the SP1 domain of CA-SP1. The invention is also directed to a vector comprising said polynucleotides, a host cell comprising said vector and a method of producing said polypeptides, comprising incubating said host cell in a medium and recovering said polypeptide from the medium.

[0157] "Near" or "adjacent," as used herein in reference to polypeptides is meant to include about 50, about 25, about 20, or about 15 residues from the point of reference. For example, near may encompass about 50, about 25, about 20 or about 15 residues on either side of the HIV-1 Gag CA-SP1 cleavage site; more preferably about ten residues on either side of the HIV-1 Gag CA-SP1 cleavage site; and most preferably about seven residues on either side of the HIV-1 Gag CA-SP1 cleavage site. In reference to polynucleotides, the terms "near" or "adjacent refer to about 150, about 75, about 60, about 45, or about 30 nucleotides from the point of reference.

[0158] "Isolated" means altered "by the hand of man" from the natural state. If an composition or substance occurs in nature, it has been changed or removed from its original environment, or both, when found in its "isolated" form. Also, "isolated" nucleic acid molecule(s) of the invention is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

[0159] "Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded

regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritiated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

[0160] "Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation,

covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[0161] "Mutant" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively. A typical mutant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the mutant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical mutant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A mutant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A mutant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a mutant that is not known to occur naturally. Non-naturally occurring mutants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

[0162] Thus, the mutant, (or fragments, derivatives or analogs) of a polypeptide encoded by any one of the polynucleotides described herein may be (i) one in which at least one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (a

conserved amino acid residue(s), or at least one but less than ten conserved amino acid residues) and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which one or more of the amino acid residues includes a substituent group, (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG:Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such mutants are deemed to be within the scope of those skilled in the art from the teachings herein. Polynucleotides encoding these mutants are also encompassed by the invention. "Mutant" as used herein is equivalent to the term "variant."

[0163] Substitutions of charged amino acids with another charged amino acids and with neutral or negatively charged amino acids are included. Additionally, one or more of the amino acid residues of the polypeptides of the invention (*e.g.*, arginine and lysine residues) may be deleted or substituted with another residue to eliminate undesired processing by proteases such as, for example, furins or kexins. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al., Clin Exp. Immunol. 2*:331-340 (1967); Robbins *et al., Diabetes 36*:838-845 (1987); Cleland *et al. Crit. Rev. Therapeutic Drug Carrier Systems 10*:307-377 (1993)). Thus, the polypeptides of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

[0164] As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 3).

TABLE 3. Conservative Amino Acid Substitutions

Aromatic	Phenylalanine Tryptophan

	Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

[0165] However, in some embodiments, it is desirable to use nonconservative substitutions of amino acids. For example nonconservative substitution of amino acids is used to render a DSB sensitive virus resistant to DSB.

[0166] The polynucleotides encompassed by this invention may have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% identity with a reference sequence, providing the reference polynucleotide encodes an amino acid sequence containing a mutation in the CA-SP1 protein, said mutation which results in the decrease in the inhibition of processing of p25 to p24 by a 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid. The polynucleotides also encompassed by this invention include those mutations which are "silent," in which different codons encode the same amino acid (wobble).

[0167] "Identity" is a measure of the identity of nucleotide sequences or amino acid sequences. The term "identity" is used interchangeably with the word "homology" herein. In general, the sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques. While there exist a number of methods to measure identity between two polynucleotide or polypeptide

sequences, the term "identity" is well known to skilled artisans. Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Baxevanis and Oullette, *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins, Second Edition*, Wiley-Interscience, New York, (2001). Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCS program package (Devereux, J. *et al., Nucleic Acids Research 12*(1):387, (1984)), BLASTP, BLASTN, FASTA (Atschul, S. F. *et al., J. Molec. Biol.* 215:403, (1990)).

[0168] A polynucleotide having a nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

[0169] Similarly, by a polypeptide having an amino acid sequence having at least, for example, 95% "identity" to a reference amino acid sequence, is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid. To obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference

sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence. The reference (query) sequence may be the entire nucleotide sequence of any one of the nucleotide sequences of the invention or any polynucleotide fragment (e.g., a polynucleotide encoding the amino acid sequence of the invention and/or C terminal deletion).

[0170] Whether any particular nucleic acid molecule having at least 40%, 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, 99% identity or which are identical to, for instance, the nucleotide sequences of the invention can be determined conventionally using known computer programs such as the BESTFIT program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). BESTFIT uses the local homology algorithm of Smith and Waterman, (*Advances in Applied Mathematics 2*:482-489 (1981)), to find the best segment of homology between two sequences. When using BESTFIT or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

[0171] In a specific embodiment, the identity between a sequence of the present invention and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag et al. (*Comp. App. Biosci. 6*:237-245 (1990)). Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the reference sequence because of 5' or 3'

deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. A determination of whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of this embodiment. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score. For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence, which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected. No other manual corrections are made for the purposes of this embodiment.

[0172] The present application is directed to nucleic acid molecules having at least about 40%, 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 96%, 97%,

98% or 99% identity or which is identical to the nucleic acid sequence disclosed herein, or fragments thereof, irrespective of whether they encode a polypeptide having the disclosed functional activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having the disclosed functional activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having the disclosed functional activity include, *inter alia*: (1) isolating the variants thereof in a cDNA library; (2) in situ hybridization (e.g., "FISH") to determine cellular location or presence of the disclosed sequences, and (3) Northern Blot analysis for detecting mRNA expression in specific tissues.

[0173] As used herein, the term "PCR" refers to the polymerase chain reaction that is the subject of U.S. Pat. Nos. 4,683,195 and 4,683,202 to Mullis *et al.*, as well as improvements now known in the art. In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual,*3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[0174] The term "stringent conditions," as used herein refers to homology in hybridization, is based upon combined conditions of salt, temperature, organic solvents, and other parameters typically controlled in hybridization reactions, and well known in the art (Sambrook, *et al. supra*). The invention includes an isolated nucleic acid molecule comprising, a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the sequence complementary to the coding and/or noncoding (i.e., transcribed, untranslated) sequence of any polynucleotide or a polynucleotide fragment as described herein. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising, or alternatively consisting of: 50% formamide, 5x SSC (750 mM NaCl, 75 mM trisodium

citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20μg/ml denatured, sheared salmon sperm DNA, followed by washing in 0.1x SSC at about 65°C. Polypeptides encoded by these polynucleotides are also encompassed by the invention.

[0175] The invention also includes a virus comprising the polynucleotides of the invention, and wherein the virus includes a retrovirus comprising said polynucleotides, and wherein the retrovirus may be a member of the group consisting of HIV-1, HIV-2, HTLV-I, HTLV-II, SIV, avian leukosis virus (ALV), endogenous avian retrovirus (EAV), mouse mammary tumor virus (MMTV), feline immunodeficiency virus (FIV), or feline leukemia virus (FeLV).

[0176] The invention further includes a polypeptide containing a mutation in the CA-SP1 protein, said mutation which results in the decrease in inhibition of processing of p25 to p24 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, and also wherein said mutation is optionally located at or near the CA-SP1 cleavage site or located in the SP1 domain of SEQ ID NO: 5 or SEQ ID NO: 7 (parental polynucleotide sequences) encoding the CA-SP1 protein. Said polypeptide may be encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9, or may comprise a sequence that is selected from the group consisting of GHKARVLVEAMSQV (SEQ ID NO: 2) and SHKARILAEVMSQV (SEQ ID NO: 3). The polypeptide of this invention may further be encoded by a polynucleotide which hybridizes to a polynucleotide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9. The invention also includes a polypeptide encoded by a polynucleotide which hybridizes to SEQ NO: 5, SEQ ID NO: 7 or SEQ ID NO: 10 or 12, which contains a mutation that results in decrease in inhibition of processing of p25 to p24 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, and also wherein said mutation is optionally located at or near the CA-SP1 cleavage site or in the SP1 domain of CA-SP1. The polypeptide of this invention further includes polypeptides that are part of a chimeric or fusion protein. Said chimeric proteins may be derived from species which include, but are not limited to: primates, including simian and human; rodentia, including rat and

mouse; feline; bovine; ovine; including goat and sheep; canine; or porcine. Fusion proteins may include synthetic peptide sequences, bifunctional antibodies, peptides linked with proteins from the above species, or with linker peptides. Polypeptides of the invention may be further linked with detectable labels; metal compounds; cofactors; chromatography separation tags, such as, but not limited to: histidine, protein A, or the like, or linkers; blood stabilization moieties such as, but not limited to: transferrin, or the like; therapeutic agents, and so forth.

[0177] The invention also includes an antibody which selectively binds an amino acid sequence containing a mutation in the CA-SP1 protein that results in a decrease in the inhibition of processing of p25 (CA-SP1) to p24 (CA) by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid and also wherein said mutation is optionally located at or near the CA-SP1 cleavage site or in the SP1 domain of CA-SP1. The invention also includes an antibody which selectively binds the polypeptide having a mutation which comprises a sequence that is one of GHKARVLVEAMSQV (SEQ ID NO: 2), SHKARILAEVMSQV (SEQ ID NO: 3)., Said antibody can selectively bind the polypeptide encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9. Said antibody can also selectively bind the polypeptide encoded by a polynucleotide which hybridizes under highly stringent conditions to a polynucleotide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9. The invention also includes an antibody that selectively binds SP1, which would enable one to distinguish SP1 from CA-SP1 (p25). The invention also includes an antibody that selectively binds CA (p24), which would enable one to distinguish CA from CA-SP1. The invention also includes an antibody that selectively binds CA-SP1, which would enable one to distinguish CA from CA-SP1. The invention additionally includes an antibody that selectively binds at or near the CA-SP1 cleavage site. The antibody of this invention may be a polyclonal antibody, a monoclonal antibody or said antibody may be chimeric or bifunctional, or part of a fusion protein. The invention further includes a portion of any antibody of this invention, including single chain,

light chain, heavy chain, CDR, $F(ab')_2$, Fab, Fab', Fv, sFv, or dsFv, or any combinations thereof.

[0178] As used herein, an antibody "selectively binds" a target peptide when it binds the target peptide and does not significantly bind to unrelated proteins. The term "selectively binds" also comprises determining whether the antibody selectively binds to the target mutant sequence relative to a native target sequence. An antibody which "selectively binds" a target peptide is equivalent to an antibody which is "specific" to a target peptide, as used herein. An antibody is still considered to selectively bind a peptide even if it also binds to other proteins that are not substantially homologous with the target peptide so long as such proteins share homology with a fragment or domain of the peptide target of the antibody. In this case, it would be understood that antibody binding to the peptide is still selective despite some degree of cross-reactivity. In another embodiment, the determination whether the antibody selectively binds to the mutant target sequence comprises: (a) determining the binding affinity of the antibody for the mutant target sequence and for the native target sequences; and (b) comparing the binding affinities so determined, the presence of a higher binding affinity for the mutant target sequence than for the native indicating that the antibody selectively binds to the mutant target sequence.

[0179] The invention is further drawn to an antibody immobilized on an insoluble carrier comprising any of the antibodies disclosed herein. The antibody immobilized on an insoluble carrier includes multiple well plates, culture plates, culture tubes, test tubes, beads, spheres, filters, electrophoresis material, microscope slides, membranes, or affinity chromatography medium.

[0180] The invention also includes labeled antibodies, comprising a detectable signal. The labeled antibodies of this invention are labeled with a detectable molecule, which includes an enzyme, a fluorescent substance, a chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, an electron dense substance, and a radioisotope, or any combination thereof.

[0181] The invention further includes a method of producing a hybridoma comprising fusing a mammalian myeloma cell with a mammalian B cell that

produces a monoclonal antibody which selectively binds an amino acid sequence containing a mutation in the CA-SP1 protein, said mutation resulting in a decrease in the inhibition of processing of p25 to p24 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid and a hybridoma producing any of the monoclonal antibodies disclosed herein. The invention further includes a method of producing an antibody comprising growing a hybridoma producing the monoclonal antibodies disclosed herein in an appropriate medium and isolating the antibodies from the medium, as is well known in the art. The invention also includes the production of polyclonal antibodies comprising the injection, either one injection or multiple injections of any of the polypeptides of the inventions into any animal known in the art to be useful for the production of polyclonal antibodies, including, but not limited to mouse, rat, hamster, rabbit, goat, sheep, deer, guinea pig, or primate, and recovering the antibodies in sera produced therein. The invention includes high avidity or high affinity antibodies produced therein. The invention also includes B cells produced from the listed species to be further used in cell fusion procedures for the manufacture of monoclonal antibody-producing hybridomas as disclosed herein.

[0182] The invention is further drawn to a kit comprising the antibody or a portion thereof as disclosed herein, a container comprising said antibody and instructions for use, a kit comprising the polypeptides of this invention and instructions for use and a kit comprising the polynucleotide of the invention, a container comprising said polynucleotide and instructions for use, or any combinations thereof. These kits would include, but not be limited to nucleic acid detection kits, which may, or may not, utilize PCR and immunoassay kits. Such kits are useful for clinical diagnostic use and provide standardized reagents as required in current clinical practice. These kits could either provide information as to the presence or absence of mutations prior to treatment or monitor the progress of the patient during therapy. The kits of the invention may also be used to provide standardized reagents for use in research laboratory studies.

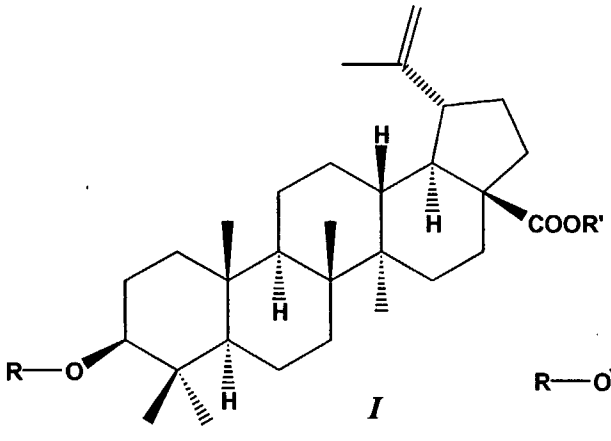
Compounds of the Invention

[0183] In one aspect, the invention is also directed to a compound, a method of using a compound, a method of identifying a compound and the like.

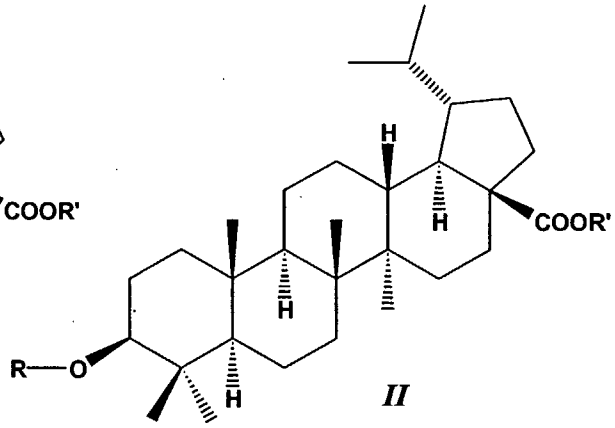
[0184] The term "a", "an" or "one", as used in the present invention may refer to either the singular or the plural. For example, "a compound" encompasses one or more compounds.

[0185] Compounds useful in the methods of the present invention include derivatives of betulinic acid and betulin that are presented in U.S. Patent Nos. 5,679,828 and 6,172,110 respectively, and in U.S. application Nos. 60/443,180 and 10/670,797, which are herein incorporated by reference. Additional useful compounds include oleanolic acid derivatives disclosed by Zhu *et al.* (*Bioorg. Chem Lett. 11*:3115-3118 (2001)); oleanolic acid and promolic acid derivatives disclosed by Kashiwada *et al.* (*J. Nat. Prod. 61*:1090-1095 (1998)); 3-*O*-acyl ursolic acid derivatives described by Kashiwada *et al.* (*J. Nat. Prod. 63*:1619-1622 (2000)); and 3-alkylamido-3-deoxy-betulinic acid derivatives, disclosed by Kashiwada *et al.* (*Chem. Pharm. Bull. 48*:1387-1390 (2000)). (All references incorporated by reference).

[0186] In some embodiments, compounds useful in the present invention include, but are not limited to those betulinic acid derivatives having the general Formula ***I*** and dihydrobetulinic acid derivatives of Formula ***II***:



I



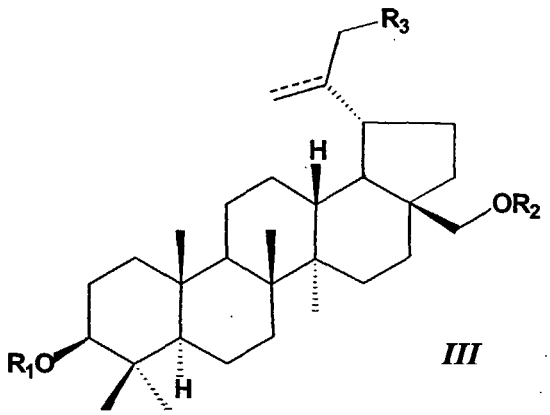
II

or a pharmaceutically acceptable salt thereof, wherein,

R is a C_2-C_{20} substituted or unsubstituted carboxyacyl,

R' is hydrogen, C_2-C_{10} substituted or unsubstituted alkyl, or aryl group. Preferred compounds are those wherein R is one of the substituents in Table 4, below, and R' is hydrogen.

[0187] In other embodiments, useful compounds include derivatives of betulin and dihydrobetulin of Formula III:



III

or a pharmaceutically acceptable salt thereof, wherein,

R_1 is a C_2-C_{20} substituted or unsubstituted carboxyacyl, or an ester thereof;

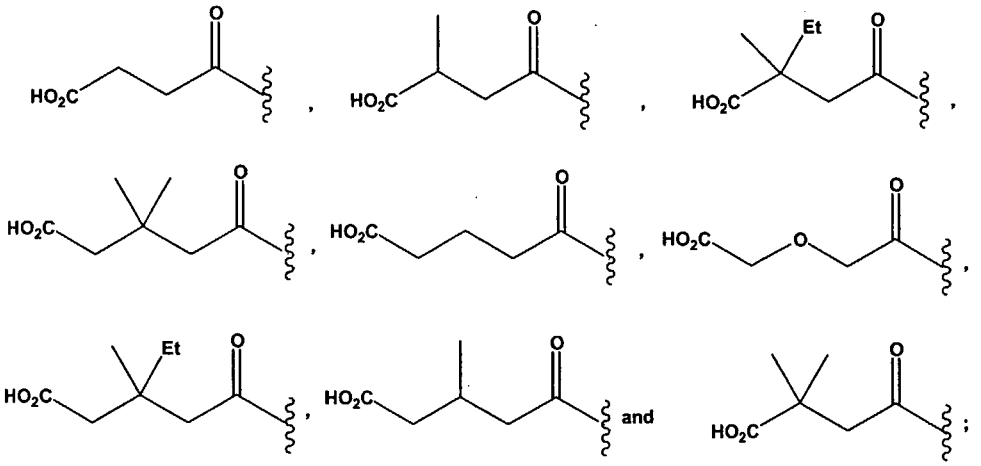
R_2 is hydrogen, $C(C_6H_5)_3$, or a C_2-C_{20} substituted or unsubstituted carboxyacyl; and

R_3 is hydrogen, halogen, amino, optionally substituted mono- or di-alkylamino, or -OR_4, where R_4 is hydrogen, C_{1-4} alkanoyl, benzoyl, or C_2-C_{20} substituted or unsubstituted carboxyacyl;

wherein the dashed line represents an optional double bond between C20 and C29.

[0188] Preferred compounds useful in the present invention are those where R_1 is one of the substituents in Table 4, R_2 is hydrogen or one of the substituents in Table 4 and R_3 is hydrogen.

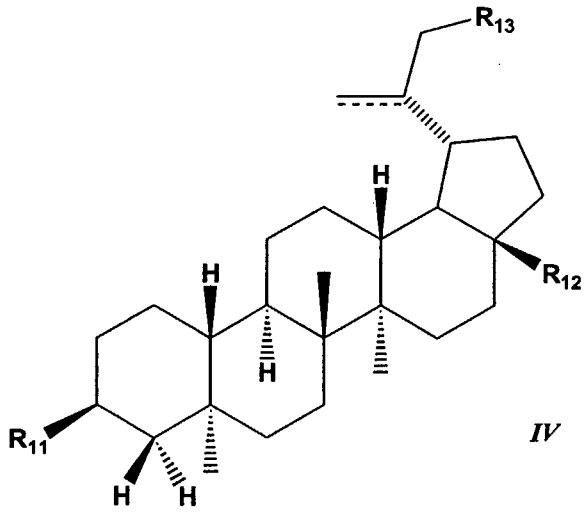
Table 4: Preferred Substituents for R, R', R_1, R_2:



[0189] More preferred compounds are 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, 3-*O*-(3',3'-dimethylsuccinyl) dihydrobetulinic acid, 3-*O*-(3',3'-dimethylsuccinyl) betulin, and 3-*O*-(3',3'-dimethylsuccinyl or glutaryl) dihydrobetulin.

[0190] A particularly preferred compound is 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid.

[0191] In some embodiments, compounds useful in the present invention are described by the Formulas *IV*, *V*, *VI* and *VII*.



IV

R_{11} = -OR_{14} or -NHR_{15};

R_{12} = $COOR_{17}$, COO^-A^+, or CH_2OR_{17}

R_{13} = -H, halogen, amino, optionally substituted mono-or di-alkylamino, or -OR_{16};

R_{14} = -H, C_2 - C_{20} substituted or unsubstituted carboxyacyl;

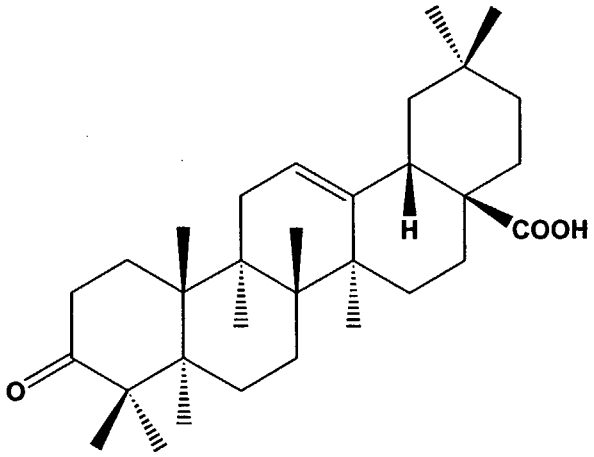
R_{15} = -H, C_2 - C_{20} substituted or unsubstituted carboxyacyl;

R_{16} = -H, C_4 - C_7 alkanoyl, benzyloyl, or C_2 - C_{20} substituted or unsubstituted carboxyacyl;

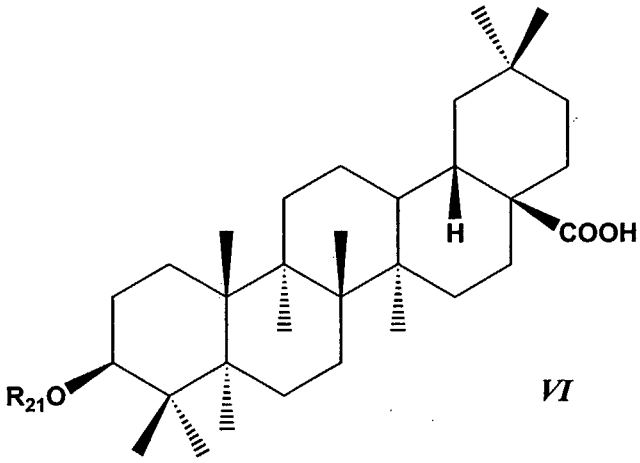
R_{17} = -H, $C(C_6H_5)_3$, or C_2 - C_{20} substituted or unsubstituted carboxyacyl;

wherein dashed line represents optional bond between C_{20} and C_{29}, and

wherein A = Na+, K^+, or other cation,



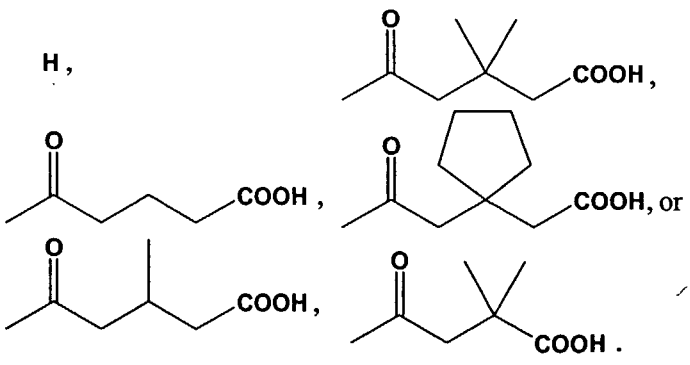
V

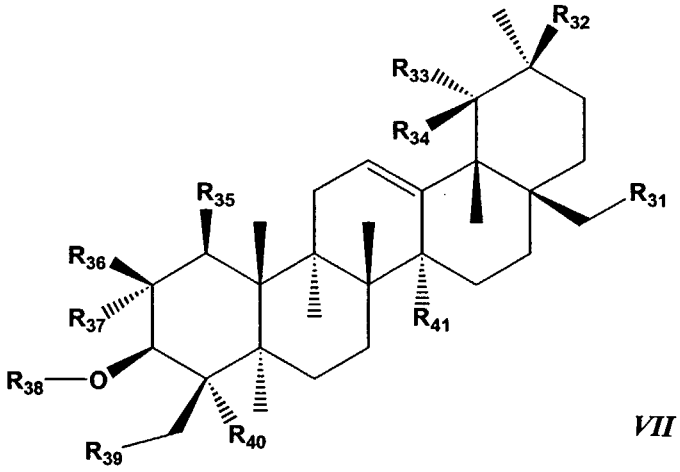


VI

wherein

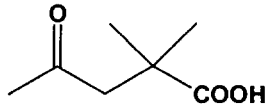
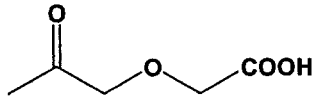
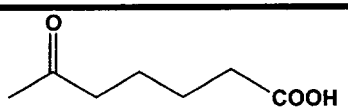
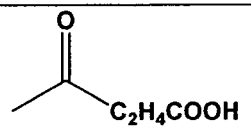
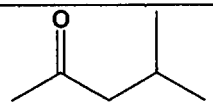
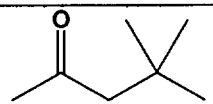
R_{21} =

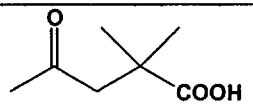
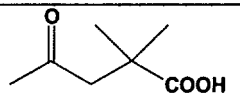




VII

	R_{31}	R_{32}	R_{33}	R_{34}	R_{35}	R_{36}	R_{37}	R_{38}	R_{39}	R_{40}	R_{41}
1	CH_2NH_2	H	H	H	H	H	H	H	H	H	H
2		H	H	H	H	H	H		H	H	H
3		H	H	H	H	H	H		H	H	H
4	COOH	H	H	H	H	H	H	H	H	H	H
5	COOH	H	H	H	H	H	H		H	H	H
6	COOH	H	H	H	H	H	H		H	H	H
7	COOH	H	H	H	H	H	H		H	H	H
8	COOH	H	H	H	H	H	H		H	H	H
9	COOH	H	H	H	H	H	H		H	H	H

	R_{31}	R_{32}	R_{33}	R_{34}	R_{35}	R_{36}	R_{37}	R_{38}	R_{39}	R_{40}	R_{41}
10	COOH	H	H	H	H	H	H		H	H	H
11	COOH	H	H	H	H	H	H		H	H	H
12	COOH	H	H	H	H	H	H		H	H	H
13	COOH	H	H	H	H	H	H		H	H	H
14	COOH	H	H	H	H	H	H		H	H	H
15	COOH	H	H	H	H	H	H		H	H	H
16	COOH	H	H	H	H	H	H	H	H	H	H
17	COOH	H	H	H	H	H	OH	H	H	OH	H
18	COOH	H	H	H	H	H	OH	H	OH	H	H
19	COOH	H	H	H	H	H	H	H	H	H	OH
20	COOH	H	OH	H	H	H	H	H	H	H	H
21	COOH	H	H	H	H	H	H	H	H	H	H

	R_{31}	R_{32}	R_{33}	R_{34}	R_{35}	R_{36}	R_{37}	R_{38}	R_{39}	R_{40}	R_{41}
22	COOH	H	H	H	H	H	OH	H	H	H	H
23	COOH	H	H	H	H	H	OH	H	OH	H	H
24	COOH	H	OH	H	H	H	OH	H	H	H	H
25	COOH	H	OH	H	H	O		H	H	H	H
26	COOH	H	OH	H	OH	O		H	H	H	H
27	COOH	H	OH	H	OH	OH	H	H	H	H	H
28	COOH	H	OH	H	OH	H	OH	H	H	H	H
29	COOH	H	OH	H	HH		OH	H	H	H	H
30	COOH	CH_3	H	H	H	H	H	H	H	H	H
31	COOH	CH_3	H	H	H	H	H		H	H	H
32	COOH	H	H	CH_3	H	H	H	H	H	H	H
33	COOH	H	H	H	H	H	H		H	H	H

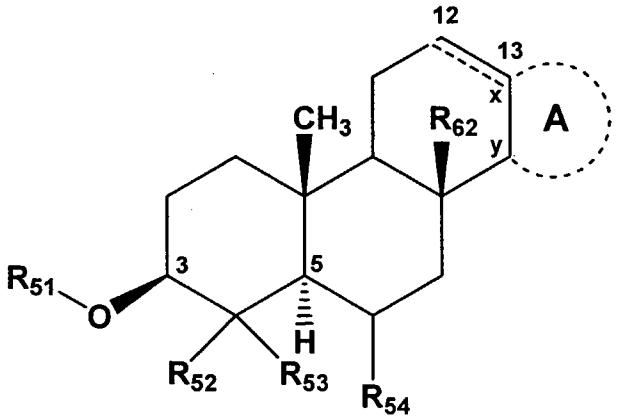
[0192] R_{38} moieties other than hydrogen are attached to R_{33} oxygen by a covalent bond to the carbonyl carbon.

[0193] Preferred compounds are those where R_{38} is not hydrogen.

[0194] In additional embodiments, any of R_{38}, R_{40} and/or R_{41} are methyl.

[0195] In some embodiments, compounds useful in the methods of the invention also include those described in U.S. Provisional Application No. 60/559,358, which is entirely incorporated by reference. In one aspect, these compounds are described by reference to the following compounds *VIII* to *XI:*

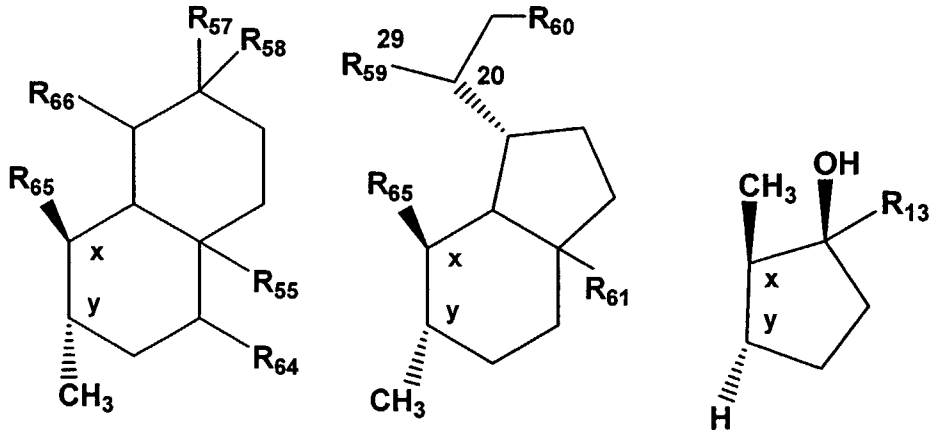
[0196] In some embodiments, compounds useful in the present invention have the general Formula *VIII*:



VIII

or a pharmaceutically acceptable salt or ester thereof:

wherein A is a fused ring of formula



or

(i) (ii) (iii)

wherein the ring carbons designated *x* and *y* in the formulas of A are the same as the ring carbons designated *x* and *y* in Formula *VIII*;

R_{51} is a carboxyalkanoyl, where the alkanoyl chain can be optionally substituted by one or more hydroxy or halo, or can be interrupted by a nitrogen, sulfur or oxygen atom, or combinations thereof;

R_{52}, R_{53} and R_{54} are independently hydrogen, methyl, halogen, or hydroxy, carbonyl or -$COOR_{66}$, wherein R_{66} is alkyl or carboxylalkyl, where the alkyl chain can be optionally substituted by one or more nydroxyl or halo, or can be interrupted by nitrogen, sulfur or oxygen atom, or combinations thereof;

R_{55} is carboxyalkoxycarbonyl, alkoxycarbonyl, alkanoyloxymethyl, carboxyalkanoyloxymethyl, alkoxymethyl or carboxyalkoxymethyl, any of which is optionally substituted by one or more hydroxy or halo, or R_{55} is a carboxyl or hydroxymethyl;

R_{56} is hydrogen, methyl, hydroxy or halogen;

R_{57} and R_{58} are independently hydrogen or C_{1-6} alkyl;

R_{59} is CH_2 or CH_3;

R_{60} is hydrogen, hydroxy or methyl;

R_{61} is methyl, methoxycarbonyl, carboxyalkoxycarbonyl, alkanoyloxymethyl, alkoxymethyl or carboxyalkoxymethyl, any of which is optionally substituted by one or more hydroxy or halo;

R_{62} is hydrogen or methyl;

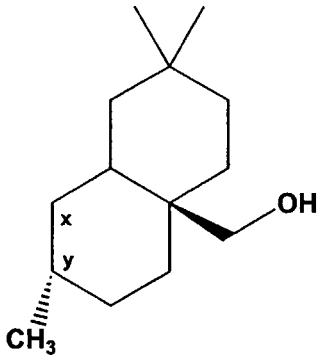
R_{63} is hydrogen or methyl;

R_{64} is hydrogen or hydroxy;

R_{65} is hydrogen if C12 and C13 form a single bond, or R_{65} is absent if C12 and C13 form a double bond; and

wherein the straight dashed line represents an optional double bond between C12 and C13 or C20 and C29;

with the proviso that when A is

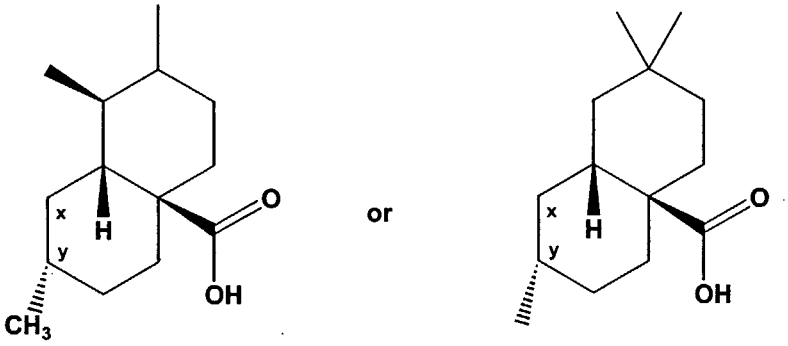


then R_{51} cannot be glutaryl or succinyl when a double bond exists between C12 and C13;

when A is (ii) and R_{61} is methyl, then R_{51} cannot be succinyl;

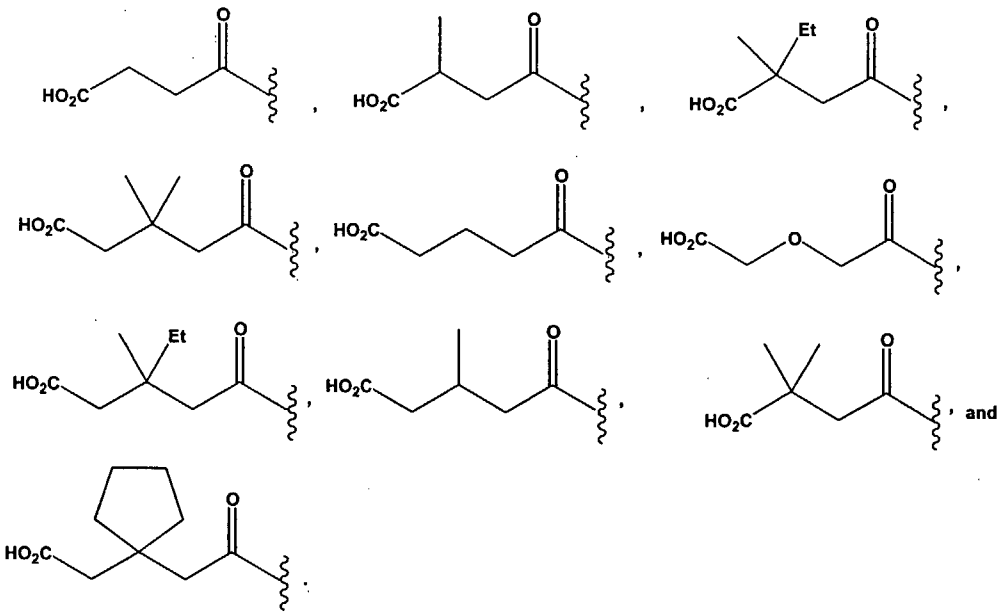
when A is (iii) and R_{52}, R_{53} and R_{63} are each hydrogen, then R_{51} cannot be succinyl; and

with the proviso that A (i) cannot be

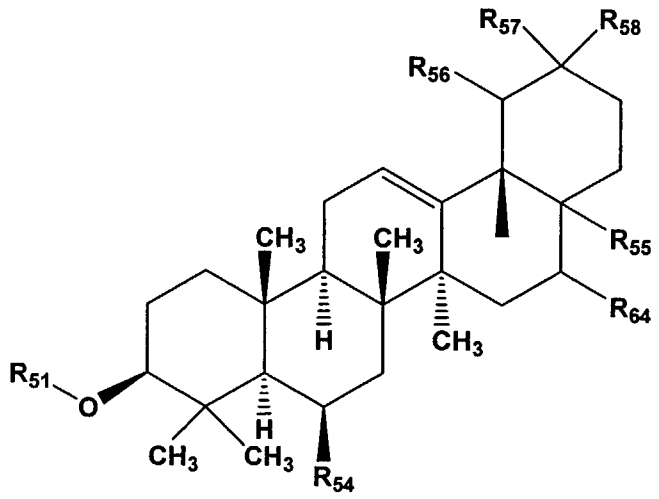


when R_{52} and R_{53} are both methyl and a double bond exists between C12 and C13.

[0197] In some embodiments, R_{51} is a carboxy(C_{2-6})alkylcarbonyl group or a carboxy(C_{2-6})alkoxy(C_{1-6})alkylcarbonyl group. Suitable groups are selected from the group consisting of:



[0198] According to the invention, in some embodiments the compounds have Formula *IX*:

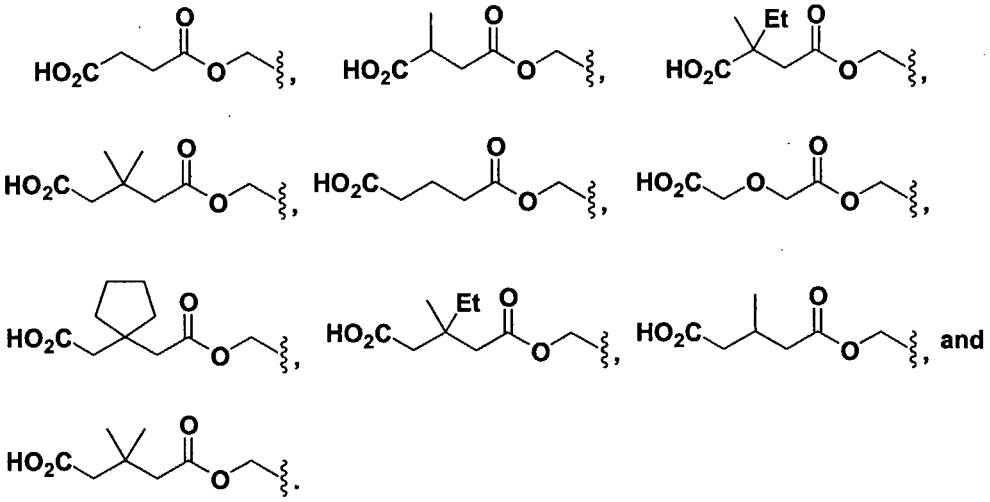


IX

wherein R_{51}, R_{54}, R_{55}, R_{56}, R_{57}, R_{58} and R_{64} are as defined above for Formula *VIII*. In one embodiment, R_{56} is β-methyl, R_{58} is hydrogen, R_{55} is hydroxymethyl and R_{51} is 3',3'-dimethylglutaryl, 3',3'-dimethylsuccinyl, glutaryl or succinyl. In another embodiment, R_{56} is hydrogen, R_{57} and R_{58} are both methyl, R_{55} is carboxyl and R_{51} is 3',3'-dimethylglutaryl, 3',3'-dimethylsuccinyl, glutaryl or succinyl.

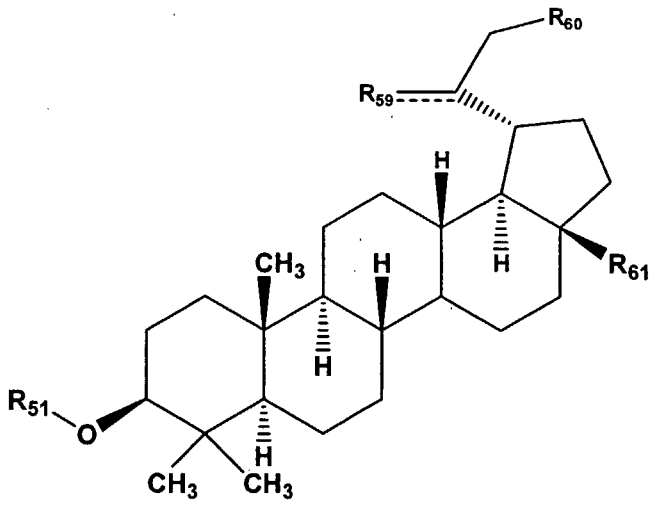
[0199] In some embodiments, R_{55} is carboxyalkoxycarbonyl, alkoxycarbonyl, alkanoyloxymethyl, carboxyalkanoyloxymethyl, alkoxymethyl or carboxyalkoxymethyl, any of which is optionally substituted by one or more

hydroxy or halo, or R_{55} is a carboxyl or hydroxymethyl. In some embodiments, R_{55} is selected from a group consisting of carboxyl, hydroxymethyl, $-CO_2(CH_2)_nCOOH$, $-CO_2(CH_2)_nCH_3$, $-CH_2OC(O)(CH_2)_nCH_3$, $-CH_2OC(O)(CH_2)_nCOOH$, $-CO(CH_2)_nCH_3$ and $-CO(CH_2)_nCOOH$. In some embodiments, R_{55} is selected from a group consisting of



[0200] In some embodiments, R_{55} is hydroxymethyl. In some embodiments, R_{55} is carboxyl. In some embodiments, n is from 0 to 20, and preferably 0 to 6. In some embodiments, n is from 1 to 10. In some embodiments, n is from 2 to 8. In some embodiments, n is from 1 to 6. In some embodiments, n is from 2 to 6.

[0201] In some embodiments, compounds useful in the present invention have the Formula *X*:



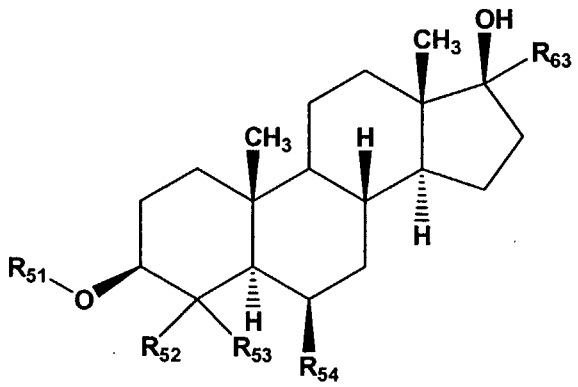
X

wherein R_{51}, R_{59}, R_{60}, and R_{61} are as defined above for Formula *VIII*. In one embodiment, R_{51} is 3',3'-dimethylglutaryl, 3',3'-dimethylsuccinyl, glutaryl or succinyl.

[0202] In some embodiments, R_{61} is methyl, methoxycarbonyl, carboxyalkoxycarbonyl, alkanoyloxymethyl, alkoxymethyl or carboxyalkoxymethyl, any of which is optionally substituted by one or more hydroxy or halo. In some embodiments, R_{61} is selected from the group consisting of methyl, $-CO_2(CH_2)_nCOOH$, $-COC(O)(CH_2)_nCH_3$, $-CO(CH_2)_nCH_3$ and $-CO(CH_2)_nCOOH$.

[0203] In some embodiments, n is from 0 to 20, or preferably 0 to 6. In some embodiments, n is from 1 to 10. In some embodiments, n is from 2 to 8. In some embodiments, n is from 1 to 6. In some embodiments, n is from 2 to 6. In some embodiments, R_{61} is methyl. In some embodiments, R_{61} is methoxycarbonyl. In some embodiments, R_{61} is selected from the group consisting of methoxymethyl and ethoxymethyl. In some embodiments, methyl groups found in R_{61} can be substituted with a halogen or a hydroxy.

[0204] In some embodiments, the compounds useful in the present invention have Formula *XI*:



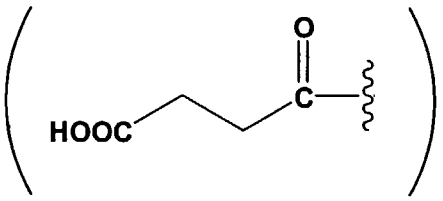
XI

wherein R_{51}, R_{52}, R_{53}, R_{54}, and R_{63} are as defined above for Formula *VIII*. In one embodiment, R_{51} is 3',3'-dimethylglutaryl, 3',3'-dimethylsuccinyl, glutaryl or succinyl. In one embodiment, both R_{52} and R_{53} are methyl.

[0205] Any triterpene which falls within the scope of Formula *VIII* can be used. According to the invention, in some embodiments the compounds of Formula *VIII* are selected from the group consisting of derivatives of uvaol, ursolic acid, erythrodiol, echinocystic acid, oleanolic acid, sumaresinolic acid,

lupeol, dihydrolupeol, betulinic acid methylester, dihydrobetulinic acid methylester, 17-α-methyl-androstanediol, androstanediol, and 4,4-dimethyl-androstanediol.

[0206] In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein R_{52} and R_{53} are both methyl. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein R_{51} is 3',3'-dimethylsuccinyl. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein R_{51} is succinyl, i.e.,

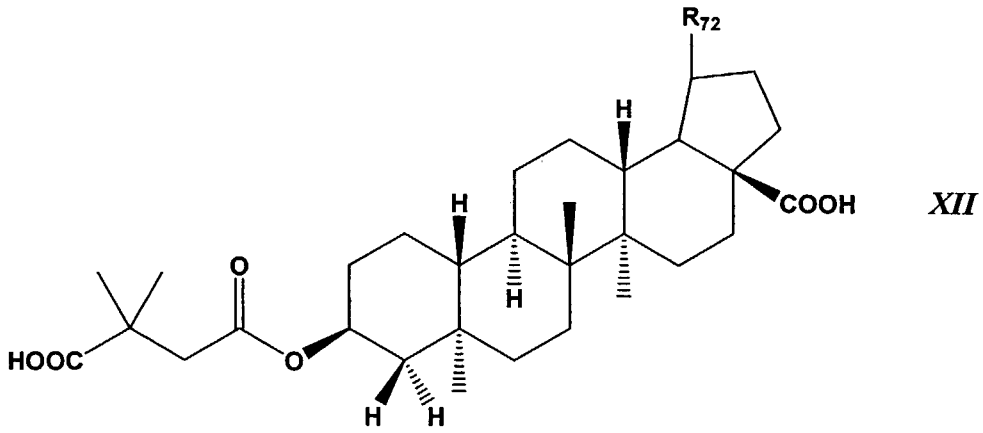


[0207] According to the invention, in some embodiments the stereochemistry of the sidechain substituents is important. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein A is (i) and R_{55} is in the β position. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein A is (i) and R_{56} is in the β position. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein A is (i) and R_{64} is in the α position. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein A is (i), R_{57} is α-methyl, and R_{58} is hydrogen. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein A is (i), R_{58} is α-methyl, and R_{57} is hydrogen. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein A is (i) and both R_{57} and R_{58} are methyl. In some embodiments, the compounds of the present invention are defined as in Formula *VIII*, wherein A is (ii) and R_{61} is in the β position.

[0208] In some embodiments, 3',3'-dimethylsuccinyl is at the C3 position. In some embodiments, the compounds of Formula *IX* are 3-*O*-(3',3'-dimethylsuccinyl)uvaol; 3-*O*-(3',3'-dimethylsuccinyl)erythrodiol; 3-*O*-(3',3'-dimethylsuccinyl)echinocystic acid or 3-*O*-(3',3'-dimethylsuccinyl) sumaresinolic acid. In some embodiments, the compounds of Formula *X* are

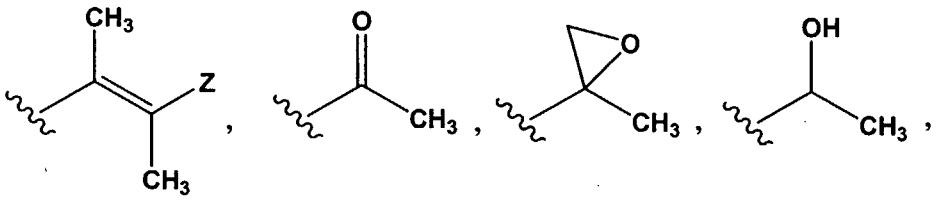
3-*O*-(3',3'-dimethylsuccinyl) lupeol; 3-*O*-(3',3'-dimethylsuccinyl) dihydrolupeol; 3-*O*-(3',3'-dimethylsuccinyl)17β-methylester-betulinic acid; or 3-*O*-(3',3'-dimethylsuccinyl)17β-methylester-dihydrobetulinic acid. In some embodiments, the compounds of Formula ***XI*** are 3-*O*-(3',3'-dimethylsuccinyl) 4,4-dimethylandrostanediol; 3-*O*-(3',3'-dimethylsuccinyl)17α-methylandrostanediol; 3-*O*-(3',3'-dimethylsuccinyl) androstanediol.

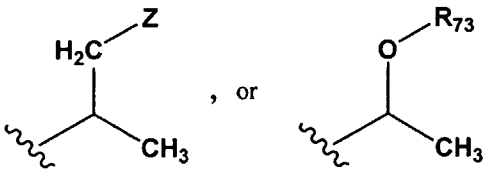
[0209] In an additional embodiment, the invention includes compounds and methods that use compounds of Formula ***XII***:



XII

where R_{72} is one of:



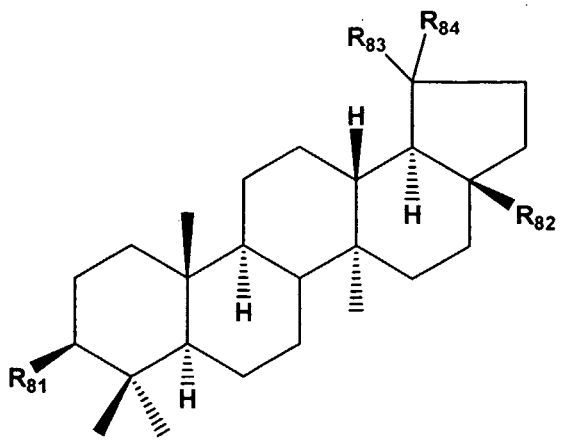


wherein

Z is hydroxy or halogen; and

R_{73} is lower alkyl, such as methyl, ethyl or propyl.

[0210] In additional embodiments, compounds useful in the present invention are betulin derivative compounds of Formula ***XIII***:

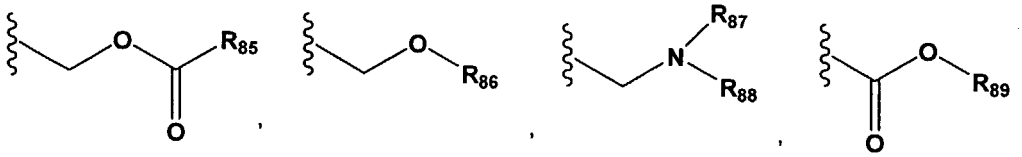


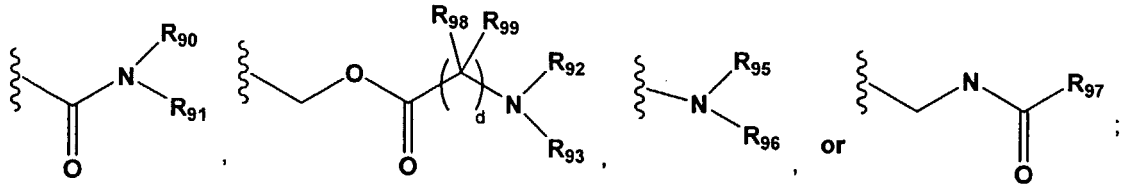
XIII

or a pharmaceutically acceptable salt or prodrug thereof, wherein:

R_{51} is C_3-C_{20} alkanoyl, carboxyalkanoyl, carboxyalkenoyl, alkoxycarbonylalkanoyl, alkenyloxycarbonylalkanoyl, cyanoalkanoyl, hydroxyalkanoyl, aminocarbonylalkanoyl, hydroxyaminocarbonylalkanoyl, monoalkylaminocarbonylalkanoyl, dialkylaminocarbonylalkanoyl, heteroarylalkanoyl, heterocyclylalkanoyl, heterocyclylcarbonylalkanoyl, heteroarylaminocarbonylalkanoyl, heterocyclylaminocarbonylalkanoyl, cyanoaminocarbonylalkanoyl, alkylsulfonylaminocarbonylalkanoyl, arylsulfonylaminocarbonylalkanoyl, sulfoaminocarbonylalkanoyl, phosphonoaminocarbonylalkanoyl, tetrazolylalkanoyl, phosphono, sulfo, phosphonoalkanoyl, sulfoalkanoyl, alkylsulfonylalkanoyl, or alkylphosphonoalkanoyl;

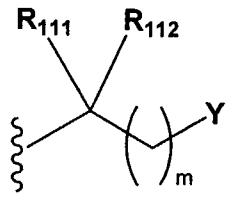
R_{82} is formyl, carboxyalkenyl, heterocyclyl, heteroaryl, $-CH_2SR_{94}$,





R_{83} is hydroxyl, isopropenyl, isopropyl, 1'-hydroxyisopropyl, 1'-haloisopropyl, 1'-thioisopropyl, 1'-trifluoromethylisopropyl, 2'-hydroxyisopropyl, 2'-haloisopropyl, 2'-thioisopropyl,

2'-trifluoromethylisopropyl, 1'-hydroxyethyl, 1'-(alkoxy)ethyl, 1'-(alkoxyalkoxy)ethyl, 1'-(arylalkoxy)ethyl; 1'-(arylcarbonyloxy)ethyl, 1'-(oxo)ethyl, 1'-(hydroxyl)-1'-(hydroxyalkyl)ethyl, 1'(oxo)oxazolidinyl, 1',2'-epoxyisopropyl, 2'-haloisopropenyl, 2'-hydroxyisopropenyl, 2'-aminoisopropenyl, or



;

wherein Y is $-SR_{111}$or $-NR_{113}R_{114}$;

R_{111} is methyl;

R_{112} is hydrogen or hydroxyl;

R_{113} and R_{114} are independently hydrogen, alkyl, alkanoyl, arylalkyl, heteroarylalkyl, arylsulfonyl or arylaminocarbonyl; or

R_{113} and R_{114} can be taken together with the nitrogen to which they are attached to form a heterocycle, wherein the heterocycle can optionally include one or more additional nitrogen, sulfur or oxygen atoms;

m is zero to three;

R_{84} is hydrogen; or

R_{83} and R_{84} can be taken together to form oxo, alkylimino, alkoxyimino or benzyloxyimino;

R_{85} is C_2-C_{20} alkyl, alkenyl, C_2-C_{20} carboxyalkyl, amino, aminoalkyl, monoalkylaminoalkyl, dialkylaminoalkyl, alkoxyalkyl, cyano, cyanoalkyl, alkylthioalkyl, cycloalkyl, cycloalkylalkyl, aryl, arylalkyl, heterocyclyl, heteroaryl, heterocyclylalkyl, heteroarylalkyl, sulfo, phosphono, sulfoalkyl, phosphonoalkyl, alkylsulfonyl, alkylphosphono, alkanoylaminoalkyl, aminocarbonylalkyl, alkylaminocarbonylalkyl, dialkylaminocarbonylalkyl, heterocyclylcarbonylalkyl, cycloalkylcarbonylalkyl, heteroarylalkylaminocarbonylalkyl, arylalkylaminocarbonylalkyl, heterocyclylalkylaminocarbonylalkyl, carboxyalkylaminocarbonylalkyl, arylsulfonylaminocarbonylalkyl, alkylsulfonylaminocarbonylalkyl, arylphosphonoaminocarbonylalkyl, alkylphosphonoaminocarbonylalkyl, or hydroxyimino(amino)alkyl;

R_{86} is hydrogen, phosphono, sulfo, cyano, alkyl, sulfoalkyl, phosphonoalkyl, alkylsulfonyl, alkylphosphono, cycloalkyl, heterocyclyl, aryl, heteroaryl, carboxyalkyl, alkoxycarbonylalkyl, or cyanoalkyl;

R_{87} or R_{88} are independently hydrogen, alkyl, aminoalkyl, monoalkylaminoalkyl, dialkylaminoalkyl, carboxyalkyl, alkoxyalkyl, alkoxyalkoxyalkyl, alkoxycarbonylaminoalkoxyalkyl, alkoxycarbonylaminoalkyl, aminoalkoxyalkyl, alkylcarbonylaminoalkyl, heterocyclyl, heterocyclylalkyl, aryl, arylalkyl, arylcarbonylaminoalkyl, or cycloalkyl, or R_{87} and R_{88} can together with the nitrogen atom to which they are attached form a heterocyclyl or heteroaryl group, wherein the heterocyclyl or heteroaryl can optionally include one or more additional nitrogen, sulfur or oxygen atoms;

R_{89} is hydrogen, phosphono, sulfo, cyano, alkyl, alkylsilyl, cycloalkyl, carboxyalkyl, alkoxycarbonyloxyalkyl, aminoalkyl, monoalkylaminoalkyl, dialkylaminoalkyl, cyanoalkyl, phosphonoalkyl, sulfoalkyl, alkylsulfonyl, alkylphosphono, aryl, heteroaryl, heterocyclyl, arylalkyl, heteroarylalkyl, heterocyclylalkyl, or dialkoxyalkyl;

R_{90} and R_{91} are independently hydrogen, hydroxyl, cyano, alkyl, amino, aminoalkyl, monoalkylaminoalkyl, dialkylaminoalkyl, carboxyl, carboxyalkyl, alkanoyloxyalkyl, alkoxyalkyl, alkoxyalkoxyalkyl, alkoxycarbonylaminoalkoxyalkyl, alkoxycarbonylaminoalkyl, aminoalkoxyalkyl, alkylcarbonylaminoalkyl, heterocyclyl, heterocyclylalkyl, aryl, arylalkyl, arylcarbonylaminoalkyl, arylsulfonyl, or cycloalkyl, or alkyl interrupted by one or more oxygen atoms, or R_{90} and R_{91} can together with the nitrogen atom to which they are attached form a heterocyclyl group, wherein the heterocyclyl can optionally include one or more additional nitrogen, sulfur or oxygen atoms;

R_{92} and R_{93} are independently hydrogen, alkyl, alkoxycarbonyl, alkoxyaminoalkyl, cycloalkyloxy, heterocyclylaminoalkyl, cycloalkyl, cyanoalkyl, cyano, sulfo, phosphono, sulfoalkyl, phosphonoalkyl, alkylsulfonyl, alkylphosphono, alkoxyalkyl, heterocyclylalkyl, or R_{92} and R_{93} can together with the nitrogen atom to which they are attached form a heterocyclyl group, wherein the heterocyclyl can optionally include one or

more additional nitrogen, sulfur or oxygen atoms, or R_{92} and R_{93} can together with the nitrogen atom to which they are attached form an alkylazo group, and b is one to six;

R_{94} is hydrogen, alkyl, alkenyl, arylalkyl, carboxyalkyl, carboxyalkenyl, alkoxycarbonylalkyl, alkenyloxycarbonylalkyl, cyanoalkyl, hydroxyalkyl, carboxybenzyl, aminocarbonylalkyl;

R_{95} and R_{96} are independently hydrogen, alkyl, alkoxycarbonyl, alkoxyaminoalkyl, cycloalkyloxy, heterocyclylaminoalkyl, cycloalkyl, cyanoalkyl, cyano, sulfo, phosphono, sulfoalkyl, phosphonoalkyl, alkylsulfonyl, alkylphosphono, alkoxyalkyl, heterocyclylalkyl, or R_{95} and R_{96} can together with the nitrogen atom to which they are attached form a heterocyclyl group, wherein the heterocyclyl can optionally include one or more additional nitrogen, sulfur or oxygen atoms, or R_{95} and R_{96} can together with the nitrogen atom to which they are attached form an alkylazo group;

R_{97} is hydrogen, alkyl, alkenyl, carboxyalkyl, amino, aminoalkyl, monoalkylaminoalkyl, dialkylaminoalkyl, alkoxyalkyl, alkoxycarbonyl, cyanoalkyl, alkylthioalkyl, cycloalkyl, cycloalkylalkyl, aryl, arylalkyl, heterocyclyl, heteroaryl, heterocyclylalkyl, heteroarylalkyl, alkanoylaminoalkyl, aminocarbonylalkyl, alkylaminocarbonylalkyl, dialkylaminocarbonylalkyl, heterocyclylcarbonylalkyl, cycloalkylcarbonylalkyl, heteroarylalkylaminocarbonylalkyl, arylalkylaminocarbonylalkyl, heterocyclylalkylaminocarbonylalkyl, carboxyalkylaminocarbonylalkyl, arylsulfonylaminocarbonylalkyl, alkylsulfonylaminocarbonylalkyl, or hydroxyimino(amino)alkyl;

R_{98} and R_{99} are independently hydrogen, methyl or ethyl, preferably hydrogen or methyl; and d is from one to six.

[0211] Alkyl groups and alkyl containing groups of the compounds of the present invention can be straight chain or branched alkyl groups, preferably having one to ten carbon atoms. In some embodiments, the alkyl groups or alkyl containing groups of the present invention can be substituted with a C_{3-7} cycloalkyl group. In some embodiments, the cycloalkyl group may include, but is not limited to, a cyclobutyl, cyclopentyl or cyclohexyl group.

[0212] Also, included within the scope of the present invention are the non-toxic pharmaceutically acceptable salts of the compounds of the present invention. These salts can be prepared *in situ* during the final isolation and purification of the compounds or by separately reacting the purified compound in its free acid form with a suitable organic or inorganic base and isolating the salt thus formed. These can include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium and amine cations including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, ethylamine, N-methyl glucamine and the like.

[0213] Also, included within the scope of the present invention are the non-toxic pharmaceutically acceptable esters of the compounds of the present invention. Ester groups are preferably of the type which are relatively readily hydrolyzed under physiological conditions. Examples of pharmaceutically acceptable esters of the compounds of the invention include C_{1-6} alkyl esters wherein the alkyl group is a straight or branched chain. Acceptable esters also include C_{5-7} cycloalkyl esters as well as arylalkyl esters, such as, but not limited to benzyl. C_{1-4} alkyl esters are preferred. In some embodiments, the esters are selected from the group consisting of alkylcarboxylic acid esters, such as acetic acid esters, and mono- or dialkylphosphate esters, such as methylphoshate ester or dimethylphosphate ester. Esters of the compounds of the present invention can be prepared according to conventional methods.

[0214] Certain compounds are listed above derivatives referred to as "prodrugs". This includes compounds within the scope of Formula ***VIII*** to ***XI***, for example. The expression "prodrug" refers to compounds that are rapidly transformed *in vivo* by an enzymatic or chemical process, to yield the parent compound of the above formulas, for example, by hydrolysis in blood. A thorough discussion is provided by Higuchi, T. and V. Stella in *Pro-drugs as Novel Delivery Systems, Vol. 14*, A.C.S. Symposium Series, and in *Bioreversible Carriers in Drug Design*, Ed. Edward B. Roche, American Pharmaceutical Association, Pergamon Press, 1987. Useful prodrugs can be esters, for example, of the compounds of Formulae ***VIII, IX, X***, and ***XI***. In

some prodrug embodiments, a lower alkyl group is substituted with one or more hydroxy or halo groups by a suitable acid. Suitable acids include, *e.g.*, carboxylic acids, sulfonic acids, phosphoric acid or lower alkyl esters thereof, and phosphonic acid or lower alkyl esters thereof. For example, suitable carboxylic acids include alkylcarboxylic acids, such as acetic acid, arylcarboxylic acids and arylalkylcarboxylic acids. Suitable sulfonic acids include alkylsulfonic acids, arylsulfonic acids and arylalkylsulfonic acids. Suitable phosphoric and phosphonic acid esters are methyl or ethyl esters.

[0215] In some embodiments, the C3 acyl groups having dimethyl groups or oxygen at the C3' position can be the most active compounds. This observation suggests that these types of acyl groups might be important to the enhanced anti-HIV activity.

[0216] In one embodiment, the invention is drawn to a method of treating HIV-1 infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is a compound of Formula ***I*** through ***XIII***

[0217] In one embodiment, the invention is drawn to a method of treating HIV-1 infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is a compound of Formula ***I*** through ***XIII***, with the exception of DSB.

[0218] In one embodiment, the invention is drawn to a method of treating HIV-1 infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is other than a compound of Formula ***I*** through ***VII***, or is other than a compound of Formula ***I*** through ***XIII***. In one embodiment, the invention is drawn to a method of treating HIV-1 infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is other than a compound of Formula ***I*** through ***XI***; or in other embodiments is other than ***I*** through ***XIII***.

[0219] In another embodiment, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is a compound of Formula Groups ***I*** through ***XIII***.

[0220] In another embodiment, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is other than a compound of Formula ***I*** through ***VII***; or in other embodiments is other than a compound of Formula ***I*** through ***XIII***.

[0221] In another embodiment, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is other than a compound of Formula ***I*** through ***XI***; or in other embodiments is other than a compound of Formula ***I*** through ***XIII***.

[0222] In another embodiment, the invention is drawn to a method of treating human blood or blood products by inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is a compound of Formula ***I*** through ***XIII***.

[0223] In another embodiment, the invention is drawn to a method of treating human blood or blood products by inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is other than a compound of Formula ***I*** through ***VII***; or in other embodiments is other than a compound of Formula ***I*** through ***XIII***.

Synthesis of Dsb and Related Compounds

[0224] Reaction of betulinic acid and dihydrobetulinic acid with dimethylsuccinic anhydride produced a mixture of 3-*O*-(2',2'-dimethylsuccinyl) and 3-*O*-(3',3'-dimethylsuccinyl)-betulinic acid and dihydrobetulinic acid, respectively. The mixtures were successfully separated by preparative scale HPLC yielding pure samples. The structures of these isomers were assigned by long-range ^{1}H-^{13}C COSY examinations.

[0225] The derivatives of betulinic acid and dihydrobetulinic acid of the present invention were all synthesized by refluxing a solution of betulinic acid or dihydrobetulinic acid, dimethylaminopyridine (1 equivalent mol), and an appropriate anhydride (2.5-10 equivalent mol) in anhydrous pyridine (5-10 mL). The reaction mixture was then diluted with ice water and extracted with $CHCl_3$. The organic layer was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed using silica gel column or semi-preparative-scale HPLC to yield the product.

[0226] Preparation of 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid: yield 70% (starting with 542 mg of betulinic acid); crystallization from MeOH gave colorless needles; mp 274°-276°C.; $[\alpha]_D^{19}$+23.5° (c=0.71), $CHCl_3$-MeOH [1:1]); Positive FABMS m/z 585 $(M+H)^+$; Negative FABMS m/z 583 $(M-H)^-$; HR-FABMS calcd for $C_{36}H_{57}O_6$ 585.4155, found m/z 585.4161; ^1H NMR (pyridine-d_5): 0.73, 0.92, 0.97, 1.01, 1.05 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.55 (6H, s, 3'-CH_3 x 2), 1.80 (3H, s, 20-CH_3), 2.89, 2.97 (each 1H, d, J=15.5 Hz, H-2'), 3.53 (1H, m, H-19), 4.76 (1H, dd, J=5.0, 11.5 Hz, H-3), 4.78, 4.95 (each 1H, br s, H-30).

[0227] 3-*O*-(3',3'-dimethylsuccinyl) dihydrobetulinic acid: yield 24.5% (starting with 155.9 mg of dihydrobetulinic acid); crystallization from MeOH-H_2O gave colorless needles; mp 291°-292°C.; $[\alpha]_D^{20}$-13.4° (c=1.1, $CHCl_3$–MeOH [1:1], ^1H NMR (pyridine-d_5): 0.85, 0.94 (each 3H, d, J=7.0 Hz; 20-$(CH_3)_2$), 0.75, 0.93, 0.97, 1.01, 1.03 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.55 (6H, s; 3'-CH_3 x 2), 2.89, 2.97 (each 1H, d, J=15.5 Hz; H-2'), 4.77 (1H, dd, J=5.0, 11.0 Hz, H-3); Anal. Calcd for $C_{36}H_{58}O_6.5/2H_2O$: C 68.43, H 10.04; found C 68.64, H 9.78.

[0228] The synthesis of 3-*O*-(3',3'-dimethylglutaryl) betulinic acid was disclosed U.S. Patent No. 5,679,828, as COMPOUND NO. 4.

[0229] 3-*O*-(3',3'-dimethylglutaryl) dihydrobetulinic acid: yield 93.3% (starting with 100.5 mg of dihydrobetulinic acid); crystallization from needles MeOH-H_2O gave colorless needles; mp 287°-289°C.; $[\alpha]_D^{20}$-17.9° (c=0.5, $CHCl_3$-MeOH[1:1]); ^1H-NMR (pyridine-d_5): 0.86, 0.93 (each 3H, d, J=6.5 Hz; 20-$(CH_3)_2$), 0.78, 0.92, 0.96, 1.02, 1.05 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 1.38, 1.39 (each 3H, s; 3'-CH_3 x 2), 2.78 (4H, m, H_2 -2' and 4'), 4.76

(1H, dd, J=4.5, 11.5 Hz; H-3). Anal. Calcd for $C_{37}H_{60}O_6$: C 73.96, H 10.06; found C 73.83, H 10.10.

[0230] The synthesis for 3-*O*-diglycolyl-betulinic acid was disclosed in U.S. Patent No. 5,679,828, as COMPOUND NO. 5.

[0231] 3-*O*-diglycolyl-dihydrobetulinic acid: yield 79.2% (starting with 103.5 mg of dihydrobetulinic acid); an off-white amorphous powder; $[\alpha]_D^{20}$-9.8° (c=1.1, $CHCl_3$ –MeOH[1:1]); ^{1}H-NMR (pyridine-d_5): 0.79, 0.87 (each 3H, d, J=6.5 Hz; 20-$(CH_3)_2$), 0.87, 0.88, 0.91, 0.98, 1.01 (each 3H, s; 4-$(CH_3)_2$, 8-CH_3, 10-CH_3, 14-CH_3), 4.21, 4.23 (each 2H, s, H_2-2' and 4'), 4.57 (1H, dd, J=6.5, 10.0 Hz, H-3); Anal. Calcd for $C_{34}H_{54}O_7.2H_2O$: C 66.85, H 9.57; found C 67.21, H 9.33.

[0232] The syntheses of 3-*O*-(3',3'-dimethylsuccinyl) betulin and 3-*O*-(3',3'-dimethylglutaryl) betulin were disclosed in U.S. Application 10/670,797.

Methods Of Inhibiting Hiv With A Compound

[0233] Methods of "inhibiting HIV" or "inhibition of HIV" as used herein, means any interference in, inhibition of, and/or prevention of HIV using the methods of the invention. As such, methods of inhibition are useful in inhibiting with the infectivity of HIV, inhibition of p25 processing, inhibition of viral maturation, formation of virions that exhibit altered phenotypes, and the like. Preferably, methods of the invention act upon p25 processing in the cells of an animal, but are not limited by that method of action.

[0234] A method of inhibiting HIV with a compound may be relevant to a method of treating HIV infection in a patient. Therefore, a method of inhibiting HIV with a compound may similarly be used to treat a patient.

[0235] The methods of inhibiting HIV-1 replication in cells of an animal includes contacting infected cells with a compound of Formula ***I*** through ***XIII***, above. Related embodiments include a method of treating a HIV-1 infectionin a patient comprising administration of a compound of Formula ***I*** through ***XIII***; a method of inhibiting p25 processing either in a cell, *in vivo,* and/or *in vitro,* by administration of a compound that inhibits said p25 processing; and a method of treating human blood or blood products by administering a compound of Formula ***I*** through ***XIII***. Also included are a method of

identifying a compound that inhibits any one of p25 processing, HIV maturation, HIV infectivity, HIV virion phenotypes and the like.

[0236] In one embodiment, the compound is a derivative of betulinic acid, betulin, or dihydrobetulinic acid or dihydrobetulin and which includes the preferred substituents of Table 4. Preferred compounds include but are not limited to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, 3-*O*-(3',3'-dimethylsuccinyl) betulin, 3-*O*-(3',3'-dimethylglutaryl) betulin, 3-*O*-(3',3'-di methylsuccinyl) dihydrobetulinic acid, 3-*O*-(3',3'-dimethylglutaryl) betulinic acid, (3',3'-dimethylglutaryl) dihydrobetulinic acid, 3-*O*-diglycolyl-betulinic acid, and 3-*O*-diglycolyl-dihydrobetulinic acid.

[0237] In one embodiment, the invention is drawn to a method inhibiting HIV-1 replication in cells of an animal by contacting infected cells with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is a compound of Formulas *I* through *XIII* above.

[0238] In one embodiment, the invention is drawn to a method of inhibiting HIV-1 replication in cells of an animal by contacting infected cells with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is compound of Formulas *I* through *XIII*, with the exception of DSB.

[0239] In one embodiment, the invention is drawn to a method of inhibiting HIV-1 replication in cells of an animal by contacting infected cells with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is one that is excluded from those of Formulas *I* through *VI*. In one embodiment, the invention is drawn to a method of treating HIV-1 infection in a patient by administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps, wherein said compound is one that is other than those of Formulas *I* through *XIII*.

[0240] In another embodiment, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a

cell, but without significantly affecting other Gag processing steps, wherein said compound is a compound of Formulas *I* through *XIII*.

[0241] In another embodiment, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affecting other Gag processing steps, wherein said compound is a compound other than those of Formulas *I* through *VI*.

[0242] In another embodiment, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affecting other Gag processing steps, wherein said compound is a compound other than those of Formulas *I* through *XIII*.

[0243] In another embodiment, the invention is drawn to a method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affecting other Gag processing steps, wherein said compound is a compound other than those of Formulas *I* through *XI*. In another embodiment, the invention is drawn to a method of treating human blood or blood products by inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is a compound of Formulas *I* through *XIII*.

[0244] In another embodiment, the invention is drawn to a method of treating human blood or blood products by inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is a compound other than those of Formulas *I* through *VI*.

[0245] In another embodiment, the invention is drawn to a method of treating human blood or blood products by inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) in a cell, but without significantly affect other Gag processing steps, wherein said compound is a compound other than those of Formulas *I* through *XIII*.

[0246] The method disclosed herein, further comprises contacting said cells with one or more drugs selected from the group consisting of anti-viral agents, anti-fungal agents, anti-bacterial agents, anti-cancer agents,

immunostimulating agents, and combinations thereof. The method may include the treatment of human blood products.

[0247] The invention may also be used in conjunction with a method of treating cancer comprising the administration to an animal of one or more anti-neoplastic agents, exposing an animal to a cancer cell-killing amount of radiation, or a combination of both.

Methods of Identifying Compounds

[0248] The invention further includes a method for identifying compounds that inhibit HIV-1 replication in cells of an animal disclosed herein. In one embodiment, said method comprises:

(a) contacting a Gag polypeptide comprising a CA-SP1 cleavage site with a test compound;

(b) adding a labeled substance that selectively binds at or near the CA-SP1 cleavage site; and

(c) measuring the binding of the test compound at or near the CA-SP1 cleavage site.

[0249] Labeled substances or molecules include labeled antibodies or labeled DSB and the label includes an enzyme, fluorescent substance, chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, electron dense substance, such as gold, osmium tetroxide, lead or uranyl acetate, and radioisotope, antibodies labeled with such substances of molecules or a combination thereof. The assays could include, but are not limited to ELISA, single and double sandwich techniques, immunodiffusion or immunoprecipitation techniques, as known in the art ("*Immunoassay Handbook, 2nd ed.*," D. Wild, Nature Publishing Group, (2001)). Said methods of identifying also could include, but are not limited to Western blot assays, colorimetric assays, light and electron microscopic techniques, confocal microscopy, or other techniques known in the art.

[0250] A method of identifying compounds that inhibit HIV replication in cells of an animal further comprises:

(a) contacting a Gag protein comprising a wild-type CA-SP1 cleavage site, with HIV-1 protease in the presence of a test compound;

(b) separately, contacting a Gag protein comprising a mutant CA-SP1 cleavage site or a protein comprising an alternative protease cleavage site with HIV-1 protease in the presence of the test compound; and

(c) comparing the cleavage of the native wild-type Gag protein to the amount of cleavage of the mutant Gag protein or to the amount of cleavage of the peptide comprising an alternative protease cleavage site.

[0251] Step (b) above is performed as a control in order to eliminate compounds that might bind directly to, and therefore inhibit, the protease enzyme. The above method also includes the method wherein the wild-type CA-SP1, mutant CA-SP1 or alternative protease cleavage site is contained within a polypeptide fragment or recombinant peptide.

[0252] The method for identifying compounds that inhibit HIV-1 disclosed herein, also includes a method wherein said peptide or protein is labeled with a fluorescent moiety and a fluorescence quenching moiety, each bound to opposite sides of the CA-SP1 cleavage site, and wherein said detecting comprises measuring the signal from the fluorescent moiety, or wherein said peptide or protein is labeled with two fluorescent moieties, each bound to opposite sides of the CA-SP1 cleavage site, and wherein said detecting comprises measuring the transfer of fluorescent energy from one moiety to the other in the presence of the test compound and HIV-1 protease and comparing said transfer of fluorescent energy to that observed when the same procedure is applied to a peptide that comprises a sequence containing a mutation in the CA-SP1 cleavage site or that comprises a sequence containing another cleavage site. Examples of fluorescence-based assays of protease activity are well known in the art. In one such example, a protease substrate is labeled with green fluorescent dye molecules, which fluoresce when the substrate is cleaved by the protease enzyme (Molecular Probes, Protease Assay Kit).

[0253] The method of comparing the cleavage, above, also includes using a labeled antibody that selectively binds CA or SP1 or CA-SP1 in order to

measure the extent to which the test compound inhibits CA-SP1 cleavage. The antibody can be labeled with a molecule selected from the group consisting of enzyme, fluorescent substance, chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, electron dense substance, and radioisotope, or combinations thereof. The method also includes the use of an antibody to a specific epitope tag sequence to selectively detect CA-SP1 (p25) or SP1, into which the amino acid sequence for that epitope tag has been engineered according to standard methods in the art. Suitable tags are well known to those of ordinary skill in the art, and include haemagglutinin epitope HA (YPYDVPDYA) (SEQ ID NO: 81), bluetongue virus epitope VP7 (QYPALT) (SEQ ID NO: 82), α-tubulin epitope (EEF), Flag (DYKDDDDK) (SEQ ID NO: 83), and VSV-G (YTDOEMNRLGK) (SEQ ID NO: 84). Examples of SP1 containing epitope tags are illustrated in Figure 17.

[0254] As an example, the sequence of the FLAG epitope tag (Sigma-Aldrich) is inserted into the p2 (SP1) region of Gag by oligonucleotide-directed mutagenesis of a Gag expression plasmid. The presence of the SP1 domain in the cell-expressed protein is then be detected using commercially available anti-FLAG monoclonal antibodies (Sigma-Aldrich). (Hopp, T.P. *Biotechnology 6*: 1204-1210 (1988)).

[0255] The method of identifying compounds that disrupt CA-SP1 cleavage also includes the addition of a compound to cells infected with HIV-1 and the detection of CA-SP1 cleavage products by lysing and analyzing the cells or the released virions. The method included in the invention can be performed using a western blot analysis of viral proteins and detecting p25 using an antibody to p25 or wherein said mixture is analyzed by performing a gel electrophoresis of viral proteins and imaging of metabolically labeled proteins, or wherein the mixture is analyzed using immunoassays that use an antibody that selectively binds p25 or an antibody that selectively binds in order to distinguish p25 from p24. The invention includes the use of an antibody to a specific epitope tag sequence inserted into the C-terminal domain of SP1 to selectively detect p25 or SP1. For example, a sandwich ELISA assay can be performed where p25 and p24 in detergent-solubilized virus are captured using

an antibody that selectively binds to the CA region of Gag, which antibody is bound to a multiple well plate. Following a washing step, bound p25 is detected using an antibody to an epitope tag inserted in SP1, which is conjugated to an appropriate detection reagent (e.g. alkaline phosphatase for an enzyme-linked immunosorbent assay). Virus released by cells treated with compounds that act via this mechanism will generally have increased levels of p25 compared with untreated virions.

[0256] The disclosed method is drawn to an antibody that selectively binds p25, or an antibody that selectively binds SP1, or an antibody to an epitope tag sequence inserted into SP1, which is labeled with a molecule selected from the group consisting of enzyme, fluorescent substance, chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, electron dense substance, and radioisotope, or combinations thereof.

[0257] "Infected cells," as used herein, includes cells infected naturally by membrane fusion and subsequent insertion of the viral genome into the cells, or transfection of the cells with viral genetic material through artificial means. These methods include, but are not limited to, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, lipid-mediated transfection, electroporation or infection.

[0258] The invention may be practiced by infecting target cells *in vitro* with an infectious strain of HIV and in the presence of test compound, under appropriate culture conditions and for varying periods of time. Infected cells or supernatant fluid can be processed and loaded onto a polyacrylamide gel for the detection of virus levels, by methods that are well known in the art. Non-infected and non-treated cells can be used as negative and positive infection controls, respectively. Alternatively, the invention may be practiced by culturing the target cells in the presence of test compound prior to infecting the cells with an HIV strain.

[0259] The invention also includes a method for identifying compounds that inhibit HIV-1 replication in the cells of an animal, comprising:

(a) contacting a test compound with wild-type virus isolates and separately with virus isolates having redued sensitivity to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid; and

(b) selecting test compounds that are more active against the wild-type virus isolate compared with virus isolates that have reduced sensitivity to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid.

[0260] This invention further includes a method for identifying compounds that act by any of the abovementioned mechanism, involving treating HIV-1 infected or transfected cells with a compound then analyzing the virus particles released by compound-treated cells by thin-sectioning and transmission electron microscopy, by standard methods well known in the art. A compound acts by the abovementioned mechanism if particles are detected that exhibit spherical condensed cores that are acentric with respect to the viral particle and/or a crescent-shaped electron-dense layer just inside the viral membrane.

[0261] For electron microscopic studies, infected cells or centrifuged virus pellets obtained from the supernatant fluid can be contacted with a fixative, such as glutaraldehyde or freshly-made paraformaldehyde, and/or osmium tetroxide or other electron microscopy compatible fixative that is known in the art. The virus from the supernatant fluid or the cells, is dehydrated and embedded in an electron-lucent polymer such as an epoxy resin or methacrylate, thin sectioned using an ultramicrotome, stained using electron dense stains such as uranyl acetate, and/or lead citrate, and viewed in a transmission electron microscope. Non-infected and non-treated cells can be used as negative and positive infection controls, respectively. Alternatively, the invention may be practiced by culturing the target cells in the presence of test compound prior to infecting the cells with an HIV strain. Maturation defects caused by the compounds of the present invention are determined by the presence of morphologically aberrant viral particles, compared with controls, as described herein.

[0262] For cell culture studies, the virus-infected cells may be observed for the formation of syncytia, or the supernatant may be tested for the presence of HIV particles. Virus present in the supernatant may be harvested to infect other naïve cultures to determine infectivity.

[0263] Also included in the invention, is a method of determining if an individual is infected with HIV-1, is susceptible to treatment by a compound that inhibits p25 processing, the method involves taking blood from the patient, genotyping the viral RNA and determining whether the viral RNA contains mutations in the CA-SP1 cleavage site.

[0264] The invention also includes a method for identifying compounds that act by the abovementioned mechanisms, involving testing by a combination of the methods disclosed herein.

[0265] HIV Gag protein and fragments thereof for use in the aforementioned assays may be expressed or synthesized using a variety of methods familiar to those skilled in the art. Gag can be produced in an *in vitro* transcription and translation system using a rabbit reticulocyte lysate. Gag expressed in this system has been shown to be processed sequentially in a pattern similar to that observed in infected cells (Pettit, S.C. *et al. J. Virol. 76*:10226-10233 (2002)). Moreover, Gag expressed by this method is capable of assembling into immature viral particles when fused to a heterologous type D retroviral cytoplasmic self-assembly domain (Sakalian, M. *et al., J. Virol. 76*:10811-10820 (2002)). The plasmid pDAB72, available from the NIH AIDS Reagent Program can be used for this purpose (Erickson-Viitanen, S. *et al., AIDS Res. Hum. Retroviruses. 5*:577-91 (1989); Sidhu M.K. *et al., Biotechniques, 18*:20, 22, 24 (1995)). Other *in vitro* transcription/translation systems based on wheat germ or bacterial lysates can also be used for this purpose. HIV Gag may also be expressed in transfected cells using a variety of commercially available expression vectors. The plasmid *p55-GAG/GFP*, available from the NIH AIDS Reagent Program, may be used to express an HIV Gag-green fluorescent protein fusion protein in mammalian cells for drug interaction studies (Sandefur, S. *et al., J. Virol. 72*:2723-2732 (1998)). This construct would permit the capture and purification of Gag fusion protein using GFP-specific monoclonal antibodies. In addition, Gag may be expressed in cells using recombinant viral vectors, such as those used in the vaccinia virus, adenovirus, or baculovirus systems. Gag can also be expressed by infecting cells with HIV or by transfecting cells with proviral DNA. Finally, Gag may

be expressed in yeast or bacterial cells transformed with the appropriate expression vectors.

[0266] In addition to Gag proteins expressed in cells or *in vitro* using cell lysates, peptides corresponding to various regions of Gag may be commercially synthesized from using standard peptide synthesis techniques.

[0267] The invention further encompasses compounds identified by the method of this invention and/or a compound which inhibits HIV-1 replication according to the methods of this invention and pharmaceutical compositions comprising one or more compounds as disclosed herein, or pharmaceutically acceptable salts, esters or prodrugs thereof, and pharmaceutically acceptable carriers.

Pharmaceutical Compositions

[0268] Compounds according to the present invention have been found to possess anti-retroviral, particularly anti-HIV, activity. The salts and other formulations of the present invention are expected to have improved water solubility, and enhanced oral bioavailability. Also, due to the improved water solubility, it will be easier to formulate the salts of the present invention into pharmaceutical preparations. Further, compounds according to the present invention are expected to have improved biodistribution properties.

[0269] In one embodiment, the compounds are those of Formula *I* through *XIII*, in another they are compounds other than the compounds of Formula *I* through *XIII*.

[0270] This invention also includes a pharmaceutical composition comprising a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but has no significant effect on other Gag processing steps, or that inhibits the maturation of virus particles released from treated infected cells, such as the compounds of Formula *I* through ***XIII***. The invention includes a pharmaceutical composition comprising one or more compounds disclosed herein, or pharmaceutically acceptable salts, esters or prodrugs thereof, and pharmaceutically acceptable carriers, wherein said compound is of Formulae ***I*** through ***XIII*** above, or preferably, wherein said compound is selected from the group consisting of 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, 3-*O*-(3',3'-

dimethylsuccinyl) betulin, 3-*O*-(3',3'-dimethylglutaryl) betulin, 3-*O*-(3',3'-dimethylsuccinyl) dihydrobetulinic acid, 3-*O*-(3',3'-dimethylglutaryl) betulinic acid, (3',3'-dimethylglutaryl) dihydrobetulinic acid, 3-*O*-diglycolyl-betulinic acid, and 3-*O*-diglycolyl-dihydrobetulinic acid. The pharmaceutical compositions according to the invention, further comprise one or more drugs selected from an anti-viral agent, anti-fungal agent, anti-cancer agent or an immunostimulating agent.

[0271] Pharmaceutical compositions of the present invention can comprise at least one of the compounds of Formulae ***I*** through ***XIII*** disclosed herein. Pharmaceutical compositions according to the present invention can also further comprise other anti-viral agents such as, but not limited to, AZT (zidovudine, RETROVIR®, GlaxoSmithKline), 3TC (lamivudine, EPIVIR®, GlaxoSmithKline), AZT+3TC, (COMBIVIR®, GlaxoSmithKline), AZT+3TC+abacavir (TRIZIVIR®, GlaxoSmithKline), ddI (didanosine, VIDEX®, Bristol-Myers Squibb), ddC (zalcitabine, HIVID®, Hoffmann-La Roche), D4T (stavudine, ZERIT®, Bristol-Myers Squibb), abacavir (ZIAGEN®, GlaxoSmithKline), tenofovir (VIREAD®, Gilead Sciences), nevirapine (VIRAMUNE®, Boehringer Ingelheim), delavirdine (Pfizer), emtricitabine (EMTRIVA®, Gilead Sciences), efavirenz (SUSTIVA®, DuPont Pharmaceuticals), saquinavir (INVIRASE®, FORTOVASE®, Hoffmann-LaRoche), ritonavir (NORVIR®, Abbott Laboratories), indinavir (CRIXIVAN®, Merck and Company), nelfinavir (VIRACEPT®, Pfizer), amprenavir (AGENERASE®, GlaxoSmithKline), adefovir (PREVEON®, HEPSERA®, Gilead Sciences), atazanavir (Bristol-Myers Squibb), fosamprenavir (LEXIVA®, GlaxoSmithKline) and hydroxyurea (HYDREA®, Bristol-Meyers Squibb), or any other antiretroviral drugs or antibodies in combination with each other, or associated with a biologically based therapeutic, such as, for example, gp41-derived peptides enfuvirtide (FUZEON®, Roche and Trimeris) and T-1249, or soluble CD4, antibodies to CD4, and conjugates of CD4 or anti-CD4, or as additionally presented herein.

[0272] Additional suitable antiviral agents for optimal use with one of the compounds of Formulae ***I*** through ***XIII*** of the present invention can include,

but are not limited to,amphotericin B (FUNGIZONE®); Ampligen (mismatched RNA) developed by Hemispherx Biopharma; ; BETASERON® (β-interferon, Chiron); butylated hydroxytoluene; Carrosyn (polymannoacetate); Castanospermine; Contracan (stearic acid derivative); Creme Pharmatex (containing benzalkonium chloride); 5-unsubstituted derivative of zidovudine; penciclovir (DENAVIR® Novartis); famciclovir (FAMVIR® Novartis); acyclovir (ZOVIRAX® GlaxoSmithKline); cytofovir (VISTIDE® Gilead); ganciclovir (CYTOVENE®, Hoffman LaRoche); dextran sulfate; D-penicillamine (3-mercapto-D-valine); FOSCARNET® (trisodium phosphonoformate; AstraZeneca); fusidic acid; glycyrrhizin (a constituent of licorice root); HPA-23 (ammonium-21-tungsto-9-antimonate); ORNIDYL® (eflornithine; Aventis); nonoxynol; pentamidine isethionate (PENTAM-300); Peptide T (octapeptide sequence; ,Peninsula Laboratories); Phenytoin (Pfizer); INH or isoniazid; ribavirin (VIRAZOLE®, Valeant Pharmaceuticals); rifabutin, ansamycin (MYCOBUTIN® Pfizer); CD4-IgG2 (Progenics Pharmaceuticals) or other CD4-containing or CD4-based molecules; Trimetrexate (Medimmune); suramin and analogues thereof (Bayer); and WELLFERON® (α-interferon, GlaxoSmithKline).

[0273] Pharmaceutical compositions of the present invention can also further comprise immunomodulators. Suitable immunomodulators for optional use with a betulinic acid or betulin derivative of the present invention in accordance with the present invention can include, but are not limited to: ABPP (Bropririmine); Ampligen (mismatched RNA) Hemispherx Biopharma; anti-human interferon-α-antibody; ; ascorbic acid and derivatives thereof; interferon-β; Ciamexon; cyclosporin; cimetidine; CL-246,738; colony stimulating factors, including GM-CSF; dinitrochlorobenzene; HE2000 (Hollis-Eden Pharmaceuticals); inteferon-γ; glucan; hyperimmune gamma-globulin (Bayer); immuthiol (sodium diethylthiocarbamate); interleukin-1 (Hoffmann-LaRoche; Amgen), interleukin-2 (IL-2) (Chiron); isoprinosine (inosine pranobex); Krestin ; LC-9018 (Yakult); lentinan (Yamanouchi); LF-1695; methionine-enkephalin; Minophagen C; muramyl tripeptide, MTP-PE; naltrexone (Barr Laboratories); RNA immunomodulator ; REMUNE®

(Immune Response Corporation),;RETICULOSE® (Advanced Viral Research Corporation); shosaikoto; ginseng; thymic humoral factor; Thymopentin; thymosin factor 5; thymosin 1 (ZADAXIN®, SciClone), thymostimulin, TNF (tumor necrosis factor, Genentech), and vitamin preparations.

[0274] Pharmaceutical compositions of the present invention can also further comprise anti-cancer therapeutic agents. Suitable anti-cancer therapeutic agents for optional use include an anti-cancer composition effective to inhibit neoplasia comprising a compound, or a pharmaceutically acceptable salt or prodrug of said anti-cancer agent, which can be used for combination therapy include, but are not limited to alkylating agents, such as busulfan, cis-platin, mitomycin C, and carboplatin antimitotic agents, such as colchicine, vinblastine, taxols, such as paclitaxel (TAXOL®, Bristol-Meyers Squibb) docetaxel (TAXOTERE®, Aventis), topo I inhibitors, such as camptothecin, irinotecan and topotecan (HYCAMTIN®, GlaxoSmithKline), topo II inhibitors, such as doxorubicin, daunorubicin and etoposides such as VP16; RNA/DNA antimetabolites, such as 5-azacytidine, 5-fluorouracil and methotrexate, DNA antimetabolites, such as 5-fluoro-2'-deoxy-uridine, ara-C, hydroxyurea, thioguanine, and antibodies, such as trastuzumab (HERCEPTIN®, Genentech), and rituximab (RITUXAN®, Genentech and Biogen-Idec), melphalan, chlorambucil, cyclophosamide, ifosfamide, vincristine, mitoguazone, epirubicin, aclarubicin, bleomycin, mitoxantrone, elliptinium, fludarabine, octreotide, retinoic acid, tamoxifen, alanosine, and combinations thereof.

[0275] The invention further provides methods for providing anti-bacterial therapeutics, anti-parasitic therapeutics, and anti-fungal therapeutics, for use in combination with the compounds of the invention and pharmaceutically-acceptable salts thereof. Examples of anti-bacterial therapeutics include compounds such as penicillins, ampicillin, amoxicillin, cyclacillin, epicillin, methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin, carbenicillin, cephalexin, cepharadine, cefadoxil, cefaclor, cefoxitin, cefotaxime, ceftizoxime, cefinenoxine, ceftriaxone, moxalactam, imipenem, clavulanate, timentin, sulbactam, erythromycin, neomycin, gentamycin, streptomycin, metronidazole, chloramphenicol, clindamycin, lincomycin,

quinolones, rifampin, sulfonamides, bacitracin, polymyxin B, vancomycin, doxycycline, methacycline, minocycline, tetracycline, amphotericin B, cycloserine, ciprofloxacin, norfloxacin, isoniazid, ethambutol, and nalidixic acid, as well as derivatives and altered forms of each of these compounds.

[0276] Examples of anti-parasitic therapeutics include bithionol, diethylcarbamazine citrate, mebendazole, metrifonate, niclosamine, niridazole, oxamniquine and other quinine derivatives, piperazine citrate, praziquantel, pyrantel pamoate and thiabendazole, as well as derivatives and altered forms of each of these compounds.

[0277] Examples of anti-fungal therapeutics include amphotericin B, clotrimazole, econazole nitrate, flucytosine, griseofulvin, ketoconazole and miconazole, as well as derivatives and altered forms of each of these compounds. Anti-fungal compounds also include aculeacin A and papulocandin B.

[0278] The preferred animal subject of the present invention is a mammal. By the term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human patients.

[0279] The term "treating" means the administering to subjects a compound of Formulae *I* through *XIII* or a compound identified by one or more assays within the present invention, for purposes which can include prevention, amelioration, or cure of a retroviral-related pathology. Said compounds for treating a subject that are identified by one or more assays within the present inventions are identified as compounds which have the ability to disrupt Gag processing, described herein.

[0280] The term "inhibits the interaction" as used herein, means preventing, or reducing the rate of, direct or indirect association of one or more molecules, peptides, proteins, enzymes, or receptors; or preventing or reducing the normal activity of one or more molecules, peptides, proteins, enzymes or receptors.

[0281] Medicaments are considered to be provided "in combination" with one another if they are provided to the patient concurrently or if the time between the administration of each medicament is such as to permit an overlap of biological activity.

[0282] In one preferred embodiment, at least one compound of Formulae *I* through *XIII* above comprises a single pharmaceutical composition.

[0283] Pharmaceutical compositions for administration according to the present invention can comprise at least one compound of Formulae *I* through *XIII* above or compounds identified by one or more assays within the present invention. Said compounds for treating a subject that are identified by one or more assays within the present inventions are identified as compounds which have the ability to disrupt Gag processing, described herein. The compounds according to the present invention are further included in a pharmaceutically acceptable form optionally combined with a pharmaceutically acceptable carrier. These compositions can be administered by any means that achieve their intended purposes. Amounts and regimens for the administration of a compound of Formulae *I* through *XIII* according to the present invention can be determined readily by those with ordinary skill in the clinical art of treating a retroviral pathology.

[0284] For example, administration can be by parenteral, such as subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, transmucosal, ocular, rectal, intravaginal, or buccal routes. Alternatively, or concurrently, administration can be by the oral route. The administration may be as an oral or nasal spray, or topically, such as powders, ointments, drops or a patch. The dosage administered depends upon the age, health and weight of the recipient, type of previous or concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

[0285] Compounds and methods of the invention are useful in additional ways. For example, such compounds may be used prophylatically, to minimize the risk of infection. In another embodiment, a compound may be used to minimize spread of the disease from an infected person.

[0286] The invention is also directed to novel methods of treating HIV in an infected individual. In one embodiment, the invention is particularly useful in stimulating an immune response in a person infected with HIV. For example, by allowing noninfectious virus to be released from infected cells, such infected cells continue to expose antigens and may be effectively targeted by the immune system or other therapies directed against such antigens. In

another example, by continuing to permit the release of noninfectious virus, an infected individual continues to develop an immune response to said virus without suffering the deleterious effects of such a virus.

[0287] The invention is also useful in expanding the scope of treatment, and offers novel means of treating disease in patients in need thereof. In another embodiment, the invention may be practiced in a patient who does not respond to other therapy for reasons other than viral resistance. For example, conventional methods of treating HIV, as known in the art, are associated with deleterious side effects. In one embodiment, the methods and compositions of the invention are useful in treating a patient without a reduction in one or more deleterious side effects. In one embodiment the invention includes a method of treating a patient with a compound that does not have a particular side effect or has less of a particular side effect.

[0288] The bioavailability of drugs is also relevant in treatment. In an embodiment, the invention may be practiced such that compounds are more effectively absorbed into infected cells. In one embodiment the invention encompasses improved methods of delivering a drug to a cell infected with HIV.

[0289] Compositions within the scope of this invention include all compositions comprising at least one compound of Formulae *I* through *XIII* above according to the present invention in an amount effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. For example, a dose may comprise 0.0001mg to 10g/kg of body weight. Typical dosages comprise about 0.1 to about 100 mg/kg body weight. The preferred dosages comprise about 1 to about 100 mg/kg body weight of the active ingredient. More preferred dosages comprise about 5 to about 50 mg/kg body weight.

[0290] Administration of a compound of the present invention can also optionally include previous, concurrent, subsequent or adjunctive therapy using immune system boosters or immunomodulators. In addition to the pharmacologically active compounds, a pharmaceutical composition of the present invention can also contain suitable pharmaceutically acceptable

carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound(s), together with the excipient.

[0291] Pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

[0292] Suitable excipients are, e.g., fillers such as saccharide, for example, lactose or sucrose, mannitol or sorbitol; cellulose preparations and/or calcium phosphates, such as tricalcium phosphate or calcium hydrogen phosphate; as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, cellulose, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents can be added such as the above-mentioned starches and also carboxymethyl starch, cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated saccharide solutions can be used, which can optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to

gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropylmethyl cellulose phthalate are used. Dyestuffs or pigments can be added to the tablets or dragee coatings, for example, for identification or in order to characterize combinations of active compound doses.

[0293] Other pharmaceutical preparations which an be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which can be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils or liquid paraffin. In addition, stabilizers can be added.

[0294] Possible pharmaceutical preparations which can be used rectally include, for example, suppositories which consist of a combination of the active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

[0295] Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions can be administered. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate, triglycerides or glycol-400. Aqueous injection suspensions that can contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension can also contain stabilizers.

[0296] Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents

commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils such as cottonseed, groundnut, corn, germ, olive, castor, and sesame oils, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

[0297] Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, cellulose, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth, and combinations thereof.

[0298] Pharmaceutical compositions for topical administration include formulations appropriate for administration to the skin, mucosa, surfaces of the lung or eye. Compositions may be prepared as a pressurized or non-pressurized dry powder, liquid or suspension. The active ingredients in non-pressurized powdered formulations may be admixed in a finely divided form in a pharmaceutically-acceptable inert carrier, including but not limited to mannitol, fructose, dextrose, sucrose, lactose, saccharin or other sugars or sweeteners.

[0299] The pressurized composition may contain a compressed gas, such as nitrogen, or a liquefied gas propellant. The propellant may also contain a surface-active ingredient, which may be a liquid or solid non-ionic or anionic agent. The anionic agent may be in the form of a sodium salt.

[0300] A formulation for use in the eye would comprise a pharmaceutically acceptable ophthalmic carrier, such as an ointment, oils, such as vegetable oils, or an encapsulating material. The regions of the eye to be treated include the corneal region, or internal regions such as the iris, lens, ciliary body, anterior chamber, posterior chamber, aqueous humor, vitreous humor, choroid or retina.

[0301] Compositions for rectal administration may be in the form of suppositories. Compositions for use in the vagina may be in the form of suppositories, creams, foams, or in-dwelling vaginal inserts.

[0302] The compositions may be administered in the form of liposomes. Liposomes may be made from phospholipids, phosphatidyl cholines (lecithins) or other lipoidal compounds, natural or synthetic, as known in the art. Any non-toxic, pharmacologically acceptable lipid capable of forming liposomes may be used. The liposomes may be multilamellar or mono-lamellar.

[0303] A pharmaceutical formulation for systemic administration according to the invention can be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulation can be used simultaneously to achieve systemic administration of the active ingredient.

[0304] Suitable formulations for oral administration include hard or soft gelatin capsules, dragees, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

[0305] The compounds described above or compounds identified by one or more assays within the present invention and have the ability to disrupt Gag processing, can also be administered in the form of an implant when compounded with a biodegradable slow-release carrier. Alternatively, the compounds of the present invention can be formulated as a transdermal patch for continuous release of the active ingredient.

[0306] The following examples are illustrative only and are not intended to limit the scope of the invention as defined by the appended claims. It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention without departing from the spirit and scope of the invention. Thus, it is intended that the present invention covers the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

Examples

Example 1

Anti-Viral Activity Against Primary HIV-1 Isolates:

[0307] A robust virus inhibition assay was used to evaluate the anti-viral activity of DSB against primary HIV-1 isolates propagated in PMBC. Briefly, serial dilutions of DSB were made in medium into 96-well tissue culture plates. 25 - 250 $TCID_{50}$ of virus and 5 x 10^5 PHA-stimulated PBMCs were added to each well. On days 1, 3 and 5 post-infection, media was removed from each well and replaced with fresh media containing DSB at the appropriate concentration. On day 7 post-infection, culture supernatant was removed from each well for p24 detection of virus replication and 50% inhibitory concentrations (IC_{50}) were calculated by standard methods.

[0308] Table 5 shows the potent anti-viral activity of DSB against a panel of primary HIV-1 isolates. DSB exhibits levels of activity similar to approved drugs that were tested in parallel. Importantly, the activity of DSB was not restricted by co-receptor usage.Table 5

		----------IC_{50} (nM)----------		
Virus Isolate #	**Co-Receptor usage**	**DSB**	**AZT**	**Nevirapine**
BZ167	X4	4.0	2.2	31.2
92HT599	X4	9.8	5.8	25.3
US1	R5	5.6	0.9	22.1
19101N*	R5	3.8	2.4	59.4
3401N*	R5/X4	12.0	17.5	32.1
92US723	R5/X4	4.6	1.2	26.8
22101N*	R5/X4	2.6	0.9	4.9
Mean		**6.1**	**4.4**	**28.8**

Table 5: Inhibitory activity (IC_{50}) of DSB and two approved drugs against a panel of primary Clade B HIV-1 isolates. Clinical HIV-1 isolates denoted by * were isolated at Panacos. All other virus isolates were obtained from the NIH AIDS Reference Repository.

Note: R5 and X4 refer to the chemokine receptors CCR5 and CXCR4 respectively.

[0309] Toxicity of DSB was analyzed by incubating with PHA-stimulated PBMC for 7 days at a range of concentrations, then determining cell viability using the XTT method. The 50% cytotoxic concentration was >30 μM, corresponding to an *in vitro* therapeutic index of approximately 5000.

Example 2

Anti-Viral Activity of DSB against Drug Resistant HIV-1 Isolates:

[0310] The activity of DSB was tested against a panel of HIV-1 isolates resistant to approved drugs. These viruses were obtained from the NIH AIDS Research and Reference Reagent Program. Assays were performed using virus propagated in PBMCs with a p24 endpoint (above), or using cell line targets (MT-2 cells) and a cell killing endpoint. The MT-2 assay format was as follows. Serial dilutions of DSB, or each approved drug, were prepared in 96 well plates. To each sample well was added media containing MT-2 cells at 3 x 10^5 cells/mL and virus inoculum at a concentration necessary to result in 80% killing of the cell targets at 5 days post-infection (PI). On day 5 post-infection, virus-induced cell killing was determined by the XTT method and the inhibitory activity of the compound was determined.

[0311] Table 6 shows the potent anti-viral activity of DSB against a panel of drug-resistant HIV-1 isolates. The results were not significantly different from those obtained with the panel of wild-type isolates (Table 5), demonstrating that DSB retains its activity against virus strains resistant to all of the major classes of approved drugs.

Table 6

			---------- IC_{50}(nM) ----------			
Virus Isolate #	**Mutation(s)**	**Co-Receptor usage**	**DSB**	**AZT**	**Nevirapine**	**Indinavir**
NRTI-resistant						
1	K70R T215Y/F	R5/X4	4.4	86.4 (54X)*	ND	9.8
2	K70R T215Y/F	R5/X4	4.2	63.4 (40X)	ND	6.1
NNRTI-resistant						
3	Y181C	X4	1.0	5.1	>3800 (>177X)	2.5
4	K103N Y181C	X4	1.3	2.0	2630 (122X)	4.5
Protease-resistant						
5	V82A	X4	5.6	13.1	ND	39.7 (12X)
6	I84V	X4	5.5	14.4	ND	32.7 (10X)
7	L10R/M46I/ L63P/V82T/I 84V	X4	12.9	3.5	ND	72.5 (23X)

Table 6: Inhibitory activity (nM IC_{50}) of DSB against a panel of drug resistant HIV-1 isolates. Assays were done in fresh PBMC with a p24 endpoint except for the NNRTI-resistant isolates that were performed in MT-2 cells with a cell viability (XTT) endpoint. *Fold Resistance.

Note: R5 and X4 refer to the chemokine receptors CCR5 and CXCR4 respectively.

Example 3

DSB Inhibits HIV-1 Replication at a Late Step in the Virus Life Cycle

[0312] To distinguish the inhibitory activity of DSB against early and late replication targets, a multinuclear activation of a galactosidase indicator (MAGI) assay was used. In this assay, the targets are HeLa cells stably expressing CD4, CXCR4, CCR5 and a reporter construct consisting of the -galactosidase gene (modified to localize to the nucleus) driven by a truncated HIV-1 LTR. Infection of these cells results in expression of Tat that drives activation of the β-galactosidase reporter gene. Expression of β-galactosidase in infected cells is detected using the chromogenic substrate X-gal. As shown in Table 7, the entry inhibitor T-20, the NRTI AZT and the NNRTI nevirapine caused significant reductions in β-galactosidase gene expression in HIV-1 infected MAGI cells due to their ability to disrupt early steps in viral replication that affect Tat protein expression. In contrast, the protease inhibitor indinavir targets a late step in virus replication (following Tat expression) and does not prevent β-galactosidase gene expression in this system. Similar results were obtained with DSB as with indinavir, indicating that DSB blocks virus replication at a time point following the completion of proviral DNA integration and synthesis of the viral transactivating protein (Table 7).

Table 7

Inhibitor	DMSO	T-20	AZT	Nevirapine	Indinavir	DSB
% Decrease (β -galactosidase expression)	0	98	82	85	10	12

Table 7: Effect of DSB and inhibitors of entry (the gp41 peptide T-20), RT (AZT and Nevirapine) and protease (indinavir) on expression of b-galactosidase in HIV-1 infected MAGI cells. The DMSO control contained no drug.

[0313] Kanamoto *et al.* (*Antimicrob. Agents Chemother., April; 45*(4):1225-30, (2002)) have also reported that DSB acts at a late step in HIV replication. However, they reported that the compound inhibits release of virus from chronically-infected cells. In contrast, our data using a variety of experimental systems indicate that DSB does not have a significant effect on virus release (e.g. Example 6).

Example 4

DSB does not Inhibit HIV-1 Protease Activity

[0314] It was previously determined that DSB had no effect on HIV-1 protease function using a cell-free fluorometric assay that characterized enzyme activity by following the cleavage of a synthetic peptide substrate. The results of these experiments indicated that at concentrations up to 50 μg/mL that DSB had no effect on protease function. As a result of the observation that DSB blocks virus replication at a late step, studies were also performed using a recombinant form of the Gag protein, which is a more relevant system than the synthetic peptide substrate used in the initial assays. The use of the recombinant Gag protein as substrate resulted in a similar experimental outcome. In these experiments DSB did not disrupt protease-mediated Gag protein processing at concentrations as high as 50 μg/mL. In contrast, as expected, the protease inhibitor indinavir blocked Gag protein processing at 5 μg/mL (Figure 1).

Example 5

DSB causes a defect in the final step of Gag processing (CA-SP1 cleavage) that has been associated with viral maturation defects

[0315] In order to better define DSB's mechanism of action, a detailed examination was undertaken of the virus produced from HIV-1- infected cell lines treated with DSB. Briefly, H9 cells chronically infected with the HIV-

1_{IIIB} isolate were treated with DSB at 1 μg/mL for a period of 48 hrs. Indinavir was used as a control. At the 48hr time-point, spent media was removed and fresh media containing compound was added. At 24, 48 and 72 hrs post fresh compound addition, both cells and supernatant were recovered for analysis. The level of virus in the culture supernatant was determined and western blots were used to characterize viral protein production in both cell-associated and cell-free virus. As observed in previous experiments, DSB did not cause a significant reduction in the amount of virus produced by chronically infected H9 cells, however, there was a defect in Gag processing in both cell-associated and cell-free virus. This defect took the form of an additional band in the western blots corresponding to p25 (Figure 2). This p25 band results from the incomplete processing of the capsid CA-SP1 precursor. DSB treatment of HIV-2 and SIV chronically infected cell lines exhibited normal Gag processing consistent with the observed lack of antiviral activity against these viruses. The Gag processing defect seen in the presence of DSB is completely distinct from that observed with the protease inhibitor indinavir (Figure 2). As discussed above, mutations at the p25 to p24 cleavage site that prevent processing are associated with defects in viral maturation and infectivity (Wiegers K. *et al., J. Virol. 72*:2846-54 (1998)).

[0316] As previously discussed (C.T. Wild et al., *XIV Int. AIDS Conf.* Barcelona, Spain, Abstract MoPeA3030, (July 2002)), abnormal p25 to p24 processing is also seen in other maturation budding defects. These include mutations in the Gag late domain (PTAP) or defects in TSG-101 mediated viral assembly that disrupt budding (Garrus, J.E *et al., Cell, 107*:55-65, (2001); Demirov, D. G. *et al., J. Virology 76*:105-117, (2002)). However, these mutations cause inhibition of virus release, while DSB treatment does not have a significant effect on virus release. The morphology of these maturation/budding mutants is also quite distinct from that following DSB-treatment (see Example 6).

[0317] In addition, mutations that interfere with viral RNA dimerization and lead to the production of immature virus with defective core structures give a similar Gag processing phenotype (Liang, C. *et al., J. Virology, 73*:6147-6151, (1999)). However, in those cases RNA incorporation is inhibited and the

morphology of particles released is distinct from those following DSB treatment (see Example 6).

Example 6

DSB treatment effects HIV-1 maturation as determined by electron microscopy (EM)

[0318] It has been demonstrated that mutations in HIV-1 Gag that disrupt p25 to p24 processing give rise to non-infectious viral particles characterized by an internal morphology distinct from normal virus (Wiegers K. *et al., J. Virol. 72*:2846-54 (1998)). To determine if virus generated in the presence of DSB exhibited this distinct morphology the following experiment was carried out.

[0319] HeLa cells were transfected with HIV-1 infectious molecular clone pNL4-3 and treated as described previously with DSB. Following treatment, DSB-treated infected cells were fixed in glutaraldehyde and analyzed by EM. The results of this analysis are shown in Figure 3.

[0320] These results are consistent with a compound that disrupts p25 to p24 processing which generates non-infectious morphologically aberrant viral particles.

[0321] 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB) is an example of a compound that disrupts p25 to p24 processing and potently inhibits HIV-1 replication. However, this compound does not inhibit PR activity, and its action is specific for the p25 to p24 processing step, not other steps in Gag processing. Furthermore, DSB treatment results in the aberrant HIV particle morphology described above.

Example 7

[0322] *In vitro* selection for HIV-1 isolates resistant to compounds that disrupt the processing of the viral Gag capsid (CA) protein from the CA-spacer peptide 1 protein precursor.

[0323] A series of experiments were performed to select for viruses resistant to inhibition by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB), an inhibitor

HIV-1 maturation. For each experiment, either NL4-3 or RF virus isolate was used to infect two cell cultures. Following infection, one culture was maintained in growth medium containing DSB, while the other culture was maintained in parallel in growth medium lacking DSB.

[0324] In one experiment, H9 cells that had been infected with RF virus were maintained in the presence or absence of increasing concentrations of DSB (0.05-1.6 μg/ml). The cells were passaged every 2-3 days with the addition of fresh drug. Virus replication was monitored by p24 ELISA every 7 days. At that time, DSB-treated cultures with high levels of p24 were passaged by co-cultivation with fresh uninfected H9 cells at a 1:1 ratio of cells in the presence of 1x or 2x the original concentration of DSB. After 8 weeks of co-cultivation, cell-free virus was collected from the culture containing DSB at a concentration of 1.6 μg/ml and used to infect fresh H9 cells. Every 7 days, virus from cultures containing high levels of p24 was passaged by cell-free infection in the presence of 1x or 2x the original concentration of DSB. After 5 weeks of cell-free passaging, virus from the culture containing 3.2 μg/ml DSB was collected and used to infect MT-2 cells. Virus replication in the MT-2 cells, was monitored by observing syncytia formation microscopically. Every 1-3 days, the cells were washed to remove input virus, and fresh drug was added to the culture under selection. Every 3-4 days, following the emergence of extensive syncytia in the culture under selection, supernatant from each culture was collected and passed through a 0.45 μm filter to remove cell debris. This filtered virus supernatant was then used to infect fresh MT-2 cells in the presence or absence of fresh drug. After 4 rounds of cell-free infection (approximately 2 weeks in culture), with the concentration of drug at 3.2 μg/ml, virus stocks were collected and frozen for further analysis.

[0325] In a second experiment, a stock of virus derived from the molecular clone pNL4-3 (5.7 x 104 TCID50) was used to infect MT-2 cells (6 x 106 cells) and cultures were maintained in the presence or absence of DSB at a concentration of 1.6 μg/ml. Every 1-3 days, the cells were washed to remove input virus, and fresh drug was added to the culture under selection. Virus replication was monitored by observing syncytia formation microscopically. Every 3-7 days, following the emergence of extensive syncytia in the culture

under selection, supernatant from each culture was collected and passed through a 0.45 μm filter to remove cell debris. This filtered virus supernatant was then used to infect fresh MT-2 cells in the presence or absence of fresh drug. After 5 rounds of cell-free infection, and every other round thereafter, the concentration of drug was doubled. After 10 rounds of cell-free infection (approximately 7 weeks in culture), when the concentration of drug reached 12.8 μg/ml, virus stocks were collected and frozen for further analysis.

Example 8

[0326] Characterization of HIV-1 isolates selected for resistance to compounds that disrupt the processing of the viral Gag capsid (CA) protein from the CA-spacer peptide 1 protein precursor.

[0327] Virus stocks derived as described above were further analyzed both phenotypically and genotypically to characterize the nature of their drug-resistance. The resistance of the viruses to 3-O-(3',3'-dimethylsuccinyl)-betulinic acid (DSB) was determined in virus replication assays. Briefly, the virus stocks were first titered in H9 cells by quantitating the levels of p24 (by ELISA) in cultures 8 days after infection with serial 4-fold dilutions of virus. Virus input was then normalized for a second assay in which each virus is cultured for 8 days in the presence of serial 4-fold dilutions of drug. The IC50 for each virus was determined as the dilution of drug that reduced the p24 endpoint level by 50% as compared to the no-drug control. In these assays, the two independently derived virus stocks resulted in IC_{50} values greater than 2 μM for DSB, as compared to an IC_{50} of 0.02 μM for virus that had been cultured in parallel in the absence of drug. In a subsequent series of experiments, the A364V mutation was engineered into the HIV-1 NL4-3 proviral DNA, which was subsequently transfected into HeLa cells. Resulting virus was collected and used to test the activity of DSB in a viral replication assay, as described above. In these assays, the DSB-resistant virus resulted in an IC_{50} value of 0.1 μM whereas wild-type NL4-3 gave an IC_{50} value of 0.01 μM.

[0328] To determine if the resistant viruses were able to escape the CA-SP1 cleavage defect caused by DSB in wild-type virus, stocks of each virus grown

in either the presence or absence of drug were analyzed by Western blot. Virus was pelleted through a 20% sucrose cushion from filtered culture supernatants that were collected 60 hr post-infection and 18 hr after the cells had been washed and fresh drug added. The viruses were lysed, and the amount of each virus was normalized by quantitating p24 levels in each sample. Western blot analysis of the viral proteins in each sample demonstrated that the drug-resistant viruses did not contain the CA-SP1 product in the presence of DSB, confirming that these viruses were resistant to the effects of the drug on this cleavage event.

[0329] Finally, to identify the genetic determinants of DSB resistance, the entire Gag and PR coding regions of the viral genomes were amplified by high-fidelity RT-PCR for sequencing. The viral RNA was purified from each virus lysate prepared as described above and digested with DNase to remove any contaminating DNA. The RT-PCR products were then gel-purified to remove any non-specific PCR products. Finally, both strands of the resulting DNA fragments were sequenced using overlapping a series of primers. Two amino acid mutations were identified that are independently capable of conferring resistance to DSB, an alanine to valine substitution in the Gag polyprotein at residue 364 in the NL4-3 isolate and at residue 366 in the RF isolate. These are the first and the third residues, respectively, downstream of the CA-SP1 cleavage site (the N-terminus of SP1). Alanine is highly conserved at each of these positions throughout all HIV-1 clades in the database.

Example 9

Determinants of Activity of the HIV-1 Maturation Inhibitor DSB Map to the Gag Protein CA-SP1 Domain.

[0330] To further define the molecular determinants of DSB activity, a series of chimeric viruses were prepared in which residues proximal to the CA-SP1 cleavage site from the DSB-sensitive virus, HIV-1, were inserted into the analogous region of the Gag protein of the DSB-resistant retrovirus, SIV.

Characterization of these SIV/HIV chimeras (SHIVs) with respect to DSB activity allowed further identification of minimal HIV-1 Gag sequences both necessary and sufficient for DSB activity.

Materials and Methods

Construction of SHIV DNA clones

[0331] Three panels of SHIVs were generated using the DSB-resistant SIVmac239 as the backbone in conjunction with residues from the Gag CA-SP1 region of the DSB-sensitive HIV-1 pNL4-3. The CA-SP1 sequences for these SHIV constructs are shown in Fig. 20. All DNA mutagenesis was carried out using the PCR-overlapping-PCR strategy. (Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51-59) and other standard molecular cloning approaches (Sambrook, J., E. F. Fritsch, and T. Maniatis (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

Cell culture and DNA transfection

[0332] HeLa cells were maintained in DMEM (Invitrogen) (supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml Streptomycin) and passaged upon confluence. All plasmid DNAs were prepared using the midiprep kit (Qiagen). HeLa cells were transfected with wild-type SIVmac239, HIV-1 pNL4-3 or SHIV proviral DNAs by employing the FuGENE 6 transfection reagent (Roche). Briefly, cells were seeded into a 6-well plate (Corning) at a concentration of 1.5×10^5 cells per well the day prior to use and allowed to reach 60 to 80% confluence on the day of transfection. For each transfection, 3 μl of FuGENE 6 was diluted into 100 μl of serum-free DMEM followed by the addition of 1 μg of DNA. After gently mixing, the mixture of DNA-lipid complexes was gently added drop-wise to the cells in 1.5 ml of complete DMEM medium. Twenty-four hours post-transfection, medium containing the DNA-FuGENE 6 complexes was removed and 1.5 ml of fresh DMEM was added to the transfected cells. At 48 h post-transfection, both cells and culture supernatant were harvested for further analysis.

SDS-PAGE and Western blot

[0333] To characterize the effect of incorporation of residues from the CA-SP1 domain of HIV-1 into SIV on viral particle production and Gag polyprotein processing Western blotting was performed. Briefly, at 48h post-transfection, culture medium containing viral particles was collected and clarified by centrifugation at 2,000 rpm and 4°C for 20 min in a Sorvall RT 6000B centrifuge. Particle-containing supernatants were then concentrated through a 20% sucrose cushion in a microcentrifuge at 13,000 rpm at 4°C for 120 min and pellets were resuspended in lysis buffer (150 mM Tris-HCl, 5% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], pH 8.0). For cell lysates, at 48 h post-transfection, cells were washed once with PBS and lysed (150 mM Tris-HCl, 5% Triton X-100, 1% deoxycholate, pH 8.0) followed by centrifugation at 13,000 rpm at 4°C for 5 min to remove nuclear fractions. Viral pellets and cell lysates were separated on a 12% NuPAGE Bis-Tris Gel (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen) followed by blocking in a PBS buffer containing 0.5% Tween and 5% dry milk powder. The membrane was incubated with anti-SIVmac251 p27 McAb (NIH AIDS Research and Reference Reagent Program) and hybridized with goat anti-mouse horseradish peroxidase (Sigma). For the membrane containing HIV-1 proteins, the membrane was incubated with immunoglobulin from HIV-1-infected patients (HIV-Ig) (NIH AIDS Research and Reference Reagent Program) and hybridized with goat anti-human horseradish peroxidase (Sigma). The immune complex was visualized with an ECL system (Amersham Pharmacia Biotech) according to the instructions provided by the manufacturer.

Effect of DSB on SHIV Gag processing

[0334] To address the effect of the Gag substitutions on the ability of DSB to inhibit CA-SP1 processing, HeLa cells were transfected with wild-type SIVmac239, HIV-1 pNL4-3 or SHIV proviral DNAs by employing the procedure described above. DSB at a concentration of 1 μg/ml or DMSO (no drug control) was maintained throughout the entire period of the culture and

SDS-PAGE/Western-Blot for analyzing viral proteins derived from these cultures was performed as described above.

[0335] Only viruses that were fully sensitive to the effects of DSB were scored as sensitive, while those with residual resistance were scored as resistant. As such, the scoring in Experiment 9 differs from previous scoring, in that viruses that were not fully resistant were scored as sensitive. For this reason, SHIV.DM was scored as DSB resistant in Experiment 9, but was previously scored as DSB sensitive.

Results

Characterization of Gag SHIVs

[0336] Three panels of HIV-1/SIV Gag chimeras were prepared (Fig. 20). Panel 1 consisted of viruses containing the SIV backbone into which residues from the HIV-1 SP1 domain had been inserted. The HIV-1 inserts in these chimeras ranged in size from a single point substitution (SHIV DA) to the complete replacement of the SIV SP1 domain with the SP1 sequence from HIV-1 (SHIV DN). Panel 2 consisted of viruses containing the same SP1 substitutions plus the inclusion of the two C-terminal CA resides from HIV-1 (LM to VL). Panel 3 SHIVs were identical to those in panel 2 except that, in addition to the substitutions in the SP1 domain and the two C-terminal CA resides from HIV-1 (LM to VL), each of these chimeras also incorporated a Q (SIV) to H (HIV-1) change at the 6^{th} position upstream (P6) from the CA-SP1 cleavage site.

[0337] The level of viral particle release from transfected cells and the Gag processing profile for each of the SHIVs were determined (Fig. 21). All panel 1 SHIVs behaved similarly with respect to particle production and Gag processing; these chimeric viruses exhibited near wild-type levels of particle production and a normal Gag processing profile compared with the parental SIV. The majority of chimeras in panel 2 were characterized by normal Gag processing profiles, whereas the cellular expression of SHIVs FC and 11 was somewhat reduced (Fig. 21A). For all panel 2 SHIVs, the amount of virus production relative to cell-associated expression was comparable to the parental SIV. In contrast to the panel 1 and 2 SHIVs, most of the chimeras in

panel 3 exhibited defects in Gag processing. Of these SHIVs, only GH, GI and 23 exhibited a normal Gag processing profile. It is clear from these results that the Q to H change six residues upstream from the CA-SP1 cleavage site affects the ability of SIV PR to process the chimeric Gag proteins.

[0338] Results from SHIV panel 2, comprised of viruses containing HIV-1 residues in both the SP1 and CA domains, are shown in Figure 21. As described previously, this panel of SHIVs is identical to panel 1 except that in addition to the substitutions in the SP1 domain, each of these chimeras also incorporates the two HIV-1 CA C-terminal residues (VL from HIV-1 replaces LM from SIV). As with the viruses in panel 1, the chimeras in panel 2 were characterized by normal Gag processing profiles (Fig. 21) and, while the cellular expression of SHIVs FC and 11 was somewhat reduced (Fig. 21A), the proportion of virus found in the supernatant of cells transfected with DNA encoding these SHIVs was proportional to the level of viral release observed for all panel 2 viruses.

[0339] Results from SHIV panel 3 with viruses containing HIV-1 residues in both the Gag SP1 and CA domains are shown in Fig. 21. This panel of SHIVs is identical to those in panel 1 except that in addition to the substitutions in the SP1 domain, each of these chimeras also incorporates the two C-terminal resides from HIV-1 (LM from SIV to VL from HIV-1) plus a Q (SIV) to H (HIV-1) change at the 6^{th} position upstream from the CA-SP1 cleavage site. Unlike the first two panels, most of the chimeras in panel 3 exhibited defects in Gag processing (Fig. 21). Of these SHIVs, only GH, GI and 23 exhibited a normal Gag processing profile. It is clear from these results that the Q to H change six residues upstream from the CA-SP1 cleavage site has a significant affect on the ability of SIV protease to process the resulting chimeric Gag protein.

Sensitivity of Gag SHIVs to DSB

[0340] Each of the SHIVs in panels 1, 2 and 3 were characterized for their sensitivity to DSB. As described above, DSB disrupts HIV-1 CA-SP1 cleavage leading to the release of non-infectious viral particles that exhibit aberrant core morphology (Li, F., R. Goila-Gaur, K. Salzwedel, N. R. Kilgore,

M. Reddick, C. Matallana, A. Castillo, D. Zoumplis, D. E. Martin, J. M. Orenstein, G. P. Allaway, E. O. Freed, and C. T. Wild. (2003) PA-457: a potent HIV inhibitor that disrupts core condensation by targeting a late step in Gag processing. *Proc. Natl. Acad. Sci. USA 100*:13555-13560.). In the current study, SHIV-expressing cells were cultured in the presence of DSB at a concentration of 1μg/ml for a period of 48hrs. At the end of that time virus was harvested from the culture supernatant and the Gag processing profile for each chimeric virus was analyzed and compared to Gag protein processing in the absence of compound (Fig. 22).

[0341] As can be seen in Fig. 22, none of the SP1 SHIVs (panel 1) exhibited sensitivity to DSB. Even SHIV DN, which contains the complete HIV-1 SP1 domain, exhibited a normal Gag processing profile in the presence of compound at concentrations in excess of the tissue culture determined IC_{50}. (Fig. 22) (Li *et al.*). The results from the panel 1 chimeras demonstrate that the determinants of DSB activity include regions of HIV-1 Gag outside of the SP1 domain. Results from the panel 2 SHIVs are identical to those observed for panel 1. Specifically, none of these CA-SP1 chimeras exhibited DSB sensitivity (Fig. 22). Since the panel 2 viruses contained the C-terminal VL amino acid residues, in addition to the SP1 sequence from HIV-1, these results demonstrate that HIV-1 Gag residues other than those immediately flanking the CA-SP1 cleavage site play a role in DSB sensitivity.

[0342] As shown in figure 22A, DSB does disrupt CA-SP1 processing for a subset of the panel 3 SHIVs. Of these chimeric viruses, SHIVs 23 and GI exhibited a level of DSB-sensitivity comparable to the prototypic HIV-1 isolate NL4-3 (Fig. 22A). In repeat experiments the magnitude of the effect of the compound on each of these viruses, as determined by the relative ratios of CA-SP1 to CA, was nearly identical. Also in panel 3, SHIV GH exhibited some level of DSB sensitivity, however, on a qualitative level, the activity observed against this virus was reduced compared to that observed with SHIVs 23 and GI. For SHIV GH the effect of DSB on Gag processing was reduced to the point where the very faint CA-SP1 band observed in the immunoblot is not apparent in Fig. 22A. A 5X increase in the amount of viral proteins loaded onto the gel enhanced the sensitivity of the Western blot assay

to a level that allowed the DSB-mediated processing defect for SHIV GH to be observed (Fig. 22B). For the remainder of the viruses in panel 3 the effect of the compound could not be determined due to sequence-related defects in Gag processing (Fig. 21B). The results from the panel 3 SHIVs indicate that the His residue at the 6th position upstream from the CA-SP1 cleavage site plays an important role in DSB sensitivity and that significant portions of both CA and SP1 beyond the immediate vicinity of the cleavage site are necessary for DSB activity.

Discussion

[0343] Three panels of SIV/HIV-1 chimeras were prepared (Fig. 20) and characterized. All panel 1 and 2 SHIVs behaved similarly with respect to the effect of SP1 or CA/SP1 substitutions on Gag protein processing, viral particle release and sensitivity to DSB (Figs. 21 and 22). Although some of the viruses in panel 2 (i.e. FD and 11) were characterized by a reduction in the level of viral particle production, this effect was most likely due to an overall reduction in the amount of virus generated by the transfected cells (Fig 21A). The fact that none of these panel 1 and 2 SHIVs were sensitive to DSB as determined by the effect of the compound on CA-SP1 processing indicates that Gag sequences outside the immediate vicinity of the HIV-1 CA-SP1 cleavage site play a critical role in DSB activity.

[0344] In contrast to the viruses in panels 1 and 2, three members of the panel 3 SHIVs exhibited some degree of DSB sensitivity. Among these viruses, SHIVs 23 and GI exhibited NL4-3-like sensitivity to DSB while SHIV GH displayed a somewhat reduced level of sensitivity to the compound. Of the additional panel 3 viruses, the effect of the CA-SP1 substitutions on Gag processing made it impossible to determine the effect of the compound on the remaining chimeras.

[0345] The effect of DSB on the Gag processing profile of these 3 panels of CA-SP1 SHIVs suggests that the determinants of compound activity include a relatively large region of Gag flanking the CA-SP1 cleavage site. Comparison of the activity of DSB against panel 2 SHIV 11 (insensitive) with panel 3 SHIV 23 (sensitive) indicates that the His residue located at the 6th CA

position upstream from the cleavage site is critical to DSB activity (Fig. 22B). Consistent with this observation, *in vitro* resistance selection studies have identified a mutation at this position that confers some level of DSB insensitivity.

[0346] The results herein provide for the existence of both high and low affinity binding sites in the CA-SP1 region. A low-affinity interaction would result in partial DSB activity (i.e., SIVm3) while high affinity binding would give full compound sensitivity (i.e., SHIV 23).

Example 10

Genotyping of Viral Isolates

[0347] As shown above, sequence polymorphisms in HIV have been demonstrated to correspond with the ability of a virus to replicate in the presence of DSB. Most sequence polymorphisms are clustered in *gag*, especially in the region encoding CA-SP1. Accordingly, genotyping of a viral isolate may be used to readily determine whether the replication of such a virus is likely to be inhibited by DSB, or any other compound that intereferes with p25 processing.

[0348] The results of such genotyping are useful in, for example, determining whether a viral infection in a patient may be treated with DSB, or any other compound that intereferes with p25 processing in a similar manner, or in determining the emergence of resistant variants during a course of treatment with DSB.

[0349] Genotyping may be performed by a number of methods. In some embodiments, genotyping is performed by sequencing.

Methods

[0350] A single frozen aliquot (approximate volume 1.2 ml) of plasma is obtained from each patient. The plasma sample is stored at -70°C until ready for processing. Each sample is identified using the three digit patient ID number.

[0351] On the day of processing, each plasma sample is thawed rapidly in a 37°C water bath and then placed on ice. A 140 µl aliquot of plasma is removed to a separate tube for nucleic acid purification using the QIAamp Mini Viral RNA Purification Kit (Qiagen). The remainder of the plasma sample is transferred to a separate tube for brief, low speed centrifugation (3 min at 8,000 rpm) to clarify the plasma. One ml of the clarified supernatant is then transferred to a fresh tube and centrifuged for 2 hr at full speed (13,000 rpm) to pellet virus. The supernatant is carefully removed using a pipet and transferred to a separate tube for storage at -70°C as a precaution against possible disruption of the viral pellet. The virus pellet is resuspended in 140 µl of PBS and stored at -70°C as a backup sample in case sufficient RT-PCR product is not obtained from the initial aliquot of non-pelleted plasma.

Table 8 HIV-1 Gag primers

HIV-1 Gag CA/SP1 primers conserved in clade B:

	Name	Sequence	Length	Tm	%GC
Forward ("+" strand) primers	F-625	cacctagaactttaaatgcatgg	23	51	39
	F-575	gcccagaagtaatacccatgttttcagc	28	62	46
	F-550	cagaaggagccaccccacaag	21	58	62
	F-525	caccatgctaaacacagtggg	21	53	52
	F-375	ggaagtgacatagcaggaactactag	26	52	46
	F-300	ccacctatcccagtaggag	19	53	58
	F-125	ggatgacagaaaccttgttggtcc	24	58	50
Reverse ("-" strand) primers (5'-3')	R+100	cctttccacatttccaacagccc	23	57	52
	R+200	cttccctaaaaaattagcctgtc	23	51	39
	R+275	ctggtggggctgttggctc	19	64	68
	R+400	gggtcgttgccaaagagtg	19	60	58
	R+450	ctgtatcatctgctcctgtatctaatag	28	52	39
	R+525	caattccccctatcatttttggtttcc	27	62	41
	R+625	cttccaattatgttgacaggtgtaggtcc	29	60	45

[0352] Following purification, viral RNA is eluted in a final volume of approximately 60 µl. Only 7 µl of this stock is used initially as a template for reverse transcription using the StrataScript First Strand Synthesis System (Stratagene). The remainder of the RNA stock is stored at -70°C as a backup. The primer for reverse transcription (R+625) anneals approximately 625 bp downstream of the CA-SP1 cleavage site. All of the primers to be used for RT-PCR and sequencing in this project have been designed to anneal to

regions of *gag* that are highly conserved among clade B HIV isolates and have been validated using plasma samples from 42 different patients.

[0353] The reverse transcription reaction is performed in a total volume of 50 µl. Only 5 µl of this reaction is used initially as a template for PCR amplification of the CA-SP1 region using the PicoMaxx High Fidelity PCR Master Mix (Stratagene). The remainder of the reaction is stored at -20°C as a backup. A two-step "nested" PCR strategy is used which has been found to provide a high yield of very clean DNA product. The forward and reverse primers for the first-round PCR amplification (F-625 and R+525) anneal approximately 625 bp upstream and 525 bp downstream of the CA-SP1 cleavage site, respectively. No product is typically visible by agarose gel analysis following this first PCR reaction.

[0354] The initial PCR reaction is performed in a total volume of 50 µl. After cycling, 5 µl of this reaction is removed and used as a template in a second-round "nested" PCR reaction using primers F-575 and R-450, which anneal to regions of *gag* that are internal to the regions to which the initial primer pair anneals. The remainder of the first-round PCR reaction is stored at -20°C as a backup. The forward and reverse primers for the second-round PCR reaction anneal approximately 575 bp upstream and 450 bp downstream of the CA-SP1 cleavage site, respectively. Five µl of the final "nested" PCR reaction is removed for analysis of DNA products by agarose gel electrophoresis. If, as expected, the reaction contains only one prominent band of the predicted size for the desired product (~1.1 kb), and the yield is estimated to be sufficient to permit sequencing (i.e. ~200 ng total), then 40 µl of the reaction is removed for purification of the DNA product using the MinElute PCR Purification Kit (Qiagen). The remaining ~5 µl of the reaction is stored at -20°C as a backup. Following elution of the purified DNA product, an appropriate volume (corresponding to at least 40 ng of DNA) is transferred to two tubes, each containing a different sequencing primer (one for each strand of the DNA). The "+" strand sequencing primer (F-300) anneals approximately 300 bp upstream of the CA-SP1 cleavage site. The "-" strand sequencing primer (R+275) anneals approximately 275 bp downstream of the CA-SP1 cleavage site. The template/primer mixture is shipped for sequencing and analysis. The

remainder of the purified DNA product is stored at -20°C as a backup. The resulting sequence analysis provides overlapping reads for each DNA strand to help resolve any ambiguities in any single sequencing reaction.

[0355] If ambiguities are found in both sequencing reactions, additional sequencing reactions are analyzed using alternate validated sequencing primers in case the problem lies in the heterogeneity of the region of *gag* to which the original sequencing primers anneal. These will include two "+" strand primers (F-375 and F-125) that anneal approximately 375 and 125 bp upstream and two "-" strand primers (R+100 and R+400) that anneal approximately 100 and 400 bp downstream of the CA-SP1 cleavage site, respectively.

[0356] If the final "nested" PCR reaction contains significant background bands when analyzed on an agarose gel (i.e. greater than approximately 10% of the total yield), or if sequencing fails to yield clean sequence, then the desired DNA product is purified by running the entire PCR reaction (re-amplified from a backup sample if necessary) on an agarose gel and excising the desired band. The DNA is then purified from the agarose using the QIAEX II Gel Extraction Kit (Qiagen) and eluted product is prepared for sequencing as described above.

[0357] If the PCR reaction fails to yield sufficient product for sequencing, then additional RT-PCR or PCR reactions could be run, if necessary, using any of the backup samples outlined above and additional validated primer sets, including four forward primers that anneal approximately 550, 375, 300, and 125 bp upstream (F-550, F-375, F-300 and F-125) and three reverse primers that anneal approximately 400, 275, and 100 bp downstream (R+ 400, R+275 and R+100) of the CA-SP1 cleavage site. For example, excellent results have been obtained using primer R+525 for reverse transcription and primers F-575 and R+450 for single-round PCR amplification. Primers F-550 and R+400 also work well for PCR amplification.

[0358] The resulting genotype is then matched with the genotype of viruses identified elsewhere herein to determine whether the virus is inhibited or is not inhibited by DSB. Further confirmation of the genotyping results may be

obtained by follow-up experiments such as by direct experiments on viral isolates.

Example 11

Genetic Change During Treatment With DSB

[0359] To determine the change in genotype of HIV-1 during a course of treatment with DSB, the genotype of the virus population in each patient prior to dosing and at the end of the study (day 28) is obtained to determine if any mutations have occurred during the course of treatment. If any mutations are identified in the end of study samples that were not present prior to dosing, then intermediate samples drawn on days 7 and 10 after dosing are also be genotyped to determine when the mutation occurred.

[0360] Using this method, the mutations that may occur in the total virus population during the course of the study are determined. A mutation will be identified as a greater than 25% variation in the amino acid designation for a given codon. Once a mutation has been identified, a chromatogram of the raw data is reviewed to determine the identities of the amino acids at that position in the minor virus populations. If none of the resistance mutations listed above are identified using these criteria, then the chromatograms from each reaction are reviewed to determine if any minor species (less than 25% of the total population) are present at any of the relevant positions.

[0361] Having now fully described this invention, it will be understood to those of ordinary skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any embodiment thereof. All patents, applications and publications cited herein are fully incorporated by reference in their entirety.

What is Claimed Is:

1. A method of treating HIV-1 infection in a patient, comprising administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps.

2. The method of claim 1, wherein said method does not significantly reduce the quantity of virions released from infected cells in said patient.

3. The method of claim 1, wherein said method has no significant effect on the amount of RNA incorporation into virions released from cells of said patient.

4. The method of claim 1, wherein said compound inhibits the maturation of virions released from cells of said patient.

5. The method of claim 1, wherein a majority of virions released from cells of said patient exhibit an altered phenotype relative to wild type virions, wherein said phenotype is selected from the group consisting of:

   (a) virions possessing spherical, electron-dense cores that are acentric with respect to the viral particle;

   (b) virions possessing crescent-shaped electron-dense layers lying just inside the viral membrane;

   (c) virions having reduced or no infectivity; and

   (d) the combination of any one of (a) through (c)

6. A method of treating HIV-1-infection in a patient, comprising administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide encoded by a polynucleotide having a sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

7. The method of claim 6, wherein said polynucleotide has a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

8. A method of treating HIV-1-infection in a patient, comprising administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), and wherein said compound binds to a polypeptide having a sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

9. The method of claim 8, wherein said polypeptide has a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

10. A method of treating HIV-1-infection in a patient, comprising administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), and wherein said compound binds to a polypeptide having a sequence selected from any one of SEQ ID NO: 121 to 443.

11. The method of any one of claims 1-10, wherein said HIV-1 infection in a patient comprises infection with an HIV-1 which does not respond to other HIV-1 therapies.

12. The method of any one of claims 1-10, wherein said patient with said HIV-1 infection does not otherwise respond to other HIV-1 therapies.

13. The method of any one of claims 1-10, further comprising administering to said patient an additional compound which is an immunomodulating agent, an anticancer agent, an antibacterial agent, an antifungal agent, an antiviral agent, or a combination thereof.

14. The method of any one of claims 1-10, wherein said patient is administered said compound in combination with at least one other anti-viral agent.

15. The method of claim 14, wherein said other anti-viral agent is selected from the group consisting of: zidovudine; lamivudine; didanosine; zalcitabine; stavudine; abacavir; nevirapine; delavirdine; emtricitabine; efavirenz; saquinavir; ritonavir; indinavir; nelfinavir; amprenavir; tenofovir; adefovir; atazanavir; fosamprenavir; hydroxyurea; AL-721; ampligen; butylated hydroxytoluene; polymannoacetate; castanospermine; contracan; creme pharmatex; CS-87; penciclovir; famciclovir; acyclovir; cytofovir; ganciclovir; dextran sulfate; D-penicillamine trisodium phosphonoformate; fusidic acid; HPA-23; eflornithine; nonoxynol; pentamidine isethionate; peptide T; phenytoin; isoniazid; ribavirin; rifabutin; ansamycin; trimetrexate; SK-818; suramin; UA001; enfuvirtide; gp41-derived peptides; antibodies to CD4; soluble CD4; CD4-containing molecules; CD4-IgG2; and combinations thereof.

16. A method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), comprising administering a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps.

17. A method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), comprising administering a compound which binds to a polypeptide encoded by a polynucleotide having a sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

18. The method of claim 17, wherein said polynucleotide has a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

19. A method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), comprising administering a compound which binds to a polypeptide having a sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

20. The method of claim 19, wherein said polypeptide has a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

21. A method of inhibiting processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), comprising administering a compound which binds to a polypeptide having a sequence selected from any one of SEQ ID NO: 121 to 443.

22. A method of treating human blood products comprising contacting said blood products with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), but does not significantly affect other Gag processing steps.

23. The method of claim 22 wherein said inhibition does not significantly reduce the quantity of virions released from infected cells treated with said compound.

24. The method of claim 22, wherein said inhibition has no significant effect on the amount of RNA incorporated into the virions released from infected cells treated with said compound.

25. The method of claim 22, wherein said compound inhibits the maturation of virions released from infected cells treated with said compound.

26. The method of claim 22, wherein a majority of virions released from treated infected cells exhibit an altered phenotype relative to wild type virions wherein said phenotype is selected from the group consisting of:

(a) virions possessing spherical, electron-dense cores that are acentric with respect to the viral particle;

(b) virions possessing crescent-shaped electron-dense layers lying just inside the viral membrane;

(c) virions having reduced or no infectivity; and

(d) the combination of any one of (a) through (c)

27. A method of treating human blood products, comprising contacting said blood products with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide encoded by a polynucleotide having a sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

28. The method of claim 27, wherein said polynucleotide has a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

29. A method of treating human blood products, comprising contacting said blood products with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide having a sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

30. The method of claim 29, wherein said polypeptide has a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

31. A method of treating human blood products, comprising contacting said blood products with a compound that inhibits processing of the viral Gag p25 protein (CA-SP1) to p24 (CA), wherein said compound binds to a polypeptide having a sequence selected from any one of SEQ ID NO: 121 to 443.

32. The method of any one of claims 1 to 10, 16 to 31, wherein said compound inhibits the interaction of HIV protease with CA-SP1.

33. The method of claim 32, wherein said compound directly inhibits the interaction of HIV protease with CA-SP1.

34. The method of claim 32, wherein said compound indirectly inhibits the interaction of HIV protease with CA-SP1.

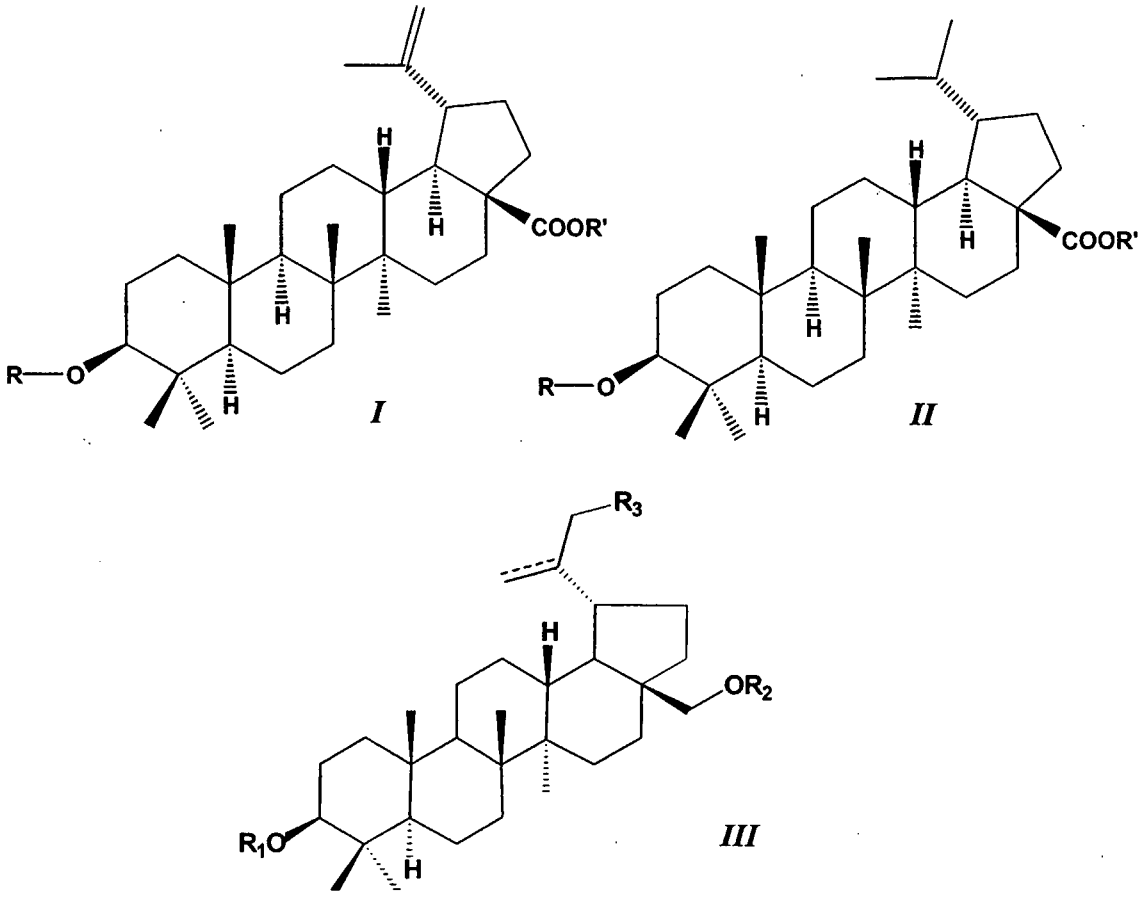
35. The method of any one of claims 1 to 10, 16 to 31, wherein said compound binds to the viral Gag protein such that interaction of HIV protease with CA-SP1 is inhibited.

36. The method of any one of claims 1 to 10, 16 to 31, wherein said compound binds at or near the site of cleavage of the viral Gag p25 protein (CA-SP1) to p24 (CA).

37. The method of any one of claims 1 to 10, 16 to 31, wherein said compound is a derivative of dimethylsuccinyl betulinic acid or a derivative of dimethylsuccinyl betulin.

38. The method of claim 37, wherein said compound is selected from the group consisting of: 3-O-(3',3'-dimethylsuccinyl) betulinic acid; 3-O-(3',3'-dimethylsuccinyl) betulin; 3-O-(3',3'-dimethylglutaryl) betulin; 3-O-(3',3'-dimethylsuccinyl) dihydrobetulinic acid; 3-O-(3',3'-dimethylglutaryl) betulinic acid; (3',3'-dimethylglutaryl) dihydrobetulinic acid; 3-O-diglycolyl-betulinic acid; 3-O-diglycolyl-dihydrobetulinic acid; and combinations thereof.

39. The method of any one of claims 1 to 10, 16 to 31, wherein said compound is not a derivative of betulin or dihydrobetulin of Formulae I to III:



or a pharmaceutically acceptable salt thereof, wherein,

R is a C_2-C_{20} substituted or unsubstituted carboxyacyl,

R' is hydrogen, C_2-C_{10} substituted or unsubstituted alkyl, or aryl group;

R_1 is a C_2-C_{20} substituted or unsubstituted carboxyacyl,

R_2 is hydrogen, $C(C_6H_5)_3$, or a C_2-C_{20} substituted or unsubstituted carboxyacyl; and

R_3 is hydrogen, halogen, amino, optionally substituted mono- or di-alkylamino, or -OR_4, where R_4 is hydrogen, C_{1-4} alkanoyl, benzoyl, or C_2-C_{20} substituted or unsubstituted carboxyacyl;

wherein the dashed line represents an optional double bond between C20 and C29.

40. A mutant lentivirus comprising a mutation in the nucleic acid sequence encoding Gag which renders the replication of said mutant lentivirus less sensitive to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB).

41. The mutant lentivirus of claim 40, wherein the Gag polypeptide of said virus does not bind to 3-O-(3',3'-dimethylsuccinyl) betulinic acid.

42. The mutant lentivirus of claim 40, wherein said mutation further renders said mutant lentivirus less sensitive to inhibition of processing of p25 (CA-SP1) to p24 (CA) by DSB.

43. The mutant lentivirus of claim 40, comprising a mutation in the nucleotide sequence encoding the Gag p25 protein (CA-SP1).

44. The mutant lentivirus of claim 43, which encodes a Gag polypeptide wherein one or more amino acids are deleted from, or substituted in the amino acid sequence in the CA-SP1 region.

45. The mutant lentivirus of claim 40, wherein said mutation occurs in the nucleotide sequence encoding an amino acid sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

46. The mutant lentivirus of claim 40, wherein said mutation occurs in the nucleotide sequence encoding the amino acid sequence selected from group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

47. The mutant lentivirus of claim 40, wherein said mutation occurs in the nucleotide sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

48. The mutant lentivirus of claim 40, wherein said mutation occurs in the nucleotide sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

49. The mutant lentivirus of claim 40, wherein said mutation in CA-SP1 occurs in the codon encoding the amino acid selected from the group consisting of:

(a) Amino acid 1 (Glycine) in GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(b) Amino acid 2 (Histidine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(c) Amino acid 6 (Valine or Isoleucine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(d) Amino acid 7 (Leucine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(e) Amino acid 8 (Alanine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(f) Amino acid 10 (Alanine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(g) Amino acid 11 (Methionine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(h) Amino acid 12 (Serine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) Amino acid 13 (Glutamine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(j) Amino acid 14 (Valine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(k) Amino acid 15 (Threonine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(l) Amino acid 16 (Asparagine) in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);and

(m) Amino acid 45 (Alanine) in HKARILAEAMSQVTNPATIMIQKGNFRNQRKTVKCFNC GKEGHIA (SEQ ID NO: 120).

50. The mutant lentivirus of claim 40, wherein said mutation in the CA-SP1 sequence results in the change in the encoded amino acid selected from the group consisting of:

(a) Glycine in GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(b) Histidine to Glutamine at amino acid 2 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO:117);

(c) Histidine to Tyrosine at amino acid 2 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(d) Valine or Isoleucine to Leucine at amino acid 6 in in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(e) Leucine to Methionine at amino acid 7 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(f) Alanine to Valine at amino acid 8 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(g) Alanine to Valine at amino acid 10 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(h) Methionine to Leucine at amino acid 11 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) Serine to Lysine at amino acid 12 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(j) Glutamine to Glutamic acid at amino acid 13 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(k) Valine to Alanine at amino acid 14 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(l) Threonine to Leucine at amino acid 15 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(m) Asparagine to Alanine at amino acid 16 in SHKARILAEAMSQVTN (SEQ ID NO: 116) or GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(n) Alanine to Threonine at amino acid 45 in HKARILAEAMSQVTNPATIMIQKGNFRNQRKTVKCFNC GKEGHIA (SEQ ID NO: 525).

51. The mutant lentivirus of claim 40, wherein said mutation occurs in the polynucleotide encoding the amino acid sequence selected from any one of SEQ ID NO: 121 to 443.

52. The mutant lentivirus of any one of claims 40 to 51, which is a mutant HIV-1.

53. A recombinant non-HIV-1 retrovirus the replication of which is inhibited by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB).

54. The recombinant non-HIV-1 retrovirus of claim 53, wherein DSB inhibits the processing of the viral Gag p25 protein (CA-SP1) to p24 (CA) but does not significantly inhibit other Gag processing steps.

55. The recombinant non-HIV-1 retrovirus of claim 53, wherein the Gag polypeptide of said virus binds to 3-O-(3',3'-dimethylsuccinyl) betulinic acid.

56. The recombinant non-HIV-1 retrovirus of claim 53 which contains a p25 (CA-SP1) protein comprising an amino acid sequence at least about 70% identical to the group consisting of

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

57. The recombinant non-HIV-1 retrovirus of claim 56 which contains a p25 (CA-SP1) protein comprising an amino acid sequence selected from the group consisting of

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

58. The recombinant non-HIV-1 retrovirus of claim 53 which comprises a polynucleotide encoding CA-SP1, which is at least about 70% identical to a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

59. The recombinant non-HIV-1 retrovirus of claim 53 which comprises a polynucleotide encoding CA-SP1, which has a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

60. The recombinant non-HIV-1 retrovirus of claim 53 which contains a p25 (CA-SP1) protein comprising an amino acid sequence selected from any of SEQ ID NO: 121 to 443.

61. The recombinant non-HIV-1 retrovirus of claim 53 having a nucleotide sequence at least about 70% identical to the sequence selected from the group consisting of:

(a) SEQ ID NO: 90;

(b) SEQ ID NO: 92;

(c) SEQ ID NO: 94;

(d) SEQ ID NO: 96; and

(e) SEQ ID NO: 98;

62. The recombinant non-HIV-1 retrovirus of claim 53 having a nucleotide sequence selected from the group consisting of:

(a) SEQ ID NO: 90;

(b) SEQ ID NO: 92;

(c) SEQ ID NO: 94;

(d) SEQ ID NO: 96; and

(e) SEQ ID NO: 98.

63. The recombinant non-HIV-1 retrovirus of claim 53, which is derived from a virus selected from the group consisting of HIV-2, HTLV-I, HTLV-II, SIV, avian leukosis virus (ALV), endogenous avian retrovirus (EAV), mouse mammary tumor virus (MMTV), feline immunodeficiency virus (FIV), Bovine immunodeficiency virus (BIV), caprine arthritis encephalitis virus (CAEV), Visna-maedi virus, and feline leukemia virus (FeLV).

64. The recombinant non-HIV-1 retrovirus of claim 63 which is derived from a virus selected from the group consisting of group consisting of:

(a) SIV;

(b) FIV;

(c) EAIV;

(d) BIV;

(e) CAEV; and

(f) Visna-Maedi virus.

65. An animal model of lentivirus infection comprising a suitable non-human animal host infected with the recombinant non-HIV-1 retrovirus of claim 53.

66. The animal model of claim 65, wherein said non-human animal host is selected from the group consisting of :

(a) a cat;

(b) a monkey;

(c) an ape;

(d) a horse;

(e) a sheep;

(f) a goat;

(g) a cow;

(h) a mouse;

(i) a rat; and

(j) a bird.

67. A method of making a recombinant non-HIV-1 lentivirus sensitive to inhibition by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB), comprising replacing the polynucleotide sequence encoding CA-SP1 from a non-HIV-1 lentivirus that is not sensitive to DSB with the sequence encoding CA-SP1 of a DSB-sensitive HIV-1 virus.

68. The method of claim 67, wherein the polynucleotide sequence encoding CA-SP1 in said recombinant non-HIV-1 lentivirus comprises a sequence at least about 70% identical with a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

69. The method of claim 68, wherein the polynucleotide sequence encoding CA-SP1 in said recombinant non-HIV-1 lentivirus comprises a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

70. The method of claim 67, wherein the CA-SP1 protein of said recombinant DSB-sensitive non-HIV-1 lentivirus comprises an amino acid sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

71. The method of claim 67, wherein the CA-SP1 protein of said DSB-sensitive recombinant non-HIV-1 lentivirus comprises an amino acid sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

72. The method of claim 67, wherein the CA-SP1 protein of said recombinant DSB-sensitive non-HIV-1 lentivirus has an amino acid sequence selected from any of SEQ ID NO: 121 to 443.

73. The method of claim 67, comprising:

(a) deleting from the genome of said lentivirus the nucleotides which corresponds to nucleotides 1370-1413 from SEQ ID NO: 18; and

(b) inserting nucleotides 1370-1413 from SEQ ID NO: 18 or nucleotides 1857-1899 of SEQ ID NO: 19 into said region of said non-HIV-1 lentivirus.

74. A recombinant non-HIV-1 lentivirus produced by the method of any one of claims 67 to 73.

75. An antibody that selectively binds at or near the CA-SP1 cleavage site of Gag.

76. The antibody of claim 75, which binds an amino acid sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119).

77. The antibody of claim 75, which binds to an amino acid sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119).

78. The antibody of claim 75, which binds to an amino acid sequence selected from any of SEQ ID NO: 121 to 443.

79. The antibody of claim 75, which selectively binds a polypeptide containing a mutation in HIV CA-SP1 polypeptide which results in a decrease in the inhibition of processing of p25 (CA-SP1) to p24 (CA) by 3-O-(3'3'-dimethylsuccinyl) betulinic acid.

80. The antibody of claim 79, wherein said mutation is located at or near the CA-SP1 cleavage site or in the SP1 domain of CA-SP1.

81. The antibody of claim 79, wherein said polypeptide is selected from the group consisting of:

(a) GHKARVLVEAMSQV (SEQ ID NO: 2);

(b) SHKARILAEVMSQV (SEQ ID NO: 3);

(c) SQKARILAEAMSQVTN;

(d) GQKARVLAEAMSQVTN;

(e) SYKARILAEAMSQVTN;

(f) GYKARVLAEAMSQVTN;

(g) SHKARLLAEAMSQVTN;

(h) GHKARLLAEAMSQVTN;

(i) SHKARIMAEAMSQVTN;

(j) GHKARVMAEAMSQVTN;

(k) SHKARILAEVMSQVTN;

(l) GHKARVLAEVMSQVTN;

(m) SHKARILVEAMSQVTN;

(n) GHKARVLVEAMSQVTN;

(o) SHKARILAEALSQVTN;

(p) GHKARVLAEALSQVTN;

(q) SHKARILAEAMLQVTN;

(r) GHKARVLAEAMLQVTN;

(s) SHKARILAEAMSEVTN;

(t) GHKARVLAEAMSEVTN;

(u) SHKARILAEAMSQATN;

(v) GHKARVLAEAMSQATN;

(w) SHKARILAEAMSQVLN;

(x) GHKARVLAEAMSQVLN;

(y) SHKARILAEAMSQVTA;

(z) GHKARVLAEAMSQVTA; and

(aa) HKARILAEAMSQVTNPATIMIQKGNFRNQRKTVKCFNC GKEGHIT

82. The antibody of claim 79, which selectively binds an amino acid sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 3.

83. The antibody of claim 75, which is selected from the group consisting of:

(a) an antibody that selectively binds SP1 but not CA-SP1;

(b) an antibody that selectively binds CA-SP1 but not CA; and

(c) an antibody that selectively binds CA but not CA-SP1.

84. The antibody of claim 75 that inhibits the binding of 3-O-(3',3'-dimethylsuccinyl) betulinic acid to Gag.

85. An isolated CA-SP1 polypeptide which is at least about 70% identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

86. The isolated CA-SP1 polypeptide of claim 85, which is identical to a sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

87. An isolated CA-SP1 polypeptide, encoded by a polynucleotide sequence at least about 70% identical to a sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

88. The isolated CA-SP1 polypeptide of claim 87, encoded by a polynucleotide sequence selected from the group consisting of:

(a) about nucleotides 1243-1435 of SEQ ID NO: 18;

(b) about nucleotides 1729-1920 of SEQ ID NO: 19;

(c) about nucleotides 1344-1435 of SEQ ID NO: 18;

(d) about nucleotides 1828-1920 of SEQ ID NO: 19;

(e) about nucleotides 1370-1413 of SEQ ID NO: 18;

(f) about nucleotides 1857-1899 of SEQ ID NO: 19

(g) about nucleotides 1372-1419 of SEQ ID NO: 18;

(h) about nucleotides 1858-1905 of SEQ ID NO: 19;

(i) about nucleotides 1372-1434 of SEQ ID NO: 18; and

(j) about nucleotides 1858-1920 of SEQ ID NO: 19.

89. An isolated polypeptide from the CA-SP1 region of HIV-1, consisting of an amino acid sequence selected from any one of SEQ ID NO: 121 to 443.

90. An isolated polypeptide from the HIV CA-SP1 polypeptide which contains a mutation that results in a decrease in inhibition of processing of p25 by 3-O-(3',3'-dimethylsuccinyl) betulinic acid.

91. The isolated polypeptide of claim 90, wherein said mutation is located in the CA-SP1 cleavage site or in the SP1 domain.

92. The isolated polypeptide of claim 90, which is encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9.

93. The isolated polypeptide of claim 90, containing a mutation in that results in a decrease in inhibition of processing of p25 by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid; wherein said mutation occurs in the nucleic acid sequence encoding a polypeptide selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

94. The isolated polypeptide of claim 90 selected from the group consisting of:

(a) GHKARVLVEAMSQV (SEQ ID NO: 2);

(b) SHKARILAEVMSQV (SEQ ID NO: 3);

(c) SQKARILAEAMSQVTN;

(d) GQKARVLAEAMSQVTN;

(e) SYKARILAEAMSQVTN;

(f) GYKARVLAEAMSQVTN;

(g) SHKARLLAEAMSQVTN;

(h) GHKARLLAEAMSQVTN;

(i) SHKARIMAEAMSQVTN;

(j) GHKARVMAEAMSQVTN;

(k) SHKARILAEVMSQVTN;

(l) GHKARVLAEVMSQVTN;

(m) SHKARILVEAMSQVTN;

(n) GHKARVLVEAMSQVTN;

(o) SHKARILAEALSQVTN;

(p) GHKARVLAEALSQVTN;

(q) SHKARILAEAMLQVTN;

(r) GHKARVLAEAMLQVTN;

(s) SHKARILAEAMSEVTN;

(t) GHKARVLAEAMSEVTN;

(u) SHKARILAEAMSQATN;

(v) GHKARVLAEAMSQATN;

(w) SHKARILAEAMSQVLN;

(x) GHKARVLAEAMSQVLN;

(y) SHKARILAEAMSQVTA;

(z) GHKARVLAEAMSQVTA; and

(aa) HKARILAEAMSQVTNPATIMIQKGNFRNQRKTVKCFNC GKEGHIT.

95. The isolated polypeptide of claim 90, encoded by an isolated polynucleotide which hybridizes under stringent conditions to a polynucleotide selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, and 10.

96. The isolated polypeptide of claim 90, which is part of a chimeric or fusion protein.

97. An isolated polynucleotide which encodes an amino acid sequence containing a mutation in an HIV Gag p25 protein (CA SP1), said mutation resulting in a decrease in inhibition of processing of p25 (CA-SP1) to p24 (CA) by 3-O-(3',3'-dimethylsuccinyl) betulinic acid.

98. The isolated polynucleotide of claim 97, wherein said decrease in inhibition of processing of p25 is due to a decrease in inhibition of the interaction of HIV-1 protease with Gag.

99. The isolated polynucleotide of claim 97, wherein said decrease in inhibition of processing of p25 is due to a decrease in the binding of 3-O-(3',3'-dimethylsuccinyl) betulinic acid to Gag.

100. The isolated polynucleotide of claim 97, wherein said decrease in inhibition of processing of p25 is due to a decrease in the binding of DSB at or near the CA-SP1 cleavage site of Gag.

101. The isolated polynucleotide of claim 97, wherein said mutation is located at or near the CA-SP1 cleavage site or in the SP1 domain of CA-SP1.

102. The isolated polynucleotide of claim 97, wherein said mutation is located at or near the polynucleotide encoding the amino acid sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

103. The isolated polynucleotide of claim 97 selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 9.

104. The isolated polynucleotide of claim 97, having at least about 95% identity to a polynucleotide selected from the group consisting of SEQ ID NO: 4, and SEQ ID NO: 6.

105. The isolated polynucleotide of claim 97, having at least about 80% identity to a polynucleotide selected from the group consisting of SEQ ID NO: 8 and SEQ ID NO: 9.

106. The isolated polynucleotide of claim 97, having at least about 95% identity to a polynucleotide selected from the group consisting of SEQ NO: 5 and SEQ ID NO: 7.

107. The isolated polynucleotide of claim 97, having at least about 80% identity to a polynucleotide of SEQ ID NO: 10.

108. A vector comprising the isolated polynucleotide of claim 97.

109. A host cell comprising the vector of claim 108.

110. A method of producing a polypeptide comprising incubating the host cell of claim 109 in a medium and recovering the polypeptide from said medium.

111. A method of identifying a compound that inhibits HIV-1 replication in cells of an animal, comprising:

(a) contacting a polypeptide comprising a CA-SP1 cleavage site with a test compound;

(b) adding a labeled substance that selectively binds at or near the CA-SP1 cleavage site; and

(c) measuring the binding of said labeled substance to said polypeptide.

112. The method of claim 111, wherein said compound binds at or near the CA-SP1 cleavage site.

113. The method of claim 111, wherein said compound inhibits the interaction of HIV-1 protease with the CA-SP1 cleavage site.

114. The method of claim 111, wherein said polypeptide comprising a CA-SP1 cleavage site is a polypeptide fragment or recombinant peptide.

115. The method of claim 111, wherein said labeled substance is a labeled antibody specific for CA-SP1; and wherein said measuring comprises measuring the change in the amount of labeled antibody bound to the protein in the presence said test compound compared with a control.

116. The method of claim 111, wherein the labeled substance is 3-O-(3',3'-dimethylsuccinyl) betulinic acid, and wherein the method comprises measuring the change in the amount of labeled 3-O-(3',3'-dimethylsuccinyl) betulinic acid bound to the protein in the presence of test compound, compared with a control.

117. The method according to claim 111, wherein the label on said labeled substance is an enzyme, a fluorescent substance, a chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, an electron dense substance, radioisotope, or a combination thereof.

118. A method for identifying a test compound that inhibits HIV-1 replication in the cells of an animal comprising:

(a) contacting a polypeptide comprising a CA-SP1 cleavage site, with a protease in the presence of a test compound; and

(b) detecting the interaction of protease with said polypeptide.

119. The method of claim 118, wherein said protease consists essentially of HIV-1 protease.

120. The method of claim 118, wherein said polypeptide is labeled with two fluorescent moieties, each bound to opposite sides of the CA-SP1 cleavage site, and wherein said detecting comprises measuring the transfer of fluorescent energy from one moiety to the other in the presence of the test compound.

121. The method of claim 118, wherein the effect of the test compound on cleavage of said polypeptide is detected by measuring the binding of an antibody to at least one of SP1, p24 (CA), or CA-SP1 (p25).

122. The method of claim 121, wherein said labeled antibody is labeled with a molecule selected from the group consisting of enzyme, fluorescent substance, chemiluminescent substance, horseradish peroxidase, alkaline phosphatase, biotin, avidin, electron dense substance, radioisotope, and combinations thereof.

123. The method of claim 118 comprising:

(a) contacting a first polypeptide comprising an HIV-1 wild-type CA-SP1 cleavage site, with HIV-1 protease in the presence of said test compound;

(b) contacting a second polypeptide, comprising a mutant CA-SP1 cleavage site or an alternative protease cleavage site with HIV-1 protease in the presence of said test compound; wherein the cleavage of said mutant cleavage site or alternative protease

cleavage site is not substantially inhibited by 3-O-(3',3'-dimethylsuccinyl) betulinic acid;

(c) detecting the interaction of HIV-1 protease with said first and second peptides; and

(d) comparing the cleavage of said first polypeptide to the cleavage of said second polypeptide.

124. The method of claim 123, wherein the wild-type CA-SP1, mutant CA-SP1, or alternative protease cleavage site region is contained within a polypeptide fragment or recombinant peptide.

125. The method of claim 123, wherein said polypeptide is labeled with a fluorescent moiety and a fluorescence quenching moiety, each bound to opposite sides of the CA-SP1 cleavage site, and wherein said detecting comprises measuring the signal from the fluorescent moiety.

126. The method of claim 123, wherein at least one of said first or said second polypeptide further comprises an epitope tag.

127. The method of claim 123, wherein (a) and (b) occur in a cell.

128. The method of claim 123, wherein said detecting the interaction of said compound with said first and second polypeptides comprises gel electrophoresis.

129. A method for identifying compounds that inhibit HIV-1 replication in the cells of an animal comprising:

(a) contacting a test compound with cells infected with a first virus, the replication of which is sensitive to inhibition by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB);

(b) contacting a test compound with cells infected with a second virus having significantly reduced sensitivity to DSB;

(c) determining the phenotype of treated cells infected with the first virus and the second virus; and

(d) selecting a test compound that is more active against the first virus compared with second virus.

130. The method of claim 129, wherein said phenotype is selected from the group consisting of:

(a) viral replication;

(b) cleavage of CA-SP1; and

(c) production of an altered virion phenotype.

131. A method for identifying a compound that inhibits HIV-1 replication in the cells of an animal, comprising:

(a) applying a test compound to cells infected with a lentivirus, the replication of which is sensitive to inhibition by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB); and

(b) analyzing a lysate derived from said infected cells to determine whether cleavage of the CA-SP1 protein has occurred.

132. The method of claim 131, wherein said lysate is obtained by lysing at least one of said

(a) infected cells; or

(b) the released viral particles.

133. The method of claim 131, wherein said analyzing to determine whether cleavage of the CA-SP1 protein has occurred comprises measuring the presence or absence of p25.

134. The method of claim 131, wherein said analyzing to determine whether cleavage of the CA-SP1 protein has occurred comprises performing a western blot of viral proteins and detecting p25 using an antibody to p25.

135. The method of claim 131, wherein said analyzing to determine whether cleavage of the CA-SP1 protein has occurred comprises performing a gel electrophoresis of viral proteins and imaging of metabolically labeled proteins.

136. The method of claim 131, wherein said analyzing to determine whether cleavage of the CA-SP1 protein has occurred comprises performing an immunoassay.

137. The method of claim 133, wherein said immunoassay comprises:

(a) capturing p25 and p24 on a substrate using an antibody that selectively binds 24;

(b) detecting the presence or absence of p25 on the substrate with an antibody that selectively binds p25.

138. The method of claim 131, wherein an epitope tag sequence is inserted into SP1, and wherein said analyzing comprises selective detection of p25 using an antibody to the epitope tag.

139. A method of identifying a compound that inhibits HIV-1 replication in the cells of an animal comprising: applying a test compound to cells infected with a lentivirus, the replication of which is sensitive to

inhibition by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB), and thereafter analyzing virus particles released by the cells for an altered virion phenotype.

140. The method of claim 139, wherein said analyzing involves transmission electron microscopy.

141. The method of claim 139, wherein said altered phenotype is selected from the group consisting of:

(a) virions possessing spherical, electron-dense cores that are acentric with respect to the viral particle;

(b) virions possessing crescent-shaped electron-dense layers lying just inside the viral membrane;

(c) virions having reduced or no infectivity; and

(d) the combination of any one of (a) through (c).

142. The method of any one of claims 129, 131 or 139, wherein said lentivirus, the replication of which is sensitive to inhibition by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB), is HIV-1.

143. The method of any one of claims 129, 131 or 139, wherein said lentivirus, the replication of which is sensitive to inhibition by 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB), is a recombinant non-HIV-1 lentivirus comprising the nucleotide encoding the CA-SP1 from HIV-1, and wherein said lentivirus is derived from a lentivirus selected from the group consisting of.

(a) SIV;

(b) FIV;

(c) EAIV;

(d) BIV;

(e) CAEV;

(f) Visna-Maedi virus; and

(g) HIV-2.

144. The method of claim 131, wherein said virus having significantly reduced sensitivity to DSB is selected from the group consisting of:

(a) SIV;

(b) FIV;

(c) EAIV;

(d) BIV;

(e) CAEV;

(f) Visna-Maedi virus;

(g) HIV-2; and

(h) mutant HIV-1.

145. A method of identifying a compound, comprising:

(a) measuring disease progression in treated animals infected with a lentivirus, wherein said lentivirus is sensitive to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB);

(b) measuring disease progression in untreated animals infected with said lentivirus,

(c) comparing the response of said treated and said untreated animals.

146. A method of identifying a compound, comprising

(a) administering said compound to animals infected with a first lentivirus, wherein the replication of said first lentivirus is inhibited by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB);

(b) administering said compound to animals infected with a second lentivirus, wherein the replication of said second lentivirus is not substantially inhibited by 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB);

(c) measuring and comparing disease progression in animals infected with said first lentivirus, compared with animals infected with said second lentivirus.

147. A method of identifying a compound, comprising measuring the interaction of said compound with an amino acid sequence selected from the group consisting of:

(a) KNWMTETFLVQNANPDCKTILKALGPAATLEEMMTA CQGVGGPSHKARILAEAMSQVTNSATIM (SEQ ID NO: 21);

(b) KNWMTETLLVQNANPDCKTILKALGPGATLEEMMTA CQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 22);

(c) TACQGVGGPSHKARILAEAMSQVTNSATIM; (SEQ ID NO: 23);

(d) TACQGVGGPGHKARVLAEAMSQVTNPATIM (SEQ ID NO: 24);

(e) SHKARILAEAMSQV (SEQ ID NO: 25);

(f) GHKARVLAEAMSQV (SEQ ID NO: 26)

(g) SHKARILAEAMSQVTN (SEQ ID NO: 116);

(h) GHKARVLAEAMSQVTN (SEQ ID NO: 117);

(i) SHKARILAEAMSQVTNSATIM (SEQ ID NO: 118); and

(j) GHKARVLAEAMSQVTNPATIM (SEQ ID NO: 119)

148. A method of determining if a patient is infected with HIV-1 that is susceptible to treatment by a compound that inhibits p25 processing, comprising:

(a) obtaining a specimen from said patient;

(b) obtaining viral nucleic acid encoding CA-SP1 from the specimen;

(c) genotyping said viral nucleic acid;

(d) determining if the nucleic acid encodes a CA-SP1 variant, the processing of which is sensitive to inhibition by to 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid (DSB).

149. A method of treating a disease in a patient in need thereof comprising:

(a) identifying a compound which inhibits the processing of viral Gag p25 protein (CA-SP1) to p24 (CA), but has no significant effect on other Gag processing steps;

(b) obtaining regulatory approval for the sale and use of said compound;

packaging the compound for sale and treatment of a disease in a patient in need thereof.