INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

A61K 39/395 (2006.01)  A61P 31/18 (2006.01)
A61K 35/12 (2006.01)  A61P 31/12 (2006.01)

International Application Number:
PCT/US2014/032832

International Filing Date:
3 April 2014 (03.04.2014)

Filing Language:
English

Publication Language:
English

Priority Data:
US. 61/808,097  3 April 2013 (03.04.2013)

Applicant:

Inventors:

Agents:
BOULWARE, Margaret, A. et al; Boulware & Valoir, Three Riverway, Suite 950, Houston, TX 77056 (US).

Designated States (unless otherwise indicated, for every kind of national protection available):

Designated States (unless otherwise indicated, for every kind of regional protection available):

Published:
— with international search report (Art. 21(3))
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
— with sequence listing part of description (Rule 5.2(a))

Title: EXPRESSION OF HIV INHIBITORS BY MESENCHYMAL STEM CELLS

Abstract: A composition of matter and method of treating HIV with mesenchymal stem cells (MSC) is disclosed. Specifically, MSCs are transduced with vectors incorporating an anti-viral fusion inhibitor, such as a C46-derived peptide. The transduced MSCs are capable of expression the inhibitor and preventing HIV virus-cell fusion.
EXPRESSION OF HIV INHIBITORS BY MESENCHYMAL STEM CELLS

PRIOR RELATED APPLICATIONS

[0001] This disclosure claims priority to US 61/808097, filed on April 3, 2013 and incorporated by reference in its entirety herein.

FEDERALLY SPONSORED RESEARCH STATEMENT

[0002] Not applicable.

REFERENCE TO MICROFICHE APPENDIX

[0003] Not applicable.

FIELD OF THE DISCLOSURE

[0004] The present disclosure relates generally to methods of treating human immunodeficiency virus (HIV), and specifically, to the use of transduced mesenchymal stem cells to treat HIV.

BACKGROUND OF THE DISCLOSURE

[0005] Human immunodeficiency virus (HIV) is a slowly replicating retrovirus that leads to progressive failure of the immune system in humans, thus opening the door for life-threatening opportunistic infections and cancers to thrive. Infection with HIV results in CD4+ T cells depletion through a number of mechanisms including: apoptosis of uninfected bystander cells; direct viral killing of infected cells; and, killing of infected CD4+ T cells by CD8 cytotoxic lymphocytes that recognize infected cells. The loss of the CD4+ T cells causes Acquired Immune Deficiency Syndrome (AIDS). The loss of immune function from HIV and AIDS has led to the death of over 25 million people from AIDS worldwide.

[0006] Many therapies exist to manage but not cure HIV. Antiretroviral therapies (ART), for example, control viremia, a condition where viruses enter the bloodstream and have access to the rest of the body. The available anti-HIV
drugs target different phases of the HIV life cycle, wherein each drug is classified according to the phase it inhibits. However, a standard ART consists of at least three antiretroviral drugs used in combination such that multiple phases of the HIV life cycle are inhibited and replication is suppressed as much as possible.

While showing promise in HIV and AIDS treatment, ART does have its drawbacks. Over 30 different types of drugs are available for ART, but only a few combinations may work for a given patient. A patient may have to cycle through multiple combinations and dosage levels before finding one that works. Multiple pills must be taken one or more times a day on a specific schedule to decrease a patient's viral load. Any deviation from the treatment schedule can result in mutations of the HIV/AIDS strain that may be resistant to the current drug combination. Furthermore, these medications may cause side effects, such as insulin resistance or lipid abnormalities, which also require additional medication in combination with the ART. Thus, the ART drug regimens are complex and expensive, require life-long intervention with potential side effects.

Like most viruses, HIV has a viral envelope. Embedded in HIV's viral envelope is a protein known as Env, which consists of a cap made of three molecules called glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the virus structure into the viral envelope. Because this glycoprotein complex enables the virus to attach to and fuse with target cells to initiate infection, the glycoproteins are targeted by treatments and vaccines.

Enfuvirtide (fuzeon, or T-20) is an FDA-approved drug in a new class of fusion inhibitor that disrupts the HIV-1 molecular machinery at the final stage of fusion with the target cell. Enfuvirtide targets the gp41 glycoprotein during fusion. Specifically, gp41 undergoes conformational changes that allow fusion of HIV-1 to target cells. Enfuvirtide binds to gp41, thus preventing the creation of an entry pore for the virus, keeping it out of the cell.

However, this injectable drug has significant side effects and drug delivery issues as most patients develop some local injection site reaction. Furthermore, enfuvirtide has a short half-life, which requires twice daily administration, thus antagonizing the local injection site reaction. The
subcutaneous application of enfuvirtide is also a distinct disadvantage in patients who are already burdened by complex oral therapy.

Thus, there exist a need for novel therapies that are less complex and more accommodating. Ideally, the new therapies will have limited side effects and a longer half-life.

**SUMMARY OF THE DISCLOSURE**

The present disclosure provides a novel composition of MSCs transduced with one or more retroviral and/or lentiviral vectors expressing anti-viral fusion inhibitors of the HIV infection and methods of use. In accordance with this discovery, the disclosure also provides a method of inhibiting HIV infection using transduced MSC expressing these inhibitors.

Multipotent stem cells, such as mesenchymal stem cells (MSCs), are stem cells capable of differentiating into cells specific to tissues and organs containing these cells and are involved in fetal, neonatal and adult periods of growth and development, but also in the maintenance of homeostasis of adult tissue and the functioning of inducing regeneration upon tissue damage. The variety of cell types that MSCs can differentiate into is illustrated in FIG. 1.

MSCs have been proposed for the treatment of numerous illnesses in part because these cells have been shown to migrate to sites of inflammation. Unlike most other human adult stem cells, MSC can be obtained in quantities appropriate for clinical applications, making them good candidates for use in tissue repair. Furthermore, they also have antiproliferative, immunomodulatory and anti-inflammatory effects and are low immunogenic. Although the mechanism underlying the immunosuppressive effects of MSCs has not been clearly defined, their immunosuppressive properties have already been exploited in the clinical setting. Therefore, MSCs have implications for treatment of allograft rejection, graft- versus-host disease, rheumatoid arthritis, autoimmune inflammatory bowel disease and other disorders in which immunomodulation and tissue repair are required.
In addition to tissue repair, MSCs have also been used in certain virus treatments. WO20 10053350 describes the use of MSCs for inhibition of a hepatitis viral infection. Here, cells replicating the virus are contacted with either a liquid containing MSCs or with an exudate of the MSCs. However, MSCs have never been used to express inhibitors of viruses such as HIV.

In the present disclosure, it was determined that MSCs can be used as "trojan bioreactors" to carry and express HIV inhibitors at local sites of infection. The MSCs were transduced with retroviral and lentiviral vectors packaged with antiviral fusion inhibitors of the HIV infection. The transduced MSCs were able to express the inhibitors and maintain differentiation abilities. In addition to inhibiting HIV fusion, these transduced MSC are expected to reduce the general immune activation frequently observed in patients with HIV infection as has been shown by MSC treatment in other disease indications.

In one embodiment, the present disclosure provides transduced MSCs expressing certain inhibitors to treat HIV. Specifically, HIV anti-viral fusion inhibitors are incorporated into delivery vehicles such as retroviruses and lentiviruses. These delivery vehicles are introduced into the MSCs, wherein the cells under go transduction. The transduced MSC are then able to express the HIV peptide inhibitors while retaining important characteristics such as the capacity for osteogenic, adipogenic, and chondrogenic differentiation.

In another embodiment, a method of treating human immunodeficiency virus by administering mesenchymal stems cells expressing anti-viral fusion inhibitors of HIV infection is disclosed. The MSCs will provide long-term, continuous expression of the inhibitors without the side effects associated with currently available injectable HIV inhibitors, such as injection-site irritation and difficulties in handling.

In another embodiment, the present disclosure provides a method of inhibiting HIV infection by administering conditioned medium from transduced MSC expressing anti-viral fusion inhibitors.

Additionally, the above methods can be combined with an anti-HIV drug treatment. The MSCs can be administered before, during or after the antiviral
drugs are administered. Preferably, the MSCs are administered after the antiviral
drugs.

In some embodiments, the transduced MSCs are administered directly to a subject. In other embodiments, the MSCs or conditioned media is mixed with an appropriate pharmaceutical carrier for administration. Preferred routes of administration are intravenous, subcutaneous and intraperitoneal injections.

Multiple HIV anti-viral fusion inhibitors can be used with the present composition and methods. Peptide inhibitors that block different points in the HIV replication and fusion mechanism by targeting CCR5, CD4-binding, gp41 and envelope can be used in the above embodiments. In addition to, or in place of, peptide inhibitors, neutralizing antibodies can be packaged into the vectors. Neutralizing antibodies defend a cell from an antigen or infectious body by inhibiting or neutralizing any effect it has biologically.

Exemplary peptide inhibitors are C46 peptides, which are derived from the HPV2 gene of gp41. Secretable antiviral entry inhibitory (SAVE) peptides for C46 such as those in US20110027240, which is incorporated herein in its entirety, can also be used. For instance, a customized C46 peptide:

WMEWDREINNYTSLIHSIESQNNQQEKEQELLELDKWaslwnWF (SEQ NO. 1) was used in some embodiments of the present disclosed methods. T-20:

YTSLIHSIEESQNNQQEKeQELLELDKWaslwnWF (SEQ No. 2), provided through the NIH AIDS Reagent Program, division of AIDS, NIAID, NIH: T-20, fusion Inhibitor from AIDS, NIAID, is another possible peptide inhibitor. Such SAVE peptides are known to disrupt the fusion mechanism by blocking the gp41 viral envelope protein. Other peptide inhibitors of HIV replication, in addition to the C46 SAVE peptides, would be acceptable in the presently disclosed composition and method.

The sequence of the peptides inhibitors are incorporated into retroviral and lentiviral vectors before being transduced in the MSCs. Success of the transduction is confirmed by analyzing the MSCs using commonly acceptable practices to confirm expression of the peptide inhibitors. Once expression is
confirmed, the MSCs can be injected into a person who has HIV, who has been exposed to HIV, or who is at risk for exposure to HIV.

[0025] The inhibitors can be packaged into the retroviral and lentiviral vectors using any known method in the art, typically, using purchased kits. Invitrogen offers lentiviral packaging kits, as does InvivoGen. Additionally, markers such as green fluorescence protein can also be packaged to facilitate confirmation of expression and determine transduction efficiency.

[0026] The MSCs for the present methods can be allogeneic or autologous, depending on the stage of infection and degree of immune deficiency. All acceptable methods of extraction of MSC can be used in the present methods. Preferably, MSCs derived from adipose tissue are utilized due to the large number of cells per volume of sample. Furthermore, adipose tissue is much easier to access than other sources such as bone marrow, which is particularly important for immune-compromised subjects. However, because MSCs can be expanded in culture before being transduced, other sources of MSCs, such as bone marrow, can be utilized.

[0027] The described composition and treatment methods can be applied to animals, preferably mammals, and most preferably, to humans.

[0028] In the context of the disclosure, "HIV" includes HIV-1 and HIV-2 types. Further included are all natural HIV strains (quasi-species or isolates). In this respect, it is understood that HIV strains include all known HIV strains as listed in the Los Alamos National Laboratory HIV Sequence Database.

[0029] As used herein, "mesenchymal stem cells" means any cells that meet the criteria set forth by The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT). Under this criteria, MSCs: 1) are plastic-adherent when maintained under standard culture conditions (especially in the presence of human or fetal bovine serum); 2) have the capacity for osteogenic, adipogenic, and chondrogenic differentiation; 3) express CD73, CD90, and CD105; and 4) lack expression of the hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD19, CD79, and human leukocyte antigen-DR.
The term "autologous" as used herein shall be taken to mean from the same individual.

The term "allogeneic" as used herein shall be taken to mean from an individual separate from the individual being treated.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims or the specification means one or more than one, unless the context dictates otherwise.

The term "about" means the stated value plus or minus the margin of error of measurement or plus or minus 10% if no method of measurement is indicated.

The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or if the alternatives are mutually exclusive.

The terms "comprise", "have", "include" and "contain" (and their variants) are open-ended linking verbs and allow the addition of other elements when used in a claim.

The phrase "consisting of" is closed, and excludes all additional elements.

The phrase "consisting essentially of" excludes additional material elements, but allows the inclusions of non-material elements that do not substantially change the nature of the disclosure.

The following abbreviations are used herein:

<table>
<thead>
<tr>
<th>ABBREVIATION</th>
<th>TERM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART</td>
<td>Antiretroviral therapies</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>FACs</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Rh</td>
<td>Rhesus</td>
</tr>
<tr>
<td>BMS</td>
<td>Bone marrow MSCs</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
</tbody>
</table>

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Illustration of potential cell types upon MSC differentiation.
FIG. 2 shows a diagram of the HIV fusion mechanism. From Drugs Fut. 1999, 24(12): 1355.

FIG. 3 shows the design of a number of vectors.

FIG. 4 shows a determination of transduction level using GFP+ cells.

FIG. 5 shows GFP+ levels by flow cytometry.

FIG. 6 shows confirmation of a transduced RbMS Differentiation Assay.

FIG. 7A-B display western blots of MSCs transduced with various HIV peptide inhibitors packaged in virus vectors, wherein RbMS cells are in 7A and 293T cells are in 7B.

FIG. 8 displays a single round infection/inhibition assay in CEMxl74 cells using conditioned media from MSC transduced with different inhibitors.

DETAILED DESCRIPTION

The disclosure describes novel transduced mesenchymal stems cells (MSC) that express HIV virus-cell fusion inhibitors and a method of use to treat HIV. Specifically, mesenchymal stems cells (MSC) are transduced with one or more retroviral and/or lentiviral vectors expressing antiviral fusion inhibitors of HIV is disclosed. The transduced MSCs express the inhibitors without losing important MSC characteristics such as the ability to differentiate.

Methods of using the novel composition can comprise treating an animal, and preferably a human, suffering from an HIV infection wherein a peptide inhibitor or neutralizing antibody is incorporated into a viral or nonviral vector, which is then transduced into a MSC such that the MSC can express the inhibitor. The transduced MSCs are then administered to a subject alone or in combination with anti-retroviral therapies. The MSCs can be autologous or allogenic and taken from any source including bone marrow or adipose tissue. Preferred viral vectors are lentivirus, retrovirus, or any combination thereof. Exemplary inhibitors include one or more of secreted antiviral entry inhibitory (SAVE)
peptides, C46, maC46 or neutralizing antibodies. The MSCs can be administered intravenously, subcutaneously, or intraperitoneally.

[0049] FIG. 2 displays the fusion mechanism by which HIV infects new cells. HIV fusion with a target cell starts with the attachment of gpl20 to the N-terminal domain of the CD4+ receptors (B). Once attached, gpl20 undergoes a conformational change that unmasks the binding site for the cell co-receptor (C). Once the virus is bound, a conformational change exposes the N-terminal hydrophobic region of gp41 to the CD4+ cell membrane and becomes the "pre-hairpin intermediate". Gp41 undergoes a conformation change (D), also referred to as a coiled-coil bundle, to bring the virus and cell membranes together, mediating fusion of virus and cell (E).

[0050] In one aspect of the present disclosure, to inhibit the fusion of HIV, MSCs are transduced with HIV peptide inhibitors. Many HIV peptide inhibitors exist including maC46 and C46 peptides as well as neutralizing antibodies. The HIV entry inhibitor maC46 is a membrane-anchored peptide derived from the second heptad repeat of the HIV-1 transmembrane glycoprotein gp41. C46 peptides are members of the new fusion inhibitor class of antiretroviral drugs that efficiently block infection of new cells by interfering with the function of gp41. The C46 peptides can be membrane-bound or secreted.

[0051] The peptide inhibitors are incorporated into retroviral or lentiviral vectors, which act as gene delivery vehicles for transducing the MSCs with the peptide inhibitors. Exemplary vectors include murine leukemia (MLV) and self-inactivating lentiviral vectors (HSRT), however other viral or non-viral vectors systems would also be applicable. FIG. 3 displays a few of the virus vectors that have incorporated the peptide inhibitors, particularly the maC46 and SAVE peptide sequences. The vectors can also contain a fluorescence marker, such as GFP, to aid in monitoring the success of the transduction.

[0052] After being transduced, the MSC can be analyzed to confirm expression of the peptide inhibitors using fluorescent microscopy, PCR, differentiation assays and other commonly used techniques.
The present disclosure is exemplified with respect to the examples and figures below. However, this is exemplary only, and the disclosure can be broadly applied to incorporate any HIV fusion inhibitor for all phases of the HIV life cycle and can be used for any animal. The following experiments and examples are intended to be illustrative only, and not unduly limit the scope of the appended claims.

**EXAMPLE 1**

Gene transfer and expression was performed by the following steps:

**Cells:** Human Bone Marrow stem cells (HuMSCs), Rhesus Bone Marrow Stem cells (RhMSCs), and Rhesus Adipose stem cells (RhACSs) were plated at 10,000/cm² in alpha-MEM/20% FBS with no antibiotics the day before transduction.

For transduction, the plated cells were exposed to various MOI of the control vectors, such as LZRS-GFP (19) or HRST-cmvGFP (38), or the therapeutic vectors, such as T42 (unknown), T60 (22), and M218 (unknown), containing the C46 peptide sequences shown in FIG. 3. Then, the plates were centrifuged at 1000 rpm for 1-2 hours. After which, the MSCs were cultured at 37°C and 5% CO₂ for two days. After Day 2, cells were returned to standard culture conditions, wherein the cells were re-suspended in complete conditioned media and filter through a 70 mm cell strainer.

**Expression Confirmation:** Fluorescent microscopy and flow cytometry were used to assess the expression of the C46 peptide using GFP. Real time PCR was also performed.

As shown in FIG. 4, cells were observed under fluorescent microscope to confirm transduction and expression of GFP in transduced cells. The upper panels are, from left, non-transduced (NT) MSC, rhASC with phase contrast, and GFP expression overlayed onto the phase contrast image. Lower panels show overlay of GFP expression in HuMSCs, RhMSCs, and RhACSs transduced with HRST-cmvGFP. As seen, the GFP is readily expressed in the human and rhesus stem cells.
[0059] FIG. 5 displays the flow cytometry Fluorescence-activated cell sorting (FACs) results for HuMSCs, RhMSCs, and RhACSs. For this analysis, cells were trypsinized and washed with 1% mouse serum. After the wash, cells were fixed with 4% paraformaldehyde and analyzed for GFP expression by flow cytometry. The NT control population was set as background fluorescence.

[0060] The HRST-GFP showed the highest expression levels in all three populations by FACs analysis. The LZRS-transduced MSC showed slightly elevated levels of GFP expression in all three MSCs populations.

[0061] Gene transfer and expression was detectable up to 69% in MSCs by fluorescent microscopy and flow cytometry.

[0062] The real time PCR results for the vector backbone, shown below, detected between 5-25% of the human MSCs, rhesus MSCs, and HEK 293 cells contained the Psi packaging element in T60 and M218. Both T60 and M218 are murine leukemia virus based vectors.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>C46 Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T60 (%)</td>
</tr>
<tr>
<td>Hu MSC</td>
<td>3.9</td>
</tr>
<tr>
<td>Rh MSC</td>
<td>12.5</td>
</tr>
<tr>
<td>Rhs ASC</td>
<td>10.6</td>
</tr>
<tr>
<td>293T</td>
<td>10.0</td>
</tr>
</tbody>
</table>

[0063] Differentiation assays were also performed to confirm transduced RhMSCs to test that the inhibitors do not disturb MSCs differentiation and results are shown in FIG. 6. The osteogenic, adipogenic, and chondrogenic differentiation abilities of the MSCs were taken at 21 days for the M218, T60, HRST-GFP and T42 vector. As seen in FIG. 6, the MSCs retained their ability to differentiate into multiple cells, regardless of the vector or peptide inhibitor used. This shows that MSCs have not lost their differentiation abilities after being transduced.

[0064] Results: The HIV 1-based lentiviral vector HRST-cmvGFP was superior to LZRS-GFP in both the frequency of gene transfer and the level of expression. Furthermore, real-time PCR data showed a transduction rate of 5-25% in the MSCs for MLV C46 vectors.
EXAMPLE 2

[0065] The above method was applied to a larger variety of cells.

[0066] **Cells:** Most cells were obtained from the NIH AIDS Reagent Program. The NIH AIDS Reagent Program is a worldwide resource for state-of-the-art HIV research materials, some of which are not yet commercially available. CEMx174 cells (NIH AIDS Reagent Program Cat no. 272), expressing CD4, T and B cell markers, were obtained because they are easily infected with HIV virus. Second T-cell line "PM-1" (NIH AIDS Reagent Program Cat no. 3038), permissive for growth of macrophage and T cell tropic viruses, a subclone of HuT78 (Human T cell lymphoma) expressing CD4, CXCR4, and CCR5, was obtained. All T-cell lines were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium.

[0067] Human TZM-bl cell (NIH AIDS Reagent Program Cat no. 8129), previously designated JC53-bl (clone 13) is a HeLa cell line. The parental cell line (JC.53) stably expresses large amounts of CD4 and CCR5. The TZM-bl cell line was generated from JC.53 cells by introducing separate integrated copies of the luciferase and B-galactosidase genes under control of the HIV-1 promoter. All media were supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 10,000 U/mL penicillin/streptomycin.

[0068] Rhesus macaque bone marrow/adipocyte derived mesenchymal stem cells were isolated and maintained in complete culture medium, 20% FBS MEM a (Minimum Essential Medium a), with l-glutamine, fetal bovine serum (Atlanta Biologicals Premium Select FBS), Penicillin G (10,000 units/mL), and streptomycin sulfate (10,000 µg/mL) in solution of 0.85% NaCl (Invitrogen/GIBCO).

[0069] Human embryonic kidney cell line 293T was obtained from the ATCC, and was maintained in Dulbecco's modified Eagle's medium (DMEM).

[0070] **Bone marrow and adipose derived mesenchymal stem cells isolation and culture:** Bone marrow-derived mesenchymal stem cells were isolated by discontinuous density gradient centrifugation following the Tulane Center for Stem Cell Research and Regenerative Medicine protocol. Briefly, LSM
(Lymphocyte Separation Medium, ICN50494, MP Biomedicals, No.:0850494) one-step separation medium was underlayered beneath the bone marrow mixture. The sample was centrifuged at 1400 rpm (~ 400 g) for 40 min. The low-density mononuclear cells were collect at the white interface and washed with fresh media. After the last centrifugation, the supernatant discard and a lysis buffer (ACK lysing buffer, Gibco, catalog no. 10492-01) was used to eliminate residual red blood cells.

The collected cells were plated at a concentration of ~1-2 million cells in a 15 cm tissue culture dish and incubate at 37°C in 5% CO₂. The complete culture medium was changed the next day (24 hours later) and replace with fresh complete medium. When the plate was about 70-80% confluent (~ 14 days), cells were trypsinized and frozen at P0 in Liquid N₂ until they were expand to PI or frozen as a pellet for DNA and protein analysis.

Adipose-derived stem cells were isolated according to the Tulane Center for Stem Cell Research and Regenerative Medicine protocol. Briefly, the adipose tissue was washed extensively with a 5 % Phosphate Buffer Solution (PBS 5% p/s) before being minced into small pieces using a sterile scalpel. The pieces were placed in a 15 cm dish with 0.075%, Collagenase Type I (Gibco, catalog no. 17100-017) in PBS. Collagenased tissues were incubated for 30 min at 37°C at 5% CO₂. The liquid portion was removed and the plate was washed several times with PBS 2% p/s, and centrifuge to obtain a pellet. After lysing with ACK Lysing Buffer (Gibco, catalog no. 10492-01), the adipose-derived cells were washed and incubated for 10 min on ice to lyse any red cells. The cells were again washed with 20 ml PBS 2% p/s, wherein the supernatant was discard. The cell pellets were re-suspended in complete conditioned media and filter through a 70 mm cell strainer.

Collected cells were plated into the proper size culture dish depending on the size of cell pellet and incubate at 37°C at 5% CO₂. The media was changed the next day and replaced with fresh media. When the plate was about 70-80% confluent, aspirate the medium. Cells were frozen and stored in liquid N₂ until they used to expand to PI, and then were frozen a pellet for DNA and protein analysis.
Peptide cloning and mesenchymal stem cells transduction: The C46 peptide used in the present experiment was SEQ No. 1.

C46 peptides driven by Murine leukemia virus (MLV) and lentivirus (M218, T60, and T42) and control vectors (LZRS-GFP and HRST-cmv-GFP) were transformed using one shot stbl3 kit with competent cells (Invitrogen, Cat. No. C7373-03) followed by manufacture's procedure. After sitting overnight, the colonies were selected and a single colony was grown in LB medium plus ampicillin overnight. DNA mini prep was performed with PureLink® Genomic DNA Mini Kit (Invitrogen, Catalog No. 1820-01).

Transient transduction of 293T cells with MLV and lentiviral vectors were completed by the calcium phosphate precipitation method. The calcium phosphate transduction method is used for introducing DNA into mammalian cells and is based on forming a calcium phosphate-DNA precipitate. Calcium phosphate facilitates the binding of the DNA to the cell surface. DNA then enters the cell by endocytosis.

15μg of MLV vector plasmid was used for LZRS-GFP and LZRS-T60 and 2ug of LZRS-M218 was used for transfection of the Phoenix (amphotropic) packaging cell line, a cell line bases on HEK 293T cells. They constitutively express the MLV Gag/Pol and amphotropic envelope genes. Addition of the vector genome will generate replication defective MLV-based retroviral particles in the supernatant. For the HIV-based lentiviral vectors, 13ug of HRST-GFP or T42 vector DNA was transfected along with the HIV packaging plasmids [pHDM-Hgm2 (gag/pol), pRC/Rev, pHDM-Tat lb, envelope HIV-1 JRFL or VSV-G (pHDM.G)] or packaging vector (M438).

Cell culture supernatants containing replication defective lentiviral particles were collected from 24 to 48 hours post-transduction, filtered (0.22 μm pore size) and stored at -80 °C until use.

Transduction of mesenchymal stem cells with lentiviral and MLV vectors: 1x10^5 cells/well of MSCs were plated in 6 well-plate a day before transduction. A desired amount of viral supernatant was added into each well, such as 1 ml of non-concentrated viral supernatant or 100ul of concentrated viral
supernatant. MSCs were transduced with MLV vector and lentiviral vector supernatants by spinoculation (lhr, 1200 rpm, 31°C) then incubated at 37°C in 5% CO₂.

At day 2, the viral supernatant was removed and fresh 20% FBS alpha-MEM with no antibiotics was added to the MSCs. This media was change with 20% FBS alpha-MEM with no antibiotics at day 4. At day 6-7, depending on cell density, cells were prepared for fluorescent microscopy, flow cytometry, and quantitative proviral integrations by real-time PCR. Transduction efficacies were determined 3 days after transduction by flow cytometry.

Detection of secreted version of C peptides: To determine the secreted C46 peptides in mesenchymal cell culture supernatants and cell lysates, a western blot analysis was performed and is shown in FIG. 7A and B. Human HIV-1 gp41 monoclonal antibody (2F5) was obtained from the NIH AIDS Reagent Program. 2F5 allowed the binding of the antibody to all available C peptides.

The secreted peptides were apparent at the 5.82 kD level. This shows that the peptide inhibitors are being secreted by the MSCs and can be available for HIV treatments.

Preparation of HIV pseudotyped particles: Day before transduction, 9x10⁶ cells of 293T cells were plated on 15cm culture dish for a total of 10 plates. Additional plates were prepared for VSV-G control. 80%< of confluency was confirmed under microscopy on the day of transduction.

For the transient transfection, HRST-GFP was mixed with pHDM-Hgm2 (gag/pol), pRC/Rev, pHDM-Tat lb, and envelope gene (either HIV-1 JRFL envelope or the VSV-G control envelope, pHDM.G). All the listed plasmid vectors were gift from Dr. Richard C. Mulligan, Harvard Gene Therapy Institute except JRFL envelope plasmid was provided by Dr. Dorothee von Laer from Innsbruck Medical University. 2.5M CaCl₂ and 2X HBS solution was added equal ratio. Final volume of 1ml of transduction mixture was applied into each culture dish. After 6 hours later, fresh 10% DMEM medium was replaced. Viral supernatant was collected 24-48hrs after transduction and filtered through 0.22um size and treated with 5X PEG-it virus precipitation solution (PEG-it™
Virus Precipitation Solution (5*), SBI, Cat. # LV810A-1/ LV825A-1) for 48-72 hrs. Yellow beige colored pellets were confirmed and, following manufacture's protocol, the virus was concentrated by centrifuge at 1500g for 30 min at 4°C. Virus pellets were collected and resuspended in PBS solution and stored at -80°C until used.

[0085] **Viral single round infection/inhibition assay of pseudotyped HIV-1 system by C46 transduced in MSCs:** In order to mimic the HIV infection system, HRST (CMV-GFP) was packaged with an HIV envelope, JRFL. The CD4+ cell line CEMxI74 were treated with conditioned medium (CM) from the control (HRST-GFP, LZRS-GFP) or C46 (T-60, M218, and T-42) transduced MSC and infected with various MOI of concentrated pseudo HIV-1 viral-like particles produced by calcium phosphate method. After 48 hrs, cells were harvested and fixed with 4% PFA. Samples were analyzed for GFP(+) by BD FAC Verse™ flow cytometry at Tulane National Primate Research Center. GFP (+) flow analysis was conducted by FlowJo version 10 program.

[0086] The resulting single round infection/inhibition assay is shown in FIG. 8. The CM from the control cells had the highest level of GFP expression (infection with the pseudoviral particles). Treatment with CM from the C46-transduced MSCs blocked infection. The level of GFP expression in the CEMxI74 cells was graphed relative to the expression in the control population. Thus, the conditioned media from C46-transduced MSCs were able to block infection with HIV-1 pseudovirus in a single round inhibition assay.

[0087] **Mesenchymal stem cell differentiation assay:** To test that the inhibitors do not disturb MSCs differentiation, osteogenic, adipogenic, and chondrogenic differentiation assay with StemPro® Osteogenesis Differentiation Kit, Gibco A1007201, A1007001, and A1007101 was performed on LZRS-GFP, LZRS-T60, HRST-GFP, M218, T42, and non-transduced RhMSCs. Assay procedure was followed manufacture's protocol. Briefly, cell number to be seeded for assay was $5 \times 10^3$ cells/cm$^2$ and $1 \times 10^4$ cells/cm$^2$ into 12 wells for osteogenic and adipogenic differentiation respectively. $1.6 \times 10^7$ viable cells/ml was plated by seeding 5-$\mu$l droplets of cell solution in the center of multi-well plate wells for classical stain for chondrogenic differentiation. Following around 21 days culture
depends on cells growth, osteogenic, adipogenic, and chondrogenic
differentiation were stained by alizarin red S, oil red o, and safranin o solution
respectively. Stained cells were observed and taken pictures under bright field
light microscope.

EXEMPLARY 3

The MSCs that expressed HIV inhibitor peptides in Experiments 1 and 2
can be combined with antiviral drugs in vitro to assess the synergetic effects.
PMPA (Tenofovir disoproxil fumarate, TDF or tenofovir) and FTC (Emtriva or
Emtricitabine) are exemplary antiviral drugs whose synergetic effects with the
above MSCs can be measured in an in vitro cell culture model.

For instance, CEMx174 cells can be infected with various MOI of
Simian-HIV 89.6p or HIV strains. Then, MSCs can be treated with Mitomycin C,
which is used to generate mitotically inactive the MSC feeder cells. The
CEMx174 cells can then be co-cultured with the treated MSC for up to 14 days
with or without additional antiviral drugs (e.g. 2uM of PMPA and 1.5uM FTC or
others). The HIV replication in the CEMx174 cells can be monitored using p27
flow cytometry or ELISA, or RT-PCR, or other method. Inhibition of viral
replication will be quantified by limiting dilution assay.

Alternatively, CEMx174 cells can be treated with conditioned medium
from the control (HRST-GFP, LZRS-GFP) or C46 (T-60, M218, or T-42)
transduced MSCs and combined with the PMPA and FTC (or other standard HIV
drug). Then, CEMx174 cells are exposed to the HIV viral-like particles.
Inhibition of the HIV infection of the CEMx174 cells is measured by GFP
expression.

Additionally, the order of treatment can be varied such that infected
CEMx174 cells are treated with the transduced MSCs before the antiviral drugs
or co-administered with the antiviral drugs.
EXAMPLE 4

[0092] Animal model testing using the MSCs that expressed HIV inhibitor peptides in Experiments 1 and 2 alone or in combination with antiviral drugs can be performed with small and large animal models.

5 [0093] An exemplary small animal model is an immunodeficient mouse model. Table 1 lists many available mouse models and their advantages or disadvantages indicating that certain mouse models may be better candidates for animal testing.

<table>
<thead>
<tr>
<th>Humanized Mouse Model</th>
<th>Cellular composition in reconstituted hu-mice</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Hu-PBL SCID           | T and B cells                               | • Easy to generate  
                         |                                             | • Can immediately be used after transfer of PBLs | • No multilineage haematopoiesis  
                         |                                             | | • Limited time fram for experiments  
                         |                                             | • Strong activation of T-cells               | • Emergence of xeno-reactive T-cells (GVHD)  
                         |                                             | | |
| Thy/Liv SCID hu       | T cells                                     | • Organoid of fetal thymus/liver tissue with sustained T-cell lymphopoiesis  
                         | Single positive, double positive and double negative Thymocytes  
                         | • Valuable to study certain pathogenic aspects | • Surgical skills needed  
                         |                                             | | • Human fetal tissue needed               | • No multilineage hematopoiesis              | |
|                       |                                             |                         | • Lack of CCR5 expression on intrathymic T progenitor cells | |
| Rag2 ""yö""           | T, B cells, Monocytes, Macrophages, NK cells, and DCs | • Long term multilineage hematopoiesis  
<pre><code>                     |                                             | • Specific antibody response to recall | Delay between transplantation of human CD34+ cells and development of lymphoid systems of ~15weeks |
</code></pre>
<table>
<thead>
<tr>
<th>Models</th>
<th>Characteristic</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOG or NSG</td>
<td>T, B cells, Monocytes, Macrophages, NK cells, and DCs</td>
<td>Higher reconstitution levels as compared to Rag mice</td>
</tr>
<tr>
<td>NOD/SCID-hu BLT &amp; NOD/SCID y&lt;sup&gt;c&lt;/sup&gt;− (NSG) BLT</td>
<td>T, B cells, Monocytes, Macrophages, NK cells, and DCs</td>
<td>Generation of adaptive immune responses</td>
</tr>
</tbody>
</table>

[0094] The therapeutic potential of the stem cells in the humanized mouse models can be measured by analyzing the degree of human cell engraftment by flow cytometry of the peripheral blood mononuclear cells after they have been stained for panhuman markers such as CD45, CD3, CD4, CD8, CD14, and CD19.

[0095] Non-human primate models can be used for the large animal model testing. Such primate models would allow for the use of simian-HIV (S-HIV) infections. The S-HIV viral load in plasma of the animals can be measured using real time PCR. The therapeutic potential of the stem cells can be determined by analyzing the blood and tissue samples from infected primates for biodistribution of MSCs, toxicity and peptide measurement.

[0096] While certain novel features of this disclosure shown and described above are pointed out in the annexed claims, the disclosure is not intended to be limited to the details specified, since a person of ordinary skill in the relevant art will...
understand that various omissions, modifications, substitutions and changes in the forms and details of the disclosure illustrated and in its operation may be made without departing in any way from the spirit of the present disclosure. No feature of the disclosure is critical or essential unless it is expressly stated as being "critical" or "essential."

Furthermore, the above experiments and examples are not intended to limit the scope of the disclosure composition or methods to certain cells or animals.

The following references are incorporated by reference in their entirety.

WO2010053350
US20100226976
US20110027240
CLAIMS

1. A pharmaceutical composition for treating human immunodeficiency virus (HIV) comprising mesenchymal stem cells (MSC), wherein said MSCs express HIV antiviral fusion inhibitors.

2. The pharmaceutical composition of claim 1, wherein said antiviral fusion inhibitor is a peptide or antibody.

3. The pharmaceutical composition of claim 1, wherein said HIV antiviral fusion inhibitors is one or more of secreted antiviral entry inhibitory (SAVE), C46, or maC46 peptides, or neutralizing antibodies.

4. A method of treating human immunodeficiency virus (HIV) comprising:
   a) incorporating a HIV peptide inhibitor into a viral vector to form a gene delivery vector;
   b) transducing a mesenchymal stem cells (MSC) with said gene delivery vector, wherein said MSC expresses the HIV peptide inhibitor after transduction; and
   c) administering said transfected MSCs to a subject with HIV.

5. The method of claim 4, wherein said HIV peptide inhibitors is one or more of secreted antiviral entry inhibitory (SAVE) peptides, C46, maC46 or neutralizing antibodies.

6. The method of claim 4, wherein said MSCs are injected intravenously.

7. The method of claim 4, wherein said MSCs are injected subcutaneously.

8. The method of claim 4, wherein said MSCs are injected intraperitoneally.

9. The method of claim 4, wherein said subject is an animal.

10. The method of claim 4, wherein said subject is a human.

11. The method of claim 4, further comprising administering anti-retroviral therapies
to said subject.

12. A method of treating human immunodeficiency virus (HIV) comprising:

a) administering antiviral drugs and

b) administering any of the compositions in claims 1-3.

13. The method of claim 12, wherein one or more compositions from claims 1-3 are administered.

14. The method of claim 12, wherein the antiviral drugs are administered before the compositions in claims 1-3.

15. The method of claim 12, wherein the antiviral drugs are administered after the compositions in claims 1-3.

16. The method of claim 12, wherein steps a) and b) are administered to an animal.

17. The method of claim 12, wherein steps a) and b) are administered to a human.
FIGURE 2

A "Viral membrane"

V3

gp120

gp41

HIV

B "Adsorption"

V3

gp120

gp41

CD4

CD4+ cell

HIV

C "gp120-CD4-coreceptor" corceptor

V3

gp120

gp41

N peptide

fusion peptide

HIV

D "Pre-hairpin intermediate"

CD4+ cell

gp120

gp41

C peptide

HIV

E "Hairpin"

CD4+ cell

Fusion

Viral entry

HIV
FIGURE 3

LZRS-GFP

M218

T-60

HRST-cmv GFP

T-42

maC46 = S C46 H M

SAVE = S C46 F C46

LTR: viral long terminal repeat
wPRE: woodchuck hepatitis post-transcriptional regulatory element
cppt: central polyuridine tract
Ψ: retroviral Psi packaging element
RRE: Rev-responsive element
CMV: HIV-1 LTR/enhancer
SFFV: spleen focus-forming virus
GFP: green fluorescent protein
ΔLTR: ΔLTR

C46: HIV fusion inhibitory peptide derived from the N-terminus of gp41 (aa 4294-438 of HIV-1HXB2 env)
H: hinge / membrane proximal region C-terminus of the large/IIb domain of gp41 (aa 674-685 of HIV-1HXB2 env)
F: furin protease
M: transmembrane domain
FIGURE 7A

Samples are whole cell fraction
M: marker
1. HIV-env-transfected 293T
2. T-20 treated rhBMS
3. T-60 rhBMS
4. M218 rhBMS
5. T-42 rhBMS
6. T-60 rhBMS
7. M218 rhBMS
8. NT rhBMS
M: marker

FIGURE 7B

Samples are whole cell fraction
M: marker
1. HIV-env-transfected 293T
2. T60-293T
3. M218-293T
4. T42-293T
5. NT 293T
6. VSV-transfected-293T
7. T20 MAGI
M: marker
FIGURE 8

![Graph showing relative infection rate (%) vs. HIV-1 infectious pseudo viruses](image-url)
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

   a. a sequence listing filed or furnished
      - [ ] on paper
      - [ ] in electronic form

   b. time of filing or furnishing
      - [ ] contained in the international application as filed
      - [ ] filed together with the international application in electronic form
      - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
INTERNATIONAL SEARCH REPORT

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.: 4-17
   because they relate to subject matter not required to be searched by this Authority, namely:
   Claims 4-17 pertain to a method for treatment of the human by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.

2. □ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  

Remark on Protest

□ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

□ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

□ No protest accompanied the payment of additional search fees.
# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/395(2006.01)i, A61K 35/12(2006.01)i, A61P 31/18(2006.01)i, A61P 31/12(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 39/395; C12N 15/12; C12N 15/867; A61K 39/21; A61K 48/00; A61K 39/12; C07K 14/765; A61K 35/12; A61P 31/18; A61P 31/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: human immunodeficiency virus (HIV), mesenchymal stem cell (MSC), antiviral fusion inhibitor, secreted antiviral entry inhibitor (SAVE), C46

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 2011-0027240 A1 (LAEK, DOROTHEE VON et al.) 03 February 2011</td>
<td>1-3</td>
</tr>
<tr>
<td></td>
<td>See paragraph [0055]; and claims 1-2, 13, 18.</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Kimpel Janine et al., &quot;Survival of the fittest: positive selection of CD4+ T cells expressing a membrane-bound fusion inhibitor following HIV-1 infection&quot; PLoS ONE, 2015, Vol. 9, No. 5, e12357. See the abstract; and page 7, right-column.</td>
<td>1-3</td>
</tr>
<tr>
<td>A</td>
<td>See claim 1.</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>US 2006-0241027 A1 (HAUSER, RANS-PETER et al.) 26 October 2006</td>
<td>1-3</td>
</tr>
<tr>
<td>A</td>
<td>See claim 1.</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Egelhofer, Marc et al., &quot;Inhibition of human immunodeficiency virus type 1 entry in cells expressing gp41-derived peptides&quot;, Journal of Virology, 2004, Vol. 78, No. 2, pp. 568-575. See the abstract; and page 572, right-column.</td>
<td>1-3</td>
</tr>
</tbody>
</table>

### Further documents are listed in the continuation of Box C.

### See patent family annex.

* Special categories of cited documents:
  * "A" document defining the general state of the art which is not considered to be of particular relevance
  * "E" earlier application or patent but published on or after the international filing date
  * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  * "O" document referring to an oral disclosure, use, exhibition or other means
  * "P" document published prior to the international filing date but later than the priority date claimed

### Date of the actual completion of the international search
27 August 2014 (27.08.2014)

### Date of mailing of the international search report
27 August 2014 (27.08.2014)

### Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongna-ro, Seo-gu, Daegu Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

### Authorized officer
CHOI, Sung Hee
Telephone No. +82-42-481-8740

Form PCT/ISA/210 (second sheet) (July 2009)
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EP 2240508 A1</td>
<td>20/10/2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wo 2009-087110 A1</td>
<td>16/07/2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wo 2009-087110 A8</td>
<td>26/08/2010</td>
</tr>
</tbody>
</table>