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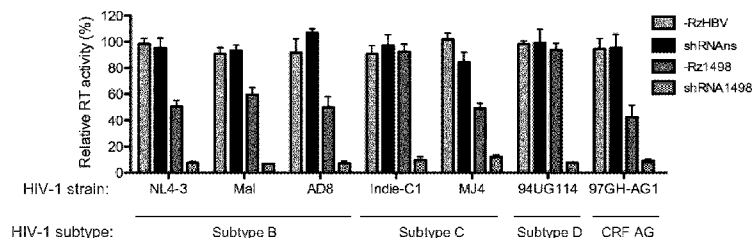


FIG. 6B

(57) Abstract: Antisense nucleic acid molecules, such as ribozymes, shRNA and siRNA, targeting the Gag region of HIV-1 open reading frame, and uses thereof for inhibiting HIV-1 replication and infection, are disclosed. The antisense nucleic acid molecules more specifically target a sequence corresponding to about nucleotide 1495 to about nucleotide 1526, or nucleotide 1497 to about nucleotide 1521, of HIV-1 clone pNL4-3.

ANTISENSE-BASED SMALL RNA AGENTS TARGETING THE GAG OPEN READING FRAME OF HIV-1 RNA

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application Serial No. 61/869,852
5 filed on August 26, 2013, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present invention generally relates to the treatment of Human Immunodeficiency
Virus 1 (HIV-1) infection.

BACKGROUND ART

10 Over 30 small molecules are available for the treatment of Human Immunodeficiency
Virus 1 (HIV-1) infection, targeting the viral proteins reverse transcriptase (RT), protease and
integrase, as well as the cellular entry co-receptor, CCR5¹. Although treatment of HIV-1 with
combination small molecule therapy is effective in preventing Acquired Immune Deficiency
Syndrome (AIDS), it is not able to eradicate the virus and is associated with a number of short-
15 and long-term side effects². Alternative therapeutic strategies for long-term viral suppression
with low adverse effects are needed.

Small RNAs represent a growing class of molecules with the potential to complement
or replace current therapies. They are being evaluated for use in *ex vivo* gene therapy³ and with
advances that have been made in their systemic delivery⁴, may soon be evaluated for use in
20 combination drug therapy. Many small RNAs, including antisense oligonucleotides (ASOs),
ribozymes (Rzs), decoys, aptamers, small nuclear (sn) RNAs, and small interfering (si) or short
hairpin (sh) RNAs have been designed with diverse target sites in the HIV-1 replication cycle⁵.
Antisense-based RNAs (ASOs, Rzs, snRNAs, sh/siRNAs) can be designed to target HIV-1
RNA, and several therapeutic candidates have been described.

25 Rzs targeting HIV-1 RNA have been made by modifying hammerhead, hairpin⁶ and
bacterial RNase P⁷ motifs. The HDV Rz represents an alternative small Rz motif, that has
evolved to function in human cells and has the potential to be used for the development of
therapeutic Rzs⁸. To improve the specificity of the HDV Rz for its target RNA, the SOFA
(Specific Qn/oEf Adaptor) module was engineered^{9,10} (**FIG. 1A**). Several SOFA-HDV-Rzs have
30 been identified with the potential to target human^{11,12}, viral^{9,13,14} and bacterial¹⁵ RNAs, including
three Rzs that we have evaluated targeting the overlapping Tat/Rev coding sequence of HIV-1
RNA¹⁶.

Optimal hammerhead Rz target sites in HIV-1 RNA have been identified using libraries
of Rzs with randomized binding arms^{17,18} and a library of Rzs targeting highly conserved
35 sequences¹⁹. Using different methods and datasets to estimate sequence conservation, sets of

optimal siRNAs²⁰ or shRNAs^{21,22} have been identified and two of these studies have reported their conservation estimates in 19 to 21 nt frames^{20,22}. Estimates have also been reported at the nucleotide (nt) level to identify or characterize Rz²³, snRNA²⁴ and shRNA²⁵ target sites.

The present description refers to a number of documents, the content of which is
5 herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present invention relates to the following items 1 to 23:

1. An antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1495 to about nucleotide 1526 of HIV-1 clone pNL4-3 (GenBank accession
10 No. M19921.2), or the complement thereof.
2. The antisense nucleic acid molecule of item 1, which is directed against a sequence corresponding to about nucleotide 1497 to about nucleotide 1521 of HIV-1 clone pNL4-3 (GenBank accession No. M19921.2), or the complement thereof.
3. The antisense nucleic acid molecule of item 1, which is a ribozyme.
- 15 4. The antisense nucleic acid molecule of item 3, wherein said ribozyme is a Specific On/Off Adaptor (SOFA) Hepatitis Delta Virus (HDV) ribozyme.
5. The antisense nucleic acid molecule of item 4, wherein said ribozyme comprises: a recognition domain (RD) comprising the sequence TTCCTGT, a biosensor (Bs) domain comprising the sequence AAGGGTACTA, and a blocker (BI) domain comprising the sequence
20 GGAA.
6. The antisense nucleic acid molecule of item 4, wherein said ribozyme comprises the sequence of SEQ ID NO:142.
7. The antisense nucleic acid molecule of item 1 or 2, which is a short hairpin RNA (shRNA).
- 25 8. The antisense nucleic acid molecule of item 6, wherein the shRNA is encoded by a nucleic acid comprising one of the following stem sequences (i) to (xii):
 - (i) 5'-GCAGGAACTACTAGTACCCT-3' (SEQ ID NO: 93)
3'-CGTCCTTGATGATCATGGGA-5' (SEQ ID NO: 118);
 - (ii) 5'-ATAGCAGGAACTACTAGTAC-3' (SEQ ID NO: 87)
30 3'-TATCGTCCTTGATGATCATG-5' (SEQ ID NO: 119);
 - (iii) 5'-TAGCAGGAACTACTAGTACC-3' (SEQ ID NO: 89)
3'-ATCGTCCTTGATGATCATGG-5' (SEQ ID NO: 120);
 - (iv) 5'-AGCAGGAACTACTAGTACCC-3' (SEQ ID NO: 91)
3'-TCGTCCTTGATGATCATGGG-5' (SEQ ID NO: 121);
 - 35 (v) 5'-CAGGAACTACTAGTACCCTT-3' (SEQ ID NO: 95)
3'-GTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 122);
 - (vi) 5'-GGAACCTACTAGTACCCTTCA-3' (SEQ ID NO: 99)

3'-CCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 123);

(vii) 5'-GCAGGAACTACTAGTACCC-3' (SEQ ID NO: 105)

3'-CGTCCTTGATGATCATGGG-5' (SEQ ID NO: 124);

(viii) 5'-GCAGGAACTACTAGTACCCTT-3' (SEQ ID NO: 107)

3'-CGTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 125);

(ix) 5'-GCAGGAACTACTAGTACCCTTCA-3' (SEQ ID NO: 109)

3'-CGTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 126);

(x) 5'-GCAGGAACTACTAGTACCCTTCAGG-3' (SEQ ID NO: 111)

3'-CGTCCTTGATGATCATGGGAAGTCC-5' (SEQ ID NO: 127);

(xi) 5'-GCAGGAACTACTAGTACCCTTCAGGAA-3' (SEQ ID NO: 113)

3'-CGTCCTTGATGATCATGGGAAGTCCTT-5' (SEQ ID NO: 128); or

(xii) 5'-GCAGGAACTACTAGTACCCTTCAGGAACA-3' (SEQ ID NO: 115)

3'-CGTCCTTGATGATCATGGGAAGTCCTTGT-5' (SEQ ID NO: 129);

or an analog thereof.

9. The antisense nucleic acid molecule of item 7, wherein the shRNA comprises a 3'-overhang.

10. The antisense nucleic acid molecule of item 8, wherein the shRNA is encoded by a nucleic acid comprising one of the following sequences (i) to (xii):

(i) GCAGGAACTACTAGTACCCTACTCGAGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 130);

(ii) ATAGCAGGAACTACTAGTACGCTCGAGGGTACTAGTAGTTCCTGCTATTT (SEQ ID NO: 131);

(iii) TAGCAGGAACTACTAGTACCGCTCGAGGGGTACTAGTAGTTCCTGCTATT (SEQ ID NO: 132);

(iv) AGCAGGAACTACTAGTACCCACTCGAGAGGGTACTAGTAGTTCCTGCTTT (SEQ ID NO: 133);

(v) CAGGAACTACTAGTACCCTTGCTCGAGGAAGGGTACTAGTAGTTCCTGTT (SEQ ID NO: 134);

(vi) GGACTACTAGTACCCTTCACCTCGAGCTGAAGGGTACTAGTAGTTCCTT (SEQ ID NO: 135);

(vii) GCAGGAACTACTAGTACCCACTCGAGAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 136);

(viii) GCAGGAACTACTAGTACCCTTGCTCGAGGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 137);

(ix) GCAGGAACTACTAGTACCCTTCACCTCGAGCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 138);

(x) GCAGGAAGTACTAGTACCCTTCAGGTCTCGAGTCCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 139);

(xi) GCAGGAAGTACTAGTACCCTTCAGGAAGCTCGAGGTTCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 140); or

5 (xii) GCAGGAAGTACTAGTACCCTTCAGGAACATCTCGAGTTGTTCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 141);

or an analog thereof.

11. The antisense nucleic acid molecule of item 1 or 2, which is a small interfering RNA (siRNA).

10 12. The antisense nucleic acid molecule of item 10, wherein the siRNA comprises one of the following pair of sequences (i) to (iv):

(i) si1497 sense: AGCAGGAACUACUAGUACCCUUCdAdG (SEQ ID NO: 75)

si1497 antisense: UAUCGUCCUUGAUGAUGAUGGGAAGUC (SEQ ID NO: 76);

(ii) si1498 sense: GCAGGAACUACUAGUACCCUUCAdGdG (SEQ ID NO: 77)

15 si1498 antisense: AUCGUCCUUGAUGAUGAUGGGAAGUCC (SEQ ID NO: 78);

(iii) si1499 sense: CAGGAACUACUAGUACCCUUCAGdGdA (SEQ ID NO: 81)

si1499 antisense: UCGUCCUUGAUGAUGAUGGGAAGUCCU (SEQ ID NO: 82); or

(iv) si1498-29 sense: GCAGGAACUACUAGUACCCUUCAGGAA (SEQ ID NO: 79)

si1498-29 antisense: dAdTCGUCCUUGAUGAUGAUGGGAAGUCCUU (SEQ ID NO:

20 80);

or an analog thereof.

13. A vector comprising a nucleic acid encoding the antisense nucleic acid molecule of any one of items 1 to 12.

14. A cell comprising the antisense nucleic acid molecule of any one of items 1 to 12 and/or the vector of item 13.

15. A composition comprising (a) the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13 and/or the cell of item 14; and (b) an excipient.

16. A method for inhibiting HIV-1 replication in a cell, the method comprising contacting said cell with an effective amount of the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15.

17. A method for treating HIV-1 infection in a subject, the method comprising administering to said subject an effective amount of the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15.

18. Use of the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15, for inhibiting HIV-1 replication in a cell.

19. Use of the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15, for the manufacture of a medicament for inhibiting HIV-1 replication in a cell.

20. Use of the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15, for treating HIV-1 infection in a subject.

21. Use of the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15, for the manufacture of a medicament for treating HIV-1 infection in a subject.

22. The antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15, for inhibiting HIV-1 replication in a cell and/or treating HIV-1 infection in a subject.

23. The antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15, for the manufacture of a medicament for inhibiting HIV-1 replication in a cell and/or treating HIV-1 infection in a subject.

24. Use of the antisense nucleic acid molecule of any one of items 1 to 12, the vector of item 13, the cell of item 14 and/or the composition of item 15, as a medicament.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

In the appended drawings:

FIGs. 1A and B show a schematic representation of the SOFA-HDV-Rz and the HIV-1 RNA region used to identify SOFA-HDV-Rz target sites. **FIG. 1A:** The SOFA-HDV-Rz is illustrated in both its *OFF* and *ON* conformations. In the *OFF* conformation, the SOFA blocker (BI) base pairs with the last 4 nts of the recognition domain (RD). When the SOFA biosensor (Bs) base pairs with a specific target sequence, the RD is released from the BI sequence and binds at 3 to 5 nts upstream from the Bs binding site in the *ON* conformation. The first nt in the target site (n+1) must be a G, forming a wobble base pair with the RD U. The cleavage site is indicated with an arrow and the nt C₇₆, which can be mutated to disable the catalytic activity of the SOFA-HDV-Rz, is shown as a circle in the Rz backbone. **FIG. 1B:** the full length genomic (g), singly-spliced (ss) and doubly-spliced (ds) RNA species of HIV-1 are illustrated. Reading frames for all HIV-1 proteins are shown above the different RNAs and the 5' region, used to identify SOFA-HDV-Rz target sites, is underlined.

FIGs. 2A to C show the SOFA-HDV-Rz target site identification. **FIG. 2A:** Criteria used to identify SOFA-HDV-Rz target sites in HIV-1 RNA based on our conservation estimates at the nt level are illustrated. The number of nts between the RD and the Bs (spacer, 3-5), and the

length of the Bs (9-11), were adjusted to avoid poorly conserved positions or to reduce potential off-target effects on human RNAs. A spacer of 4 nt and Bs length of 10 nt were used as the default positioning. **FIG. 2B:** Target sites were excluded if they were not identical in HIV-1 NL4-3 or if the corresponding Rz had potential target sites in human RNAs using a cut-off score of 20 in the Ribosubstrates tool²⁶. **FIG. 2C:** Sequence conservation estimates in the 5' region of HIV-1 RNA are shown for each nt position in HIV-1 NL4-3 with the selected Rz binding sites shaded in grey. SOFA-HDV-Rzs were named according to the first nt in their binding site. The dashed line represents the separation between the 5'UTR and Gag ORF. SOFA-HDV-Rz target sites that were moderately conserved, but did not meet our conservation criteria, are indicated with an asterisk (*).

FIG. 3 shows the inhibition of HIV-1 production by SOFA-HDV-Rzs. HEK293T cells were seeded in 24 well plates and co-transfected with HIV-1 pNL4-3 plasmid DNA (75 ng) and one of the indicated psiRNA SOFA-HDV-Rz expression plasmids (750 ng). Viral production was estimated 48 h following transfection by measuring the activity of HIV-1 RT in culture supernatants. Each replicate was expressed as a percentage of the value obtained for co-transfection with the empty Rz expression plasmid tested in parallel (Relative RT activity). Rzs were evaluated in at least three independent experiments with one to three replicate transfections, data are expressed as the mean +/- standard error mean (SEM) (n=5-10). Graph Pad Prism™ was used to calculate P values for the effects of each HIV-1 specific SOFA-HDV-Rz compared to the irrelevant control (-RzHBV). Results from un-paired t-tests are shown above each SOFA-HDV-Rz that demonstrated a significant inhibition of viral production compared to the control (* P<0.05, ** P<0.01, *** P<0.001).

FIGs. 4A to C show the effects of SOFA-HDV-Rz1498 variants on HIV-1 production. **FIG. 4A:** Schematic representation of the SOFA-HDV-Rz1498 target site (T) and variants (Rz). SOFA-HDV-Rz1498A76 has a C to A mutation in the Rz backbone, -Rz1498Bs1 and -Rz1498Bs2 have 1 or 2 nt variants in the biosensor (Bs), indicated in lower case. **FIG. 4B:** Effects of each SOFA-HDV-Rz1498 variant on viral production in HEK293T cells were evaluated exactly as in **FIG. 3**. Rzs were evaluated in at least three independent experiments with one to three replicate transfections (reported as mean +/- SEM, n=6-10). The relative expression of Rz and 5S RNA loading control for the different conditions are shown below for one of two independent experiments performed in HEK293T cells seeded in a 12-well plate and co-transfected with twice the amount of DNA used for the evaluation of viral production in 24 well plates. **FIG. 4C:** Single turnover *in vitro* cleavage activities for SOFA-HDV-Rz1498, -Rz1498Bs1 and -Rz1498Bs2 were determined at different incubation times with a small substrate RNA (Rz>>>substrate). Cleavage % was measured by dividing cleaved products by cleaved + uncleaved products, quantified from bands on a gel. A nonlinear regression one phase exponential association equation with least squares (ordinary) fit was determined using

Graph Pad Prism™ for the different Rzs. All data points represent two independent experiments and are reported as mean $\pm$ SEM (n=2). The average rate constants (k_{obs}) and maximum cleavage values (F_{max}) for the SOFA-HDV-Rzs are reported in the table.

FIGs. 5A to D show the effects of shRNA1498 on HIV-1 production. **FIG. 5A:** Sequences targeted by shRNA1498 and control shRNAs targeting HIV-1 RNA (shRNA522, shRNA553, and shRNA5983) are shown in relation to conservation estimates at the nt level reported in reference # 51. **FIG. 5B:** Effects of shRNA1498 and a nonsense shRNA (shRNAs) on viral production in HEK293T cells were evaluated exactly as in **FIG. 3**. Rzs and shRNAs were evaluated in at least three independent experiments with one to three replicate transfections (reported as mean $\pm$ SEM, n=6-10). The relative intracellular expression of HIV-1 Gag polyprotein (GAG, p55), matrix-capsid intermediate (MA-CA, p39), and capsid (CA, p24) proteins as well as GAPDH loading control are shown below for one of two independent experiments performed in HEK293T cells seeded in a 12-well plate and co-transfected with twice the amount of DNA used for the evaluation of viral production. Relative band intensities for Gag and CA were calculated using Image J™ software and are expressed as a fraction of the intensity of Gag in the SOFA-HDV-RzHBV control lane. **FIG. 5C:** The potency of shRNAs was evaluated by co-transfecting HEK293T cells seeded in a 24-well plate with 100 ng of pNL4-3 DNA and 1-750 ng of shRNA expressing plasmids. For lower amounts of shRNA plasmid DNA (1-500 ng), co-transfections were topped up to 850 ng total DNA by the addition of an irrelevant plasmid (pBluescript SK+, Stratagene, La Jolla, CA). Relative RT activity measurements were log transformed and a nonlinear regression log(inhibitor) vs. response equation with least squares (ordinary) fit was determined using Graph Pad Prism™ for the different shRNAs. All data-points represent at least two independent experiments with 2-3 replicates and are reported as mean $\pm$ SEM (n=4-8). **FIG. 5D:** Combinations of SOFA-HDV-Rz and shRNA expressing plasmids were evaluated in HEK293T cells seeded in 24-well plates and co-transfected with 100 ng pNL4-3, 10 ng of shRNA expressing plasmid and 1 μ g of Rz expressing plasmid. Data were normalized to co-transfection of 100 ng pNL4-3 with 1 μ g of the empty Rz/shRNA expression plasmid and are reported as the mean $\pm$ SEM from two independent experiments performed in triplicate (n=6).

FIGs. 6A and B show the inhibition of HIV-1 production from diverse viral strains by SOFA-HDV-Rz1498 and shRNA1498. **FIG. 6A:** The sequence in and around the shRNA1498 and SOFA-HDV-Rz1498 target site is shown for HIV-1 NL4-3 (M19921), MAL (K03456), AD8 (AF004394), Indie-C1 (AB023804.1), MJ4 (AF321523), 94UG114 (U88824.1) and 97GH-AG1 (AB049811.1). The overlapping target site for shRNA1498 and SOFA-HDV-Rz1498 is indicated by the brackets, with both the RD (7 nt) and Bs (10 nt) binding sites underlined. Nt variations compared to HIV-1 NL4-3 are indicated by the arrows and the start and end positions for each sequence are shown according to their annotation in Genbank. **FIG. 6B:** The activity of the

indicated Rz and shRNA expression plasmids against the different HIV-1 strains was determined as in **FIG. 3** (n=4-12). Left bars = RzHBV; 2nd bars = shRNAs; 3rd bars = Rz1498; right bars = shRNA1498.

FIG. 7 shows gene expression changes in cells transfected with SOFA-HDV-Rz1498 and shRNA1498. HEK293T cells were seeded in 12-well plates and co-transfected with HIV-1 pNL4-3 (150 ng) and either SOFA-HDV-Rz1498, shRNA1498 or the empty Rz/shRNA expression plasmid (1.5 µg). 48 h after transfection, total RNA was harvested and analyzed by microarray. The log2 ratios of mRNAs with the greatest differential variation between SOFA-HDV-Rz1498 or shRNA1498 expressing cells and the empty expression vector expressing cells are shown. RNAs that were up- or down-regulated in both conditions are shown in circles and squares, respectively. One gene that was down-regulated by SOFA-HDV-Rz1498 and up-regulated by shRNA1498 is shown with an arrow. The gene identities and log2 ratio values are provided in **FIGs. 15A-D**.

FIGs. 8A and B show the inhibition of HIV-1 replication by SOFA-HDV-Rz1498 and shRNA1498. **FIG. 8A:** Jurkat cells expressing the indicated SOFA-HDV-Rzs and shRNAs were infected with HIV-1 pNL4-3. The average RT activity (cpm) across four independent infections performed in triplicate (n=12) is shown for days 10 and 14 following infection. Left bars = RzHBV; 2nd bars = shRNAs; 3rd bars = Rz1498A76; 4th bars = Rz1498; right bars = shRNA1498. **FIG. 8B:** Time course of a representative infection (n=3) followed out to 32 days post-infection.

FIG. 9 shows the subtype distribution of HIV-1 sequences used to calculate conservation estimates in comparison to global distribution estimates. HIV-1 Group M subtypes A-D, F-H, J, K, circulating recombinant forms (CRFs) 01_AE and 02_AG, other CRFs and unique recombinant forms (URFs) and non M-group HIV-1 sequences are illustrated over or next to their proportional representation in the Los Alamos National Laboratory (LANL) dataset used to evaluate sequence conservation (left) and global estimates reproduced from reference # 44 (right).

FIGs. 10A and B show the effect of SOFA-HDV-Rz1498 on the quality of virions produced from co-transfected HEK293T cells. **FIG. 10A:** HEK293T cells were seeded in 24 well plates and transfected exactly as in **FIG. 3**. RT activity is expressed as counts per minute for cells transfected with the empty Rz expression plasmid (psiRNA), the irrelevant SOFA-HDV-RzHBV and SOFA-HDV-Rz1498 targeting HIV-1 RNA. **FIG. 10B:** Supernatants from **FIG. 10A** were normalized by volume to the same RT activity and used to infect TZM-bl cells seeded in 12 well plates 24 h prior to infection. 48 h after infection, intracellular luciferase activity was measured (expressed as relative luciferase units, RLU). Luciferase activity is proportional to the level of Tat protein produced from viral genomes that integrated into the TZM-bl genome following infection and is a measure of viral infectivity.

FIGs. 11A and B show Gag and Capsid protein expression in cells co-transfected with HIV-1 pNL4-3 and different HIV-1 RNA specific shRNAs. HEK293T cells were seeded in 12 well plates and co-transfected with HIV-1 pNL4-3 plasmid DNA (150 ng) and one of the indicated psiRNA short hairpin (sh) RNA expression plasmids (300 ng). A nonsense (NS) shRNA was used as a control (shRNAs) and shRNAs targeting the 5'UTR (shRNA522 and shRNA553), the Gag coding sequence (shRNA1498) and the overlapping tat/rev coding sequence (shRNA5983) of HIV-1 were evaluated. Cell lysates were obtained 48 h after co-transfection. **FIG. 11A:** The relative intracellular expression of HIV-1 Gag polyprotein (Gag, p55), matrix-capsid intermediate (MA-CA, p39), and capsid (CA, p24) proteins as well as GAPDH loading control are shown. **FIG. 11B:** Relative band intensities for Gag and CA were calculated using Image J™ software and are expressed as a fraction of the intensity of Gag in the shNS control lane.

FIG. 12 shows the change in mRNA expression ratios compared to a control vector for SOFA-HDV-Rz1498 (upper panel) and shRNA1498 (lower panel) transfected HEK293T cells as detected by microarray: RNA extracts were obtained from HEK293T cells transfected with SOFA-HDV-Rz1498, shRNA1498 or the empty Rz/shRNA expression vector (psiRNA) and then analyzed using triplicate dye-swap microarray experiments. The results are expressed as Lowess log2 ratio plots comparing all detectable mRNA species from SOFA-HDV-Rz1498 or shRNA1498-transfected cells with those from empty vector-transfected cells. Each individual line represents one detectable mRNA species and differences are reflected in the magnitude of change in log2 ratio between the triplicates on the left and right halves of each plot.

FIGs. 13A and B show stable Jurkat cell lines analysis. **FIG. 13A:** 10,000 events are shown for the different cell lines, Green fluorescence levels versus Forward scatter (FSC) is plotted, showing the level of green fluorescent protein (GFP) expression and approximate cell size (FSC) for the different Jurkat cell lines. **FIG. 13B:** Live cell counts +/- SEM (n=2) out to four days after plating different cell lines at 1.0×10^5 cells/mL.

FIGs. 14A and B show that additional siRNAs directed against the targeted site inhibit HIV replication. HEK293T cells were seeded in 24 well plates and co-transfected with HIV-1 pNL4-3 plasmid DNA (100 ng) and one of the indicated siRNAs (Dharmacon) at 25 and 100 nM using Dharmafect reagent 1. Viral production was estimated 48 h following transfection by measuring the activity of HIV-1 RT in culture supernatants. Each replicate was expressed as a percentage of the value obtained for co-transfection with a 25bp nonsense siRNA (Relative RT activity). Data are expressed as the mean +/- standard error mean (SEM) (n=2-6). **FIG. 14A:** Results for 25 bp siRNAs with overlapping target sites. **FIG. 14B:** Results for 25 and 27 bp versions of si1498 targeting the Gag coding sequence of HIV-1 RNA and si5983 targeting the overlapping Tat/Rev coding sequence of HIV-1 RNA.

FIGs. 15A to 15D show the data of microarray experiments performed as triplicate dye swaps expressed as the log2 ratio of SOFA-HDV-Rz1498 (Rz1498, FIGs. 12A and 12B) and

shRNA1498 (FIGs. 12C and 12D) compared to the empty vector cotransfected cells (psiRNA). **FIG. 15A** = Genes up-regulated in Rz1498-transfected cells vs. control cells; **FIG. 15B** = Genes down-regulated in Rz1498-transfected cells vs. control cells; **FIG. 15C** = Genes up-regulated in shRNA1498-transfected cells vs. control cells; **FIG. 15D** = Genes down-regulated in shRNA1498-transfected cells vs. control cells.

FIG. 16A shows the activity of 20 bp shRNAs targeting HIV-1 NL4-3 from starting positions 1495 to 1501. HEK293T cells were seeded in 24-well plates and co-transfected with HIV-1 pNL4-3 plasmid DNA (100 ng) and one of the indicated psiRNA-shRNA expression plasmids (10 and 100 ng). Viral production was estimated 48 h following transfection by measuring the activity of HIV-1 RT in culture supernatants. Each replicate was expressed as a percentage of the value obtained for co-transfection with the empty shRNA expression plasmid tested in parallel (Relative RT activity). Data are expressed as the mean +/- standard error mean (SEM) (n=2-6).

FIG. 16B shows the activity of shRNAs with 17 to 29 bp hairpins targeting HIV NL4-3 at starting position 1498. HEK293T cells were seeded in 24-well plates and co-transfected with HIV-1 pNL4-3 plasmid DNA (100 ng) and one of the indicated psiRNA-shRNA expression plasmids (10 and 100 ng). Viral production was estimated 48 h following transfection by measuring the activity of HIV-1 RT in culture supernatants. Each replicate was expressed as a percentage of the value obtained for co-transfection with the empty shRNA expression plasmid tested in parallel (Relative RT activity). Data are expressed as the mean +/- standard error mean (SEM) (n=2-6).

DISCLOSURE OF INVENTION

Terms and symbols of genetics, molecular biology, biochemistry and nucleic acid used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like. All terms are to be understood with their typical meanings established in the relevant art. Standard techniques may be used for chemical synthesis, and chemical analysis. Certain such techniques and procedures may be found for example in "Carbohydrate Modifications in Antisense Research" Edited by Sangvi and Cook, American Chemical Society, Washington D.C., 1994; "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., 21st edition, 2005; and "Antisense Drug Technology, Principles, Strategies, and Applications" Edited by Stanley T. Crooke, CRC Press, Boca Raton, Florida.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element. Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

In the studies described herein, the present inventors have screened HIV-1 RNA for highly conserved target sites at its 5' end, from the beginning of the 5'UTR to the end of the gag ORF (FIG. 1A). SOFA-HDV-Rzs targeting a novel site in the gag ORF with significant activity against HIV-1 production were identified, and shRNAs/siRNAs targeting the same site were shown to be extremely potent and active inhibitors of viral production. Both types of molecules were able to inhibit HIV-1 production from diverse viral strains and were active against HIV-1 replication in a T lymphocyte cell model. The results provide evidence that targeting this site using antisense-based agents constitutes a suitable approach for HIV-1 therapy.

Accordingly, in a first aspect, the present invention provides an antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1495 to about nucleotide 1526 of HIV-1 clone pNL4-3 (GenBank accession No. M19921.2), or a fragment thereof, or a sequence complementary thereto. Nucleotide numbering described herein uses numbering in the reference HIV-1 clone pNL4-3 (GenBank accession No. M19921.2). Nucleotides 1495 to 1526 correspond to the sequence ATAGCAGGAAGTACTAGTACCCTTCAGGAACA (SEQ ID NO: 117). The corresponding positions/sequences (which defines the region targeted by the antisense nucleic acid molecule of the present invention) in any HIV-1 strain may be easily identified, for example by aligning the nucleotide sequence of a given HIV-1 strain with the nucleotide sequence of reference HIV-1 clone pNL4-3 (e.g., using a software for sequence alignment such as Clustal W). It will be understood that the corresponding sequences in other HIV-1 strains may not be identical to the corresponding sequence of HIV-1 clone pNL4-3 (e.g., the sequence in other HIV-1 strains may be 75, 80, 85, 90 or 95% identical to the corresponding sequence of HIV-1 clone pNL4-3), and that the sequence of the antisense nucleic acid molecule may be adapted accordingly. FIG. 6A depicts an alignment of the sequences of different HIV strains/subtypes, and shows for example that nucleotides 1497 to 1521 correspond to nucleotides 1075 to 1099 in HIV strain Mal, and nucleotides 1488 to 1512 in HIV strain Indie-C1. In an embodiment, the antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1498 to about nucleotide 1518 of HIV-1 clone pNL4-3.

In an embodiment, the antisense nucleic acid molecule comprises a sequence that can hybridize, under stringent conditions or highly stringent conditions, with the above-mentioned sequence corresponding to about nucleotide 1495 to about nucleotide 1526 of HIV-1 clone

pNL4-3, or a fragment thereof (e.g., a fragment of at least 5, 10, 15 or 20 nucleotides located with the region defined), or the complementary sequence thereof, or a corresponding sequence with at least 75, 80, 85, 90 or 95% identity or complementarity in another HIV-1 strain. Hybridization technology is well-known in the field of molecular biology. For the purpose of illustration, the hybridization condition is a stringent condition, for example, a DNA binding to the filtration membrane is hybridized in $6\times$ sodium chloride/sodium citrate (SSC) at about 45°C or more, then is washed one or more times in $0.2\times$ SSC/ 0.1% SDS at about $50\text{--}65^{\circ}\text{C}$; or is a highly stringent condition, for example, a nucleic acid binding to the filtration membrane is hybridized in $6\times$ SSC at about 45°C , then is washed one or more times in $0.1\times$ SSC/ 0.2% SDS at about 68°C ; or is other stringent hybridization conditions known in the art (See for example, Ausubel, F. M. et al., 1989, Current Protocols in Molecular Biology, Volume 1, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York, page 6.3.1-6.3.6 and 2.10.3).

In an embodiment, the antisense nucleic acid comprises a sequence that is fully complementary to the target sequence (or a fragment thereof) over the entire length of the oligonucleotide. In certain embodiments, oligonucleotides are 99% complementary to the target sequence (or a fragment thereof). In certain embodiments, the antisense nucleic acid comprises a sequence that is at least 95% complementary to the target sequence (or a fragment thereof). In certain embodiments, the antisense nucleic acid comprises a sequence that is at least 90% complementary to the target sequence (or a fragment thereof). In certain embodiments, the antisense nucleic acid comprises a sequence that is at least 85% complementary to the target sequence (or a fragment thereof). In certain embodiments, the antisense nucleic acid comprises a sequence that is at least 80% complementary to the target sequence (or a fragment thereof). In certain embodiments, an antisense compound comprises a region that is fully complementary to a target nucleic acid and is at least 80% complementary to the target nucleic acid over the entire length of the oligonucleotide. In certain such embodiments, the region of full complementarity is from about 6 to about 14 nucleotides in length.

In an embodiment, the antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1497 to about nucleotide 1521 of HIV-1 clone pNL4-3. In another embodiment, the antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1495 to about nucleotide 1524 of HIV-1 clone pNL4-3. In another embodiment, the antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1496 to about nucleotide 1526 of HIV-1 clone pNL4-3. In another embodiment, the antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1495 to about nucleotide 1520 of HIV-1 clone pNL4-3.

The term "antisense nucleic acid molecule" as used herein refers to any nucleic acid molecule, such as a short RNA molecule, capable of inhibiting the expression of a target polypeptide by, for example, inducing degradation of a RNA molecule encoding the target

polypeptide, blocking its translation and/or stopping its replication, and includes for example microRNA (miRNA), decoys, aptamers, small nuclear (sn) RNAs, ribozyme, antisense oligonucleotides (ASOs), small interfering (si) and short hairpin (sh) RNAs. An antisense nucleic acid molecule comprises or consists of an oligonucleotide at least a portion of which is complementary to the target sequence to which it is capable of hybridizing under physiological conditions. The antisense nucleic acid molecule according to the present invention may comprise modified products produced by chemically modifying the constitution moieties, such as phosphate backbone and/or ribose and/or base etc., of the molecule. The modification methods are known in the art, which can be thio-modification and/or sterol modification and/or PEG-modification and/or glyco-modification and/or LNA-modification etc., as described for example in Dykxhoorn DM *et al.*, *Annual Review of Biomedical Engineering*, 2006, Volume 8: pages 377-402 and Behlke MA *et al.*, *Molecular Therapy*, 2006, Volume 13: pages 644-670.

The antisense nucleic acid molecules described herein, may be chemically modified, for example to change (e.g., increase or decrease) intracellular stability and half-life. Such modified are herein referred to as "analogs". Possible modifications include the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate (also known as thiophosphate) linkages rather than phosphodiesterase linkages within the backbone of the molecule. In addition, one or more ribose groups may be modified to add a methyl moiety to the 2'-OH to form a 2'-methoxy moiety (referred to as 2'-O-methyl-modified). Also, the 2'-OH moiety can be linked to the 3' or 4'-carbon of ribose by a methylene or ethylene linker, typically a methylene linker to the 4'-carbon, to form a "locked nucleic acid" (see WO 98/39352 and WO 99/14226).

In certain embodiments, chemical modification also includes the use of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and other similarly modified forms of adenine, cytidine, guanine, thymine, and uridine, which are not as easily recognized by endogenous endonucleases. Examples of modified bases include uridine and/or cytidine modified at the 5-position, e.g., 5-(2-amino)propyl uridine, 5-bromo uridine; adenosine and/or guanines modified at the 8 position, e.g., 8-bromo guanosine; deaza nucleotides, e.g., 7-deaza-adenosine; and O- and N-alkylated nucleotides, e.g., N6-methyl adenosine. "Analogues" also include sequences in which one or more thymine (T) bases have been substituted for uracil (U) base and vice versa.

In certain embodiments, the sugar moiety can be modified, typically at the 2'-OH of ribose. Examples of such modifications include instances where the 2'-OH group is replaced by a group selected from H, OR, R, halo, SH, SR, NH₂, NHR, NR₂ or ON, where R is C1-C6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

Further, chemical modification can encompass modified backbones such as morpholino and/or further non-natural internucleoside linkages such as siloxane, sulfide,

sulfoxide, sulfone, sulfonate, sulfonamide, and sulfamate; formacetyl and thioformacetyl; alkene-containing; methyleneimino and methylenehydrazino; amide, and the like.

One or more nucleotides (or linkages) within the sequences described herein can be modified. For example, a 20-mer oligonucleotide may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,
5 13, 14, 15, 16, 17, 18, 19 or 20 modified nucleotides.

In an embodiment, the above-mentioned antisense nucleic acid molecule is from about 5 to about 100 nucleotides in length, in further embodiments from about 10 to about 100, from about 5 to about 50, from about 10 to about 50, from about 15 to about 50, from about 10 to about 30, from about 18 to about 29, from about 19 to about 27, from about 18 to about 25, from
10 about 19 to about 25, or from about 19 to about 23, nucleotides in length.

In an embodiment, the antisense nucleic acid molecule is a ribozyme. The term "ribozyme" refers to enzymatic RNA molecules capable of catalyzing the specific cleavage of a target RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. The
15 ribozyme comprises one or more sequences complementary to the sequence corresponding to about nucleotide 1495 to about nucleotide 1526 (e.g., 1497 to 1521) of HIV-1 clone pNL4-3 and decreases the level of the HIV-1 Gag polyprotein and/or of one or more of its processing products, such as capsid (CA). Examples of ribozymes include, for example, Hairpin ribozyme, Hammerhead ribozyme, VS ribozyme, *glmS* ribozyme and delta ribozyme derived from the
20 genome of hepatitis delta virus (HDV ribozyme). In an embodiment, the ribozyme is HDV ribozyme.

In an embodiment, the ribozyme comprises a specific On/Off Adaptor (SOFA) module, as described for example in PCT publication No. WO 2006/002547. Such module, which comprises a SOFA blocker (BI) and SOFA biosensor (Bs), is useful to increase the specificity of
25 the ribozyme by increasing the length of the recognition sequence, enabling the ribozyme only in the presence of the target RNA substrate. As shown in FIG. 1B, in the *OFF* conformation (in the absence of the target RNA), the SOFA blocker (BI) base pairs with the last 4 nts of the recognition domain (RD). When the SOFA biosensor (Bs) base pairs with a specific target sequence, the RD is released from the BI sequence and binds at 3 to 5 nts upstream from the
30 Bs binding site in the *ON* conformation. The first nt in the target site (n+1) must be a G, forming a wobble base pair with the U in RD. The cleavage site is indicated with an arrow.

Thus, in an embodiment, the ribozyme comprises (i) a first domain (recognition domain, RD) of, e.g., about 7 nucleotides comprising a sequence that is complementary to a first sequence within the sequence corresponding to about nucleotide 1495 to about nucleotide
35 1526 of HIV-1 clone pNL4-3; (ii) a second domain (SOFA biosensor, Bs) of, e.g., about 5 to about 15 nucleotides, comprising a sequence that is complementary to a second sequence (different from the first sequence) within the sequence corresponding to about nucleotide 1495

to about nucleotide 1526 of HIV-1 clone pNL4-3 and (iii) a third domain (SOFA blocker, BI) of, e.g., about 3 to about 5 nucleotides (preferably 4 nucleotides), comprising a sequence that is complementary to a sequence of the recognition domain (in an embodiment the last 3 to 5 nucleotides from the RD). In an embodiment, the first and second sequences (within the sequence corresponding to about nucleotide 1495 to about nucleotide 1526 of HIV-1 clone pNL4-3 (i.e. the target RNA) are spaced apart by at least 1 nucleotide, and preferably by about 1 to about 5 nucleotides (referred to as the "spacer" in FIG. 1B).

In an embodiment, the RD comprises a sequence that is complementary to a sequence corresponding to about nucleotide 1498 to about nucleotide 1504 of HIV-1 clone pNL4-3.

In an embodiment, the Bs comprises a sequence that is complementary to a sequence corresponding to about nucleotide 1509 to about nucleotide 1518 of HIV-1 clone pNL4-3.

In an embodiment, the BI comprises a sequence corresponding to about nucleotide 1501 to about nucleotide 1504 of HIV-1 clone pNL4-3.

In an embodiment, the ribozyme comprises: a recognition domain (RD) comprising/consisting of the sequence TTCCTGT, a biosensor (Bs) domain comprising/consisting of the sequence AAGGGTACTA, and a blocker (BI) domain comprising/consisting of the sequence GGAA.

In an embodiment, the ribozyme comprises or consists of the following sequence:
GGGCCAGCTAGTTTAAGGGTACTAGGAACAGGGTCCACCTCCTCGCGGTTTCCTGTGGGC
ATCCGTTTCGCGGATGGCTAAGGGACCCTTTCTAGCTGG (SEQ ID NO:142).

In an embodiment, the antisense nucleic acid molecule is an RNA interference agent. In an embodiment, the antisense nucleic acid molecule is a small/short interfering RNA (siRNA) molecule, i.e. a double-stranded (ds) RNA that preferably contains about 19 to 23 base pairs. The siRNA molecules may contain 3' overhangs, such as a 1- to 5-nucleotide (e.g., 2-nucleotide) 3' overhang in one or both strands. In an embodiment, the overhang is a 3'UU or 3'TT overhang. siRNAs, which are typically chemically synthesized, may be transfected directly into the cytosol of cells.

In an embodiment, the siRNA comprises or consists of one of the following pair of sequences (i) to (iv):

- (i) si1497 sense: AGCAGGAACUACUAGUACCCUUCdAdG (SEQ ID NO: 75)
si1497 antisense: UAUCGUCCUUGAUGAUGGGAAGUC (SEQ ID NO: 76);
- (ii) si1498 sense: GCAGGAACUACUAGUACCCUUCAdGdG (SEQ ID NO: 77)
si1498 antisense: AUCGUCCUUGAUGAUGGGAAGUCC (SEQ ID NO: 78);
- (iii) si1499 sense: CAGGAACUACUAGUACCCUUCAGdGdA (SEQ ID NO: 81)
si1499 antisense: UCGUCCUUGAUGAUGGGAAGUCCU (SEQ ID NO: 82);
- or
- (iv) si1498-27 sense: GCAGGAACUACUAGUACCCUUCAGGAA (SEQ ID NO: 79)

si1498-27 antisense: dAdTCGUCCUUGAUGAUGAUGGGAAGUCCUU (SEQ ID NO: 80);

or an analog thereof.

In an embodiment, the antisense nucleic acid molecule is a short hairpin RNA (shRNA) molecule, which typically comprises two complementary 19-22 bp RNA sequences linked by a short loop of 4-11 nt similar to the hairpin found in naturally occurring miRNA. Expression of shRNA in cells can be obtained by delivery of plasmids or through viral or bacterial vectors.

In an embodiment, the shRNA is encoded by a nucleic acid comprising or consisting of one of the following stem sequences (i) to (xii):

- (i) 5'-GCAGGAACTACTAGTACCCT-3' (SEQ ID NO: 93)
3'-CGTCCTTGATGATCATGGGA-5' (SEQ ID NO: 118);
- (ii) 5'-ATAGCAGGAACTACTAGTAC-3' (SEQ ID NO: 87)
3'-TATCGTCCTTGATGATCATG-5' (SEQ ID NO: 119);
- (iii) 5'-TAGCAGGAACTACTAGTACC-3' (SEQ ID NO: 89)
3'-ATCGTCCTTGATGATCATGG-5' (SEQ ID NO: 120);
- (iv) 5'-AGCAGGAACTACTAGTACCC-3' (SEQ ID NO: 91)
3'-TCGTCCTTGATGATCATGGG-5' (SEQ ID NO: 121);
- (v) 5'-CAGGAACTACTAGTACCCTT-3' (SEQ ID NO: 95)
3'-GTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 122);
- (vi) 5'-GGAAGTACTAGTACCCTTCA-3' (SEQ ID NO: 99)
3'-CCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 123);
- (vii) 5'-GCAGGAACTACTAGTACCC-3' (SEQ ID NO: 105)
3'-CGTCCTTGATGATCATGGG-5' (SEQ ID NO: 124);
- (viii) 5'-GCAGGAACTACTAGTACCCTT-3' (SEQ ID NO: 107)
3'-CGTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 125);
- (ix) 5'-GCAGGAACTACTAGTACCCTTCA-3' (SEQ ID NO: 109)
3'-CGTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 126);
- (x) 5'-GCAGGAACTACTAGTACCCTTCAGG-3' (SEQ ID NO: 111)
3'-CGTCCTTGATGATCATGGGAAGTCC-5' (SEQ ID NO: 127);
- (xi) 5'-GCAGGAACTACTAGTACCCTTCAGGAA-3' (SEQ ID NO: 113)
3'-CGTCCTTGATGATCATGGGAAGTCCTT-5' (SEQ ID NO: 128); or
- (xii) 5'-GCAGGAACTACTAGTACCCTTCAGGAACA-3' (SEQ ID NO: 115)
3'-CGTCCTTGATGATCATGGGAAGTCCTTGT-5' (SEQ ID NO: 129);

or an analog thereof.

In an embodiment, the shRNA molecules may contain 3' overhangs, such as a 1- to 5-nucleotide (e.g., 2-nucleotide) 3'-overhang in one or both strands. In an embodiment, the overhang is a 3'UU or 3'TT overhang. In an embodiment, the shRNA comprises a loop of about

4-11 nucleotides, in further embodiments about 6-10 nucleotides or about 7-9 nucleotides. In an embodiment, the loop comprises or consists of the sequence CTCGAG. In another embodiment, the loop comprises or consists of one of the following sequences: GCTCGAGG, ACTCGAGA, TCTCGAGT or CCTCGAGC.

5 In an embodiment, the shRNA is encoded by a nucleic acid comprising one of the following sequences (i) to (xii):

(i) GCAGGAACTACTAGTACCCTACTCGAGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 130);

10 (ii) ATAGCAGGAACTACTAGTACGCTCGAGGGTACTAGTAGTTCCTGCTATTT (SEQ ID NO: 131);

(iii) TAGCAGGAACTACTAGTACCGCTCGAGGGGTACTAGTAGTTCCTGCTATT (SEQ ID NO: 132);

(iv) AGCAGGAACTACTAGTACCCACTCGAGAGGGTACTAGTAGTTCCTGCTTT (SEQ ID NO: 133);

15 (v) CAGGAACTACTAGTACCCTTGCTCGAGGAAGGGTACTAGTAGTTCCTGTT (SEQ ID NO: 134);

(vi) GGA ACTACTAGTACCCTTCACCTCGAGCTGAAGGGTACTAGTAGTTCCTT (SEQ ID NO: 135);

20 (vii) GCAGGAACTACTAGTACCCACTCGAGAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 136);

(viii) GCAGGAACTACTAGTACCCTTGCTCGAGGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 137);

(ix) GCAGGAACTACTAGTACCCTTCACCTCGAGCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 138);

25 (x) GCAGGAACTACTAGTACCCTTCAGGTCTCGAGTCCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 139);

(xi) GCAGGAACTACTAGTACCCTTCAGGAAGCTCGAGGTTCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 140); or

30 (xii) GCAGGAACTACTAGTACCCTTCAGGAACATCTCGAGTTGTTCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 141);

or an analog thereof.

Reagents and kits for performing RNA interference are available commercially from for example Ambion® Inc. (Austin, TX, USA), New England Biolabs® Inc. (Beverly, MA, USA), Sigma-Aldrich® and Invitrogen® (Carlsbad, CA, USA)

35 In another aspect, the present invention provides a nucleic acid molecule (*e.g.*, DNA) comprising a sequence encoding the antisense nucleic acid molecule.

In another aspect, the present invention provides a vector or plasmid, such as a recombinant expression vector or plasmid, comprising the above-mentioned nucleic acid molecule (e.g., DNA). The nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule may be operably linked with expression controlling sequences, thereby making it possible to express the antisense nucleic acid molecule in a cell, for example a mammalian cell such as a human cell. In an embodiment, the vector is a viral vector, for example a retroviral vector, a lentiviral vector, or other vectors such as adenoviral vectors or adeno-associated vectors (AAV).

In another aspect, the present invention provides a cell (e.g., an isolated or recombinant cell) comprising the above-mentioned antisense nucleic acid molecule, nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule, and/or vector or plasmid. The cell can be in an isolated (or *ex vivo*) form, such as a cell isolated from a HIV-infected patient or a normal individual, or *in vivo*, or a cell strain cultured *in vitro*. The antisense nucleic acid molecule, nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule, and/or vector or plasmid may be introduced in the cell either *in vitro* or *in vivo*, by known methods such as transformation, transduction, transfection, and infection, such as calcium phosphate or calcium chloride co-precipitation-mediated transfection, DEAE-dextran-mediated transfection, liposome-mediated transfection, electroporation, microinjection and the like. Additionally, as noted above, the antisense nucleic acid molecule, and/or vector or plasmid may be introduced into a cell using a viral vector such as those derived from adenovirus, adeno-associated virus and lentivirus. Details of these and other techniques are known in the art, for example, as described in J. Sambrook and D.W. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press; 3rd Ed., 2001; F.M. Ausubel, Ed., *Short Protocols in Molecular Biology*, Current Protocols; 5th Ed., 2002; and Engelke, D.R., *RNA Interference (RNAi): Nuts and Bolts of RNAi Technology*, DNA Press LLC, Eagleville, PA, 2003. .

In another aspect, the present invention provides a composition (e.g., a pharmaceutical composition) comprising (i) the above-mentioned antisense nucleic acid molecule, nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule, vector/plasmid and/or cell and (ii) a carrier/excipient (e.g., a pharmaceutically acceptable carrier/excipient).

Compositions and methods for the formulation of pharmaceutical compositions depend on a number of criteria, including, but not limited to, route of administration, extent of disease, or dose to be administered.

Pharmaceutical compositions comprising antisense compounds encompass any pharmaceutically acceptable salts, esters, or salts of such esters. In certain embodiments, pharmaceutical compositions comprising antisense compounds comprise one or more

oligonucleotide, which upon administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of antisense compounds, prodrugs, pharmaceutically acceptable salts of such prodrugs, and other
5 bioequivalents. Suitable pharmaceutically acceptable salts include, but are not limited to, sodium and potassium salts.

A prodrug can include the incorporation of additional nucleosides at one or both ends of an oligomeric compound, which are cleaved by endogenous nucleases within the body, to form the active antisense oligomeric compound.

10 Lipid moieties have been used in nucleic acid therapies in a variety of methods. In certain such methods, the nucleic acid is introduced into preformed liposomes or lipoplexes made of mixtures of cationic lipids and neutral lipids. In certain methods, DNA complexes with mono- or poly-cationic lipids are formed without the presence of a neutral lipid. In certain embodiments, a lipid moiety is selected to increase distribution of a pharmaceutical agent to a
15 particular cell or tissue.

In certain embodiments, pharmaceutical compositions provided herein comprise one or more antisense compounds and one or more excipients. In certain such embodiments, excipients are selected from water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylase, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose and
20 polyvinylpyrrolidone.

In certain embodiments, a pharmaceutical composition provided herein comprises a delivery system.

Examples of delivery systems include, but are not limited to, liposomes and emulsions. Certain delivery systems are useful for preparing certain pharmaceutical compositions including
25 those comprising hydrophobic compounds. In certain embodiments, certain organic solvents such as dimethylsulfoxide are used.

In certain embodiments, a pharmaceutical composition provided herein comprises one or more tissue-specific delivery molecules designed to deliver the one or more pharmaceutical agents of the present invention to specific tissues or cell types. For example, in certain
30 embodiments, pharmaceutical compositions include liposomes coated with a tissue-specific antibody.

In certain embodiments, a pharmaceutical composition provided herein is prepared for oral administration. In certain embodiments, pharmaceutical compositions are prepared for buccal administration.

35 In certain embodiments, a pharmaceutical composition is prepared for administration by injection (e.g., intravenous, subcutaneous, intramuscular, etc.). In certain of such embodiments, a pharmaceutical composition comprises a carrier and is formulated in aqueous

solution, such as water or physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. In certain embodiments, other ingredients are included (e.g., ingredients that aid in solubility or serve as preservatives). In certain embodiments, injectable suspensions are prepared using appropriate liquid carriers, suspending agents and the like. Certain pharmaceutical compositions for injection are presented in unit dosage form, e.g., in ampoules or in multi-dose containers. Certain pharmaceutical compositions for injection are suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Certain solvents suitable for use in pharmaceutical compositions for injection include, but are not limited to, lipophilic solvents and fatty oils, such as sesame oil, synthetic fatty acid esters, such as ethyl oleate or triglycerides, and liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, such suspensions may also contain suitable stabilizers or agents that increase the solubility of the pharmaceutical agents to allow for the preparation of highly concentrated solutions.

In certain embodiments, a pharmaceutical composition is prepared for transmucosal administration. In certain of such embodiments penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

In certain embodiments, a pharmaceutical composition provided herein comprises an antisense molecule in a therapeutically effective amount. In certain embodiments, the therapeutically effective amount is sufficient to prevent, alleviate or ameliorate symptoms of a disease or to prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

In certain embodiments, the antisense molecule provided herein is formulated as a prodrug. In certain embodiments, upon *in vivo* administration, a prodrug is chemically converted to the biologically, pharmaceutically or therapeutically more active form of the antisense molecule.

In certain embodiments, the present invention provides compositions and methods for reducing the amount or activity of a target nucleic acid in a cell. In certain embodiments, the cell is in an animal. In certain embodiments, the animal is a mammal. In certain embodiments, the animal is a rodent. In certain embodiments, the animal is a primate. In certain embodiments, the animal is a non-human primate. In certain embodiments, the animal is a human.

In certain embodiments, the present invention provides methods of administering a pharmaceutical composition comprising an antisense molecule of the present invention to an animal. Suitable administration routes include, but are not limited to, oral, rectal, transmucosal, intestinal, enteral, topical, suppository, through inhalation, intrathecal, intracerebroventricular,

intraperitoneal, intranasal, intraocular, and parenteral (e.g., intravenous, intramuscular, intramedullary, and subcutaneous).

The above-mentioned antisense nucleic acid molecule, nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule, vector/plasmid, cell and/or compositions according to the present invention can be used to inhibit HIV-1 replication in a cell or a subject in need thereof, and/or prevent or treat HIV-1 infection (and/or Acquired Immune Deficiency Syndrome, AIDS as well as AIDS-related diseases, such as opportunistic infections, Kaposi's sarcoma or pneumocystic pneumonia) in a subject.

The term "treating" as used herein, includes but is not limited to, reducing, suppressing, inhibiting, lessening, or affecting the progression, severity, and/or scope of a condition, chance of re-occurrence or returning of a disease after a remission. In one embodiment, treating may include directly affecting or curing, suppressing, inhibiting, reducing the severity of, delaying the onset of, reducing symptoms associated with HIV-1 infection, or a combination thereof. In another embodiment, treating includes delaying progression, expediting remission, inducing remission, augmenting remission, speeding recovery, increasing efficacy of or decreasing resistance to alternative therapeutics, or a combination thereof. In an embodiment, "treating" means to ameliorate at least one clinical symptom or parameter of HIV infection or preventing it from worsening or preventing the transmission of HIV, e.g., from mother to child. For example, a treatment can result in a reduction in viral load, and/or an increase in number of CD4⁺ T cells ("CD4 count").

The term "preventing," as used herein, includes but is not limited to, delaying the onset of symptoms, preventing relapse to a disease, decreasing the number or frequency of relapse episodes, increasing latency between symptomatic episodes, or a combination thereof.

An "effective amount" is an amount sufficient to effect a desired therapeutic effect, e.g., a reduction in viral load, and/or an increase in number of CD4⁺ T cells. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a composition may depend on the composition selected. The compositions can be administered once, one or more times per day, and/or one or more times per week; including once every other day. In certain embodiments, the compositions will be administered two or three times per day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to treat effectively a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and any other indications present. Treatment of a subject with an effective amount of an antisense nucleic acid described herein can include a single treatment or a series of treatments.

Dosage, toxicity and therapeutic efficacy of the antisense nucleic acids can be determined, e.g., by standard pharmaceutical procedures in cell cultures or experimental

animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Antisense nucleic acids that exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to select a dose and administration schedule that minimizes severe side effects while maximizing therapeutic efficacy.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such antisense nucleic acids lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any antisense nucleic acid used in a method described herein, an effective dosage range can be estimated initially from cell culture assays. A dose can be further formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to determine more accurately useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography (HPLC).

In an embodiment, the antisense nucleic acid molecule, nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule, vector/plasmid, cell and/or compositions according to the present invention further comprises a targeting moiety, i.e. to target antisense nucleic acid molecule to HIV-infected cells or tissues. For example, the antisense nucleic acid molecule, nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule, vector/plasmid, cell and/or compositions may comprise a moiety targeting CD4⁺ T cells, macrophages and/or dendritic cells. Such moiety may be a ligand (natural ligand, antibody, RNA/DNA aptamer) that recognizes a marker (e.g., cell surface marker) expressed by HIV-infected cells.

In an embodiment, the antisense nucleic acid molecule, nucleic acid molecule (e.g., DNA) comprising a sequence encoding the antisense nucleic acid molecule, vector/plasmid, cell and/or compositions according to the present invention is used in combination with one or more additional anti-HIV agents

MODE(S) FOR CARRYING OUT THE INVENTION

The present invention is illustrated in further details by the following non-limiting examples.

Example 1: Materials and Methods

Identification of SOFA-HDV-Rz target sites.

The use of the LANL dataset to estimate sequence conservation at the nt level has been previously described⁴⁵. Briefly, a multiple sequence alignment of all complete HIV-1 sequences were downloaded from the LANL database using the QuickAlign™ tool and a consensus sequence with % conservation at each position was generated using Jalview sequence editor⁴⁷ and exported to Microsoft Excel (see reference 51). Several positions in the consensus sequence were represented in only a small number of sequences and positions that occurred in less than 10% of the sequences were removed from the raw data to facilitate target site selection. Highly conserved target sites were selected based on criteria illustrated in **FIG. 2A**. Nucleotide BLAST⁴⁸ was then used to align target sites to HIV-1 NL4-3 (M19921) and Ribosubstrates software²⁶ was used to evaluate the potential for the corresponding SOFA-HDV-Rzs to target human RNAs as previously described²⁶. Briefly, the software identifies potential target sites for SOFA-HDV-Rzs in a cDNA database allowing for variations in the length of the spacer sequence and biosensor (**FIG. 1A**). Perfectly matched target sites are assigned a value of 0, and the score increases by 10 for each wobble base pair and by 100 for each mismatch. We set a value of 20 as the cut-off for potential off-target effects, representing at least two wobble base pairs between a SOFA-HDV-Rz targeting HIV-1 RNA and a potential target site in any human RNA.

Plasmid constructs

All SOFA-HDV Rzs and shRNAs were expressed from the human RNaseP H1 promoter in the vector psiRNA-H1GFP::Zeo (InvivoGen, San Diego, CA). SOFA-HDV-Rz inserts were generated using an overlapping PCR strategy^{11,14,28} and shRNA inserts were generated by annealing complementary oligonucleotides. Sequences for shRNA522 and shRNA553 inserts were obtained from a previous study²² and using an identical design, shRNAs (non-sense, adapted from siControl²⁰), shRNA5983 (adapted from sh1⁴⁹) and shRNA1498 inserts were designed. The cloning strategies and sequences of all oligonucleotides used for the generation of plasmid inserts are provided below, variable SOFA-HDV-Rz DNA sequences are illustrated in **Table 5**. SOFA-HDV-Rz inserts were generated by PCR using DNA primers A and B (2 μM), C and D (25 nM):

Table 1: Sequences of DNA primers A to D used to generate SOFA-HDV-Rz inserts

Name	Sequence (5'-3')	SEQ ID NO
A	TATAAGTTCTGTATGAGTTCACG GAAGAC CGACCT↓ <u>CGGGCCAG</u> <u>CTAGTTT</u>	9
B	CAACAACAGTGTTCCGGATGAACTGATGCTAT GAAGACT CCAAA↓A <u>ACCAGCTAGAAAGGGTC</u>	10
C	<u>CCAGCTAGAAAGGGTCCCTTA</u> gCCATCCGCGAACGGATGCCC	11
D	TAATACGACTCACTATAGGGCCAGCTAGTTT(Bs)(BI)CAGGGTCCA <u>CCTCCTCGCGGT(RD)GGGCATCCGTTTCGCG</u>	12

Primers A and B include *Bbs*I recognition sites (bold, cut site indicated with an arrow), C and D contain a common reverse and specific forward ribozyme sequence respectively

(overlapping sequences underlined). The nucleotide in primer C that was mutated to produce a catalytically inactivate SOFA-HDV Rz is shown as lower case (G to T mutation in this study). Variable Bs, BI and RD sequences in primer D are shown in **Table 1** for each SOFA-HDV-Rz evaluated.

- 5 shRNA inserts were generated by annealing complementary sense (S) and antisense (AS) oligonucleotides (1.25 μ M each in 75 mM NaCl, 40 μ L, 2 min at 80 °C, cooled to 37 °C):

Table 2: Sequences of shRNA inserts

Name	Sequence (5'-3')	SEQ ID NO
shRNA522 S	<u>ACCTCGCCTCAATAAAGCTTGCCTT</u> CCTCGAGCAAGGCA AGCTTTATTGAGGCTT	13
shRNA522 AS	<u>CAAAAAGCCTCAATAAAGCTTGCCTT</u> GCTCGAGGAAGGC AAGCTTTATTGAGGCG	14
shRNA553 S	<u>ACCTCGTAGTGTGTGCCCGTCTGTT</u> CCTCGAGCAACAGA CGGGCACACACTACTT	15
shRNA553 AS	<u>CAAAAAGTAGTGTGTGCCCGTCTGTT</u> GCTCGAGGAACAG ACGGGCACACACTACG	16
shRNA5s S	<u>ACCTCGTACCGCACGTCATTTCGTAT</u> CCTCGAGCATACGA ATGACGTGCGGTACTT	17
shRNA5s AS	<u>CAAAAAGTACCGCACGTCATTTCGTAT</u> GCTCGAGGATACG AATGACGTGCGGTACG	18
shRNA1498 S	<u>ACCTCGCAGGAAGTACTAGTACCCTA</u> CTCGAGAAGGGTA CTAGTAGTTCCTGCTT	19
shRNA1498 AS	<u>CAAAAAGCAGGAAGTACTAGTACCCTT</u> CTCGAGTAGGGT ACTAGTAGTTCCTGCG	20
shRNA5983 S	<u>ACCTCGCGGAGACAGCGACGAAGAGG</u> CCTCGAGGCTCTT CGTCGCTGTCTCCGCTT	21
shRNA5983 AS	<u>CAAAAAGCGGAGACAGCGACGAAGAGC</u> CCTCGAGCCTCT TCGTCGCTGTCTCCGCG	22

- 10 The nucleotides flanking the core loop sequence (bold) for the HIV-1 specific shRNA522, shRNA553 and shRNA1498 are complementary to the 21st nt of their target site in case of differential processing as previously described²². *Bbs*I cut site overhangs in the complementary oligonucleotides are underlined.

- 15 SOFA-HDV-Rz and shRNA inserts were ligated into *Bbs*I (Thermo Fischer Scientific, Waltham, MA) digested psiRNA-H1GFP::Zeo (InvivoGen, San Diego, CA) expression plasmid. All constructs were confirmed by sequencing using a primer located in the H1 promoter: 5'-TCTACGGGGTCTGACGC-3' (SEQ ID NO:23)

siRNA oligonucleotides

siRNAs were custom made by Dharmacon® with the sequences depicted in Table 3:

Table 3: Sequences of siRNAs

Name	Sequence (5'-3')	SEQ ID NO
si1497 sense	AGCAGGAACUACUAGUACCCUUCdAdG	24
si1497 antisense	UAUCGUCCUUGAUGAUGAUGGGAAGUC	25
si1498 sense	GCAGGAACUACUAGUACCCUUCAdGdG	26
si1498 antisense	AUCGUCCUUGAUGAUGAUGGGAAGUCC	27
si1499 sense	CAGGAACUACUAGUACCCUUCAGdGdA	28

si1499 antisense	UCGUCCUUGAUGAUGAUGGGAAGUCCU	29
siNS sense	GUACCGCACGUCAUUCGUAUCCUdAdT	30
siNS antisense	TTCAUGGCGUGCAGUAAGCAUAGGAUA	31

Transfections

Co-transfections of HIV-1 molecular clones with Rz or shRNA expressing plasmids were performed in either 24 or 12 well plate formats as indicated in the figure legends. 24 h prior to transfection, HEK293T cells were plated at 2×10^5 cells/mL and transfections were carried out using TransIT® reagent (Mirus, Madison, WI) according to the manufacturer's instructions. Viral production was estimated 48 h following transfection by measuring HIV-1 RT activity in the culture supernatant. To account for differences in viral production between experiments, replicates for each construct evaluated were performed in parallel with the empty vector psiRNA-H1GFP::Zeo and all data are expressed as a percentage of viral production in the empty vector co-transfected cells (Relative RT activity). For each Rz or shRNA construct evaluated we also included the irrelevant control SOFA-HDV-Rz-HBV or the nonsense control shRNAs, respectively.

Cell culture

HEK293T and TZM-bl cells were maintained in Dulbecco's modified Eagle's medium with high glucose (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 50 U/mL Penicillin and 50 µg/mL Streptomycin (Life Tech., Carlsbad, CA). Jurkat T cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Hyclone, Logan, UT) supplemented with 10% heat inactivated (55°C, 30min) fetal bovine serum (Hyclone, Logan, UT), 50 U/mL Penicillin and 50 µg/mL Streptomycin (Life Tech., Carlsbad, CA).

Rz expression in HEK293T cells

Total RNA extracts were harvested from transfected cells using Trizol reagent (Life Tech., Carlsbad, CA) according to the manufacturer's instructions. 10 µg of total RNA was resolved on an 8% denaturing polyacrylamide gel, transferred to a nylon membrane (Amersham Hybond-N+, GE Healthcare, Little Chalfont, UK) and UV cross-linked. Membranes were incubated with ProbeSOFA followed by Probe5S and visualized using a Phosphor screen. Probe sequences, labelling and details on the Northern blot conditions are provided below.

Northern blot

Probe labeling and sequences: 5 pmol of the oligonucleotide ProbeSOFA or 10 pmol of the oligonucleotide Probe5S were 5'-end labeled by incubation for 1 h at 37 °C with 3 U of T4 polynucleotide kinase (Affymetrix, Santa Clara, CA) and 3.2 pmol of [γ -³²P]ATP (6000 Ci/mmol; Perkin Elmer, Waltham, MA). The quantity of [γ -³²P]ATP was doubled for the ProbeSOFA labeling reaction. The labeled oligonucleotides were purified with ProbeQuant™ G50 Micro Columns (GE Healthcare, Little Chalfont, UK) and used directly for the Northern blot, their sequences were:

ProbeSOFA: 5'-GAAAGGGTCCCTTAGCCATCCGCGAACGGATGCCC-3' (SEQ ID NO: 32)

Probe5S: 5'-AAAGCCTACAGCACCCGGTATTCCC-3' (SEQ ID NO: 33).

Northern blot conditions: Total RNA samples were dissolved in RNase-free water and quantified (Nanovue, Roche, Basel, Switzerland). For each condition, 10 µg of total RNA mixed with 2 volumes of loading buffer was resolved on 8% denaturing PAGE. The RNA was then transferred (90 min, 4 °C, 200 mA) to a nylon membrane (Amersham Hybond-N+, GE Healthcare, Little Chalfont, UK) in 0.5× TBE using a Trans-blot cell (Bio-Rad, Hercules, CA). Membranes were UV crosslinked prior to pre-hybridization at 42 °C in CHURC buffer (1% (W/V) BSA, 1 mM EDTA, 500 mM phosphate buffer and 7% (W/V) SDS). After 2 to 4 h, the radio-labeled ProbeSOFA was added, and the membranes were hybridized at 42 °C overnight. Before analysis, the membranes were washed twice, 5 min each, in wash buffer #1 (2× SSC, 0.1% SDS) and twice, 15 min each, in wash buffer #2 (0.1× SSC, 0.1% SDS), all at 42 °C. The results were visualized using a Phosphor Screen. For the Probe5S hybridization, the membranes were stripped by incubation in wash #2 at 80 °C for 20 min to remove the ProbeSOFA. Only 5 to 10% of the labeled Probe5S was used for the hybridization of one membrane.

In vitro SOFA-HDV-Rz cleavage assay

Single-turnover conditions ($R_z \gg$ substrate) were used to evaluate the catalytic activity of SOFA-HDV-Rzs as previously described²⁸. Briefly, a trace amount of 5'-end-labeled substrate (< 1 nM) was incubated at 37 °C with a final concentration of 100 nM of the selected SOFA-HDV-Rz. The cleavage reactions were initiated by the addition of $MgCl_2$ and samples were taken at different time intervals and stopped with loading buffer. Recovered samples were resolved on a 20% denaturing polyacrylamide gel, visualized using a Phosphor Screen and quantified using ImageQuant® software (Molecular Dynamics, Sunnyvale, CA). The control reaction was performed in the absence of Rzs (replaced by water) and its last time interval sample was used to subtract the background. For each time point, the percentage of cleavage was calculated (cleaved product counts over cleaved + uncleaved products counts). Details on the DNA templates used for *in vitro* transcription, RNA synthesis and labelling are provided below. The k_{obs} and F_{max} were then calculated using GraphPad Prism™ 5 for each Rz. The rate of cleavage (k_{obs}) was obtained by fitting the data to the equation $F_t = F_{max} (1 - e^{-kt})$, where F_t is the percentage of cleavage at time t , F_{max} is the maximum percent cleavage and k is the rate constant (k_{obs}).

DNA templates for in vitro transcription:

SOFA-HDV-Rz DNA templates were generated through a PCR-based strategy with DNA oligonucleotides C and D shown in Plasmid constructs. The SOFA-HDV-Rz sense primer (D) provided the T7 RNA polymerase promoter needed for subsequent *in vitro* transcription. The fully double-stranded DNA sequences were produced using *Pwo* DNA polymerase (Roche

Diagnostics). Similarly, the substrate DNA template was produced by a combination of two complementary oligonucleotides, subA and subB.

Table 4: Sequence of oligonucleotides subA and subB

Name	Sequence (5'-3')	SEQ ID NO
subA	taatacgactcactata GGGCATAGCAGGA ACTACTAGTACCCTTGGG TCGGCAGGGTCCACCTCC	34
subB	GGGTCCCTTAGCCATGCGAAGCCGCATGCCCAGGTCCGACCG CGAGGAGGTGGACCCTGCCGACCC	35

5 subA contained the T7 RNA polymerase promoter (lower case) at its 5'-end and part of a *cis*-acting HDV ribozyme (underlined sequence) at its 3'end. The subB primer is complementary (underlined sequence) to the forward primer and completes the *cis*-acting HDV ribozyme. The *cis*-acting HDV ribozyme permits the production of a precise 3'-end as described in Avis, J.M., *et al.* (2012) *Methods Mol Biol*, **941**, 83-98. The final RNA substrate, SubMin1498,
10 corresponds to the sequence in bold. All PCR reactions were ethanol precipitated prior to *in vitro* transcription.

RNA synthesis for *in vitro* transcription:

The SOFA-HDV-Rz and the substrate RNA were synthesized by run-off transcriptions as described previously (28). Briefly, transcriptions were performed in the presence of purified
15 T7 RNA polymerase (10 µg), pyrophosphatase (0.01 U, Roche, Basel, Switzerland) and PCR product (2 to 5 µM) in a buffer containing 80 mM HEPES-KOH (pH 7.5), 24 mM MgCl₂, 2 mM spermidine, 40 mM DTT and 5 mM of each NTP in a final volume of 100 µL at 37 °C for 2 h. The reaction mixtures were then treated with RQ1 DNase (Promega, Madison, WI) at 37 °C for 20 min. After phenol/chloroform extraction the RNAs were ethanol precipitated. The pellets were
20 dissolved in equal volumes of ultrapure water and loading buffer (95% formamide, 10 mM EDTA [pH 8.0], 0.025% xylene cyanol and 0.025% bromophenol blue). The samples were then fractionated through either 8% or 20% denaturing polyacrylamide gels (PAGE, 19:1 ratio of acrylamide to bisacrylamide) in buffer containing 45 mM Tris-borate (pH 7.5), 8 M urea, and 2 mM EDTA. The RNA products were visualized by ultraviolet (UV) shadowing. The bands
25 corresponding to the correct sizes for both the SOFA-HDV-Rzs and the substrate were cut out of the gel and the RNAs eluted overnight at 4 °C in elution buffer (500 mM ammonium acetate, 10 mM EDTA, 0.1% SDS). The samples were again ethanol precipitated, washed, dried and dissolved in ultrapure water. The RNA was quantified by absorbance at 260 nm and diluted to the desired concentration (Rz 1 µM and Substrate 5 µM).

30 RNA and Probes Labelling

The RNA substrate used in cleavage reactions was 5'-end labeled as described previously (28). Briefly, the purified RNA substrate was dephosphorylated by mixing 50 pmol of RNA with 1 U of Antarctic phosphatase (New England Biolabs) in a final volume of 10 µL containing the buffer provided with the enzyme, and then incubated for 30 min at 37 °C.

Incubation at 65 °C for 8 min was used to inactivate the enzyme. The 5'-end labeling reaction was performed with 5 pmol of dephosphorylated RNAs that were incubated for 1 h at 37 °C with 3 U of T4 polynucleotide kinase (USB) and 3.2 pmol of [γ -³²P]ATP (6000 Ci/mmol; New England Nuclear) in the reaction buffer provided with the enzyme. The reactions were stopped by the addition of two volumes of loading buffer prior to fractionation by 20% denaturing PAGE. The RNAs were detected by autoradiography, cut out of the gel and eluted as described in RNA synthesis for *in vitro* transcription.

HIV-1 protein expression in HEK293T cells

The detection of HIV-1 protein expression using an HIV-1 p24 antibody in HEK293T cells has been previously described⁵⁰. Briefly, 100 µg of total protein was resolved on a 10% denaturing poly-acrylamide gel and transferred to a Hybond® ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, United Kingdom). Membranes were incubated first with anti-HIVp24 (183-H12-5C) followed by anti-GAPDH (sc-32233, Santa Cruz Biotechnology, Dallas, TX), bands were visualized using ECL (GE Healthcare, Little Chalfont, United Kingdom). The relative intensity of bands was calculated using Image J densitometry software (Version 1.48, National Institutes of Health, USA). Data are expressed as Gag or CA band intensities relative to the intensity of the Gag band in the control SOFA-HDV-RzHBV (**FIG. 5B**) or shRNAs (**FIGs. 11A and B**) lanes.

Gene expression profiling

Gene expression levels relative to control transfections were analyzed by human gene expression microarrays (Atlantic Cancer Research Institute (ACRI) proprietary slides).

RNA extraction and mRNA analysis:

Total RNA extracts were harvested from transfected cells using Trizol® reagent (Invitrogen®) according to the manufacturer's instructions and purified using an RNeasy® column (Qiagen®). Quality of total RNA samples was assessed using the Experion® bioanalyzer system with RNA StdSens chips and associated reagents (BIO-RAD®). All RNA used in these experiments had an RNA Quality Index (RQI) value greater than nine. 1 µg of each total RNA sample was amplified using the Amino Allyl MessageAmp® II aRNA amplification kit and subsequently labeled with AlexaFluor® 555 or 647 (Life Technologies®). Quantity and quality of amplified aRNA was assessed using a Nanodrop spectrophotometer and the Experion® bioanalyzer. Samples were compared in triplicate dye swap experiments, with 1.5 µg of each labeled, fragmented aRNA (3 µg total per slide) hybridized to proprietary human cDNA microarray slides. These arrays consist of roughly 35000 spots, representing roughly 17000 different 50-mer oligonucleotides spotted in duplicate on Nexterion-E® epoxy microarray slides (Schott, Mainz, Germany).

Hybridizations were performed in Ambion® SlideHyb #2 buffer (Life Technologies®) at 42 °C for 16 h using the automated TECAN® 4800 Hybridization station (TECAN®). Following

hybridization, slides were scanned at 10 μ m resolution using an Axon GenePix® 4200AL scanner (Molecular Devices, Sunnyvale, CA) and gridded using SpotReader® (Niles Scientific). Fine tuning of spot rejection was subsequently done by visual inspection of the gridded image and of a scatter plot of $M = \log(532/635)$ versus $A = \log(532) + \log(635)$, special attention being paid to outliers. A GPR file was generated that was subsequently processed to flag spots with a signal to noise ratio of less than 5.

Data analysis was done with Acuity™ 4.0 (Axon Instruments, Sunnyvale, CA) and subsequently normalized using Lowess. The resulting log2 ratios of SOFA-HDV-Rz or shRNA1498 transfected cells compared to the empty vector (psiRNA) transfected cells were then further analyzed using various statistical and visual methods such as SOM (Self Organizing Maps), t-Test, PCA (Principal Component Analysis) and volcano plot.

HIV-1 infection assay

Stable Jurkat T lymphocytes were generated by electroporation of psiRNA constructs followed by selection with Zeocin (InvivoGen). The relative expression of psiRNA constructs in the stable cell populations was estimated by measuring GFP expression from the integrated vector with a FACSCalibur® flow cytometer (BD), and proliferation was determined by counting live cells by Trypan blue (Wisent) exclusion using a hemocytometer. Prior to infection, SOFA-HDV-Rz or shRNA expressing cells were plated in 6 well plates at 2×10^5 cells per well. Viral replication was monitored by measuring RT activity in culture supernatants at various days post infection.

Generation and characterization of stable Jurkat T cells

1×10^6 Jurkat T cells were electroporated at 250 mV for 10 msec (GenePulserII®, Biorad) with 15 μ g of psiRNA plasmids and cultured in 5 mL RPMI overnight. Cells were transferred to 20 mL culture media containing 600 μ g/mL Zeocin (InvivoGen®) and cultured for 4 weeks with selection media changed every 3-4 days. Cells were split at various times during the selection to maintain a high cell density in the first 2 weeks of selection, and a low cell density in the last 2 weeks of selection. At the end of the selection period cells were frozen, or cultured for an additional day in the absence of Zeocin prior to infection with HIV-1 virions. The expression of GFP in the different cell populations was determined using a FACSCalibur® flow cytometer (BD) and data analysis was performed using FlowJo® Version 8.7 (Tree Star). Cell proliferation was determined by plating cells at 1×10^5 cells/mL in a 6 well plate followed by counting live cells diluted in Trypan blue (Wisent) with a hemocytometer. Duplicate wells were counted for each cell line out to four days.

Virus infection:

A 20 mL culture of HEK293T cells was transfected with 20 μ g of HIV-1 pNL4-3 plasmid DNA. The supernatant was harvested 48 h later, cleared of cell debris by centrifugation, and stored at -80 °C in 1 mL aliquots. SOFA-HDV-Rz and shRNA expressing Jurkat T cells were

plated in 6 well plates at 2×10^5 cells/ well and infected with the HEK293T cell supernatant corresponding to 6×10^5 cpm equivalent of HIV-1 RT activity per well. Viral replication was monitored by measuring RT activity in the culture supernatants at various days post infection. All infections were performed in triplicate wells, cells were split twice a week.

5 HIV-1 RT assay

The HIV-1 RT assay used in this study was performed as previously described⁴⁵. Briefly, 5 μ l of supernatant was incubated with a polyadenylic acid template (Roche, Basel, Switzerland), an oligodT primer (Life Tech., Carlsbad, CA) and [³²P]-dTTP (3,000 Ci/mmol, Perkin Elmer, Waltham, MA) for 2 h at 37°C in 50 μ l total reaction mixture. 5 μ l of the reaction
10 mixture was then spotted onto Diethylaminoethyl (DEAE) filter mat (Perkin Elmer, Waltham, MA) and washed five times in 2X SSC buffer, followed by two washes in 95% ethanol to remove [³²P]-dTTP not incorporated into the polydT RT product. Counts per minute (cpm) were calculated for each sample using a microplate scintillation counter (Microbeta TriLux, Perkin Elmer,) and are proportional to the amount of HIV-1 RT enzyme present in the reaction mixture.

15 **Example 2: Identification of SOFA-HDV-Rz target sites in HIV-1 RNA**

HIV-1 sequence conservation was estimated to identify target sites that are relevant for the majority of HIV-1 strains. Estimates at the nt level were made (Reference #51) using all complete sequences available in the Los Alamos National Laboratory (LANL) database (1850 at
20 the time of analysis, subtype distribution shown in **FIG. 9**). These estimates were used to identify highly conserved SOFA-HDV-Rz target sites (**FIG. 2A**) that were identical in HIV-1 strain NL4-3 (**FIG. 2B**). The Ribosubstrates informatics tool²⁶ was used to exclude SOFA-HDV-Rzs targeting 12 highly conserved and 19 moderately conserved regions in HIV-1 RNA, due to their potential to target human RNAs.

25 Several highly conserved target sites were identified in the 5'LTR U5 region within the 5'UTR (**FIG. 2C**). The Gag coding sequence had much lower overall conservation; however, four highly conserved and, with some exceptions to the conservation criteria (**FIG. 2A**), nine moderately conserved target sites were identified in this region (**FIG. 2C**). Of the Rzs that we have previously evaluated targeting the Tat/Rev exon1 coding sequence of HIV-1 RNA¹⁶, the
30 target sites for Tat1 and Tev1 were highly and moderately conserved, respectively. Conservation exceptions and sequences of all target sites used in this study are illustrated, along with the DNA sequences of the corresponding SOFA-HDV-Rz variable regions, in **Table 5**.

Table 5: SOFA-HDV-Rz target sites and DNA coding sequences

HIV-1 NL4-3 target sites : 5'-3' ¹			SEQ ID NO:	SOFA-HDV-Rz DNA sequences : 5'-3' ²			SEQ ID NO (Bs)
Rz	RD	Bs		Bs	RD	BI	

HBV	-		GAGACAAGAA	AAACCAT	GTTT	56
Tat1	<u>TAGATCCTAGaCTAGAGCCCTGGAA</u>	36	CCAGGGCTCT	TAGGATT	CCTA	57
Tev1	<u>CAGGAAGAA</u> GC GG <u>AGACAgCGACGA</u>	37	GTCGCTGTCT	TTCTTCT	AGAA	58
522	<u>AAGCCTCAATAAA</u> GCTTGCCTTGAG	38	CAAGGCAAGC	TTGAGGT	TCAA	59
553	<u>AAGTAGTGTGTGCCCGTCTGTTGTG</u>	39	ACAACAGACGG	ACACTAT	GTGT	60
560	<u>GTGTGCCCGTCTGTTGTGTGACTCT</u>	40	AGTCACACAA	CGGGCAT	CCCG	61
562	<u>GTGCCCGTCTGTTGTGTGACTCTGG</u>	41	AGAGTCACAC	GACGGGT	CGTC	62
577	<u>GTGACTCTGGTAACTAGAGATCCCT</u>	42	GGATCTCTAG	CAGAGTT	TCTG	63
798	<u>GCGAGAGCGTCggTATTAAGcGGGG</u>	43	CCGCTTAATA	CGCTCTT	AGCG	64
800	<u>GAGAGCGTCggTATTAAGcGGGGGA</u>	44	CCGCTTAAT	GACGCTT	CGTC	--
854	<u>AAGGCCAGGGGGAAAGAAAcAaTAT</u>	45	ATTGTTTCTT	CCTGGCT	CAGG	65
1283	<u>cAGCCCAGAAaGTAATACCCATGTTT</u>	46	ACATGGGTAT	TCTGGGT	CAGA	66
1309	<u>CAGCATTATCAGAAaGGAGCCACCCC</u>	47	GGTGGCTCC	ATAATGT	TTAT	--
1477	<u>GaGAACCAAGGGGAAGTGAcATAGC</u>	48	ATGTCACTTC	TTGGTTT	CCAA	67
1498	<u>TAGCAGGAACTACTAGTACCCTTCA</u>	49	AAGGGTACTA	TTCCTGT	GGAA	68
1511	<u>TAGTACCCTTCAGGAACAAATAGgA</u>	50	CTATTTGTTC	AGGGTAT	CCCT	69
1593	<u>CTGGGATTAATAAAATAGTAAGAA</u>	51	CTTACTATTT	TAATCCT	ATTA	70
1617	<u>ATGTATAGCCCTaccAGCATTcTGG</u>	52	CCAGAATGCTG	GCTATAT	TAGC	71
1638	<u>cTGGACATAAgACAAGGaCCAAAgG</u>	53	CTTTGGTCCTT	TATGTCT	CATA	72
1736	<u>TTGGATGACAGAAACCTTGtTGGTC</u>	54	CCAACAAGGT	GTCATCT	TGAC	73
1827	<u>ATGATGACAGCATGtCAGGGAGTGG</u>	55	ACTCCCTGAC	TGTCATT	GACA	74

¹ The target sites of the SOFA-HDV-Rzs identified are shown with the recognition domain (RD) and the biosensor (Bs) binding sites underlined. Nucleotides with identities different in HIV-1 pNL4-3 in comparison to the consensus sequence are in lower case, those conserved in less than 80% of the sequences are shown as **bold lower case**.

5 ² The variable DNA sequences used to clone SOFA-HDV-Rzs into the psiRNA vector are shown for the RD, Bs and Blocker (BI) regions.

Example 3: SOFA-HDV Rz screen for inhibition of HIV-1 production

10 The effect of each SOFA-HDV-Rz expressing plasmid on HIV-1 production was evaluated by co-transfection with HIV-1 molecular clone pNL4-3 in HEK293T cells, using conditions similar to those reported for other Rzs^{7,18,27} and shRNAs^{21,22}. HIV-1 RT activity was measured to estimate the production of virus released into the medium of transfected cells and effects of Rzs were normalized to co-transfection of pNL4-3 with an empty Rz expression plasmid. An irrelevant Rz targeting Hepatitis B Virus RNA (SOFA-HDV-RzHBV, adapted from

15 SOFA- δ Rz-303⁹) was used as a negative control and previously described SOFA-HDV-Rzs Tat1 and Tev1¹⁶ were used as positive controls. Compared to SOFA-HDV-RzHBV, Rzs targeting both the 5'UTR and Gag coding sequences significantly inhibited viral production, with the top candidate (SOFA-HDV-Rz-1498) targeting the Gag coding sequence (**FIG. 3**).

Example 4: Antisense and mismatched variants of SOFA-HDV-Rz1498 are not effective inhibitors of HIV-1 production

To evaluate the antisense effect of SOFA-HDV-Rz1498, we generated an inactive variant (SOFA-HDV-Rz1498A76) in which a C to A mutation at position 76 in its backbone disables its cleaving capability²⁸ (**FIG. 4A**). SOFA-HDV-Rz1498A76 did not significantly inhibit HIV-1 production at similar expression levels to SOFA-HDV-Rz1498 (**FIG. 4B**), suggesting that Rz catalytic cleavage is primarily responsible for the effects of SOFA-HDV-Rz1498. No effect on the infectivity of virus from SOFA-HDV-Rz1498 expressing cells was observed (**FIG. 10**), suggesting that the Rz reduces the amount of virus produced but does not affect the quality of the virions.

SOFA-HDV-Rz1498 variants with either a single or double mutation in their biosensor (Bs) sequence were also generated to evaluate the potential for SOFA-HDV-Rz1498 to tolerate mismatches with its target (SOFA-HDV-Rz1498Bs1 and SOFA-HDV-Rz1498Bs2, **FIG. 4A**). Neither variant inhibited HIV-1 production (**FIG. 4B**), suggesting that the effect of SOFA-HDV-Rz1498 is sensitive to mismatches with its target. The mismatched Rzs had similar *in vitro* cleavage rate constants (k_{obs}) with significantly reduced maximum cleavage (F_{max}) values (**FIG. 4C**), suggesting that part of their failure to inhibit HIV-1 production in cells is related to a reduced capacity to cleave their target.

Example 5: An shRNA targeting the 1498 site is a potent inhibitor of HIV-1 production and provides an additive effect in combination with SOFA-HDV-Rz1498

To evaluate the potential for other antisense molecules targeting the SOFA-HDV-Rz1498 target site to inhibit HIV-1 production, shRNA1498 was designed. According to the conservation estimates, each nt in the shRNA1498 target site was conserved at >80% (**FIG. 5A**, Gag). Compared to a nonsense shRNA (shRNAs) as well as SOFA-HDV-Rz1498 and its controls, shRNA1498 provided a near complete inhibition of viral production (**FIG. 5B**). This inhibition correlated with a decrease in intracellular expression of the HIV-1 Gag polyprotein and one of its processing products, capsid (CA). Unexpectedly, the decrease in CA expression was much more pronounced for both the Rz and shRNA compared to their effects on Gag expression. A similar effect was observed for shRNAs targeting sequences in the 5'UTR and Tat/Rev coding sequences of HIV-1 RNA (**FIGs. 11A and B**), suggesting that it is not specific to an shRNA targeting the Gag 1498 sequence.

To compare the potency of shRNA1498 to other candidate shRNAs, shRNA522 and shRNA553 were designed, modeled after previously characterized siRNAs²⁰ and shRNAs²² targeting the 5'UTR (**FIG. 5A**, 5'UTR) and shRNA5983, modeled after a construct in clinical development targeting the Tat/Rev exon1 coding sequence²⁹ (**FIG. 5A**, Tat/Rev). All four shRNAs inhibited HIV-1 production (**FIG. 5C**). The potency of shRNA1498 was comparable to

that of shRNA553 and shRNA5983, with 50% effective concentrations (EC50s) for shRNA plasmids below 5 ng of input DNA, whereas shRNA522 was much less potent with an EC50 value of 702 ng.

To evaluate the potential for shRNA1498 and shRNA5983 to be used in combination with SOFA-HDV-Rz1498, HEK293T cells were co-transfected with HIV-1 pNL4-3 and different combinations of Rzs and shRNAs (**FIG. 5D**). To quantify the effect of the combinations, an input level of shRNA DNA that did not completely inhibit viral production in **FIG. 5C** was chosen. In combination with both shRNA1498 and shRNA5983, SOFA-HDV-Rz1498 provided an additional inhibition of HIV-1 production compared to the control Rz, SOFA-HDV-Rz-HBV. The level of inhibition was similar to its effect when co-transfected alone (50%) (**FIG. 5B**), suggesting that the Rz can provide an additive effect in combination with both an shRNA targeting the same site (shRNA1498) and an shRNA targeting a different site (shRNA5983).

Example 6: SOFA-HDV-Rz1498 and shRNA1498 inhibit viral production from diverse HIV-1 strains

As the Gag 1498 target site was shown to be accessible to both Rz and shRNA activity in HIV-1 strain pNL4-3, it was next evaluated whether this inhibition extended to diverse viral strains representing subtype B (Mal³⁰ and AD8³¹), C (Indie-C1³² and MJ4³³), D (94UG114³⁴) and circulating recombinant form (CRF) 02_AG (97GH-AG1³⁵). SOFA-HDV-Rz1498 inhibited HIV-1 production from viral strains (Mal, AD8, MJ4 and 97GH-AG1) with nt variants in proximity to their target sites compared to NL4-3 (**FIG. 6**), suggesting that the structure of the target site is equally accessible to the Rz in these strains. Consistent with results using SOFA-HDV-Rz binding site variants (**FIG. 4**, -Bs1 and -Bs2), SOFA-HDV-Rz1498 did not inhibit HIV-1 production from the strains Indie-C1 and 94UG114, which harbor a single nt variant within their Bs binding sites (**FIG. 6**). In contrast, shRNA1498 inhibited HIV-1 production from all strains suggesting that it can tolerate a single nt mismatch in its binding site at position 17 and can inhibit HIV-1 production in diverse strains.

Example 7: SOFA-HDV-Rz1498 and shRNA1498 have minimal off target effects on human RNAs

The potential for both SOFA-HDV-Rz1498 and shRNA1498 to affect the expression of human mRNAs was next evaluated in HEK293T cells co-transfected with HIV-1 pNL4-3. Prior to gene expression profiling, the inhibition of viral production was confirmed for each condition and agreed with results presented in **FIG. 5B**. Microarray experiments were performed as triplicate dye swaps and the results were expressed as the log2 ratio of SOFA-HDV-Rz1498 or shRNA1498 compared to the empty vector co-transfected cells (**FIG. 12**). All average log2 ratios were low (below 1.0) suggesting that both SOFA-HDV-Rz1498 and shRNA1498 can inhibit HIV-

1 production with minimal effects on human mRNA expression. The log2 ratios for mRNAs with the greatest extent of up- or down- regulation are illustrated in **FIG. 7** and listed in **FIGs. 15A-D**. Several of these mRNAs were found in both SOFA-HDV-Rz1498 and shRNA1498 conditions, suggesting that part of the observed changes may be target site specific.

5

Example 8: SOFA-HDV-Rz1498 and shRNA1498 inhibit HIV-1 replication in a T lymphocyte cell line

To evaluate the potential for SOFA-HDV-Rz1498 and shRNA1498 to inhibit HIV-1 replication, Jurkat cell lines were transfected with the same constructs used for their delivery to HEK293T cells, and stably transfected cells were selected in the presence of Zeocin. All cell lines had a similar distribution of GFP expression from the integrated plasmids and proliferated at similar levels (**FIG. 13A**). Following infection with HIV-1 pNL4-3, both SOFA-HDV-Rz1498 and shRNA1498 expressing cells were able to suppress viral replication compared to cells expressing SOFA-HDV-RzHBV and shRNAs (**FIG. 8A**). SOFA-HDV-Rz1498A76 expressing cells displayed a moderate inhibition, likely representing an antisense activity of the inactive Rz. SOFA-HDV-Rz1498 and shRNA1498 expressing cells maintained low levels of viral production past the peak of infection in control cell lines (**FIG. 8B**), providing evidence that the active molecules can restrict viral replication over several weeks in culture.

20

Example 9: siRNAs targeting the identified target site in the gag ORF also inhibit HIV-1 production in a dose dependent manner

To determine whether or not the identified target site in the gag ORF could be used to design active siRNAs, siRNAs based on a Dicer substrate design (IDT) were designed with different start sites around the target site.

25

Table 6: siRNAs targeting HIV NL4-3 at starting position 1497 to 1499 with 25 and 27 bp duplexes.

Target claimed:	ATAGCAGGA ACT ACTAGTACCCTTCAGGAA (NL4-3: 1495-1524)	SEQ ID NOs
1497-25	5'-AGCAGGAACUACUAGUACCCUUCdAdG-3'	75
	3'-UAUCGUCCUUGAUGAUGAUGGGAAGUC-5'	76
1498-25	5'-GCAGGAACUACUAGUACCCUUCAdGdG-3'	77
	3'-AUCGUCCUUGAUGAUGAUGGGAAGUCC-5'	78
1498-27	5'-GCAGGAACUACUAGUACCCUUCAGGAA-3'	79
	3'-dAdTCGUCCUUGAUGAUGAUGGGAAGUCCUU-5'	80
1499-25	5'-CAGGAACUACUAGUACCCUUCAGdGdA-3'	81
	3'-UCGUCCUUGAUGAUGAUGGGAAGUCCU-5'	82
Positive control		
5983-25	5'-GCGGAGACAGCGACGAAGAGCUCdAdT-3'	83
	3'-UUCGCCUCUGUCGCGUGCUUCUCGAGUA-5'	84
5983-27	5'-GCGGAGACAGCGACGAAGAGCUCAUCA	85
(published)^[1]	3'-dTdTCGCCUCUGUCGCGUGCUUCUCGAGUAGU-5'	86

^[1] Zhou, J, Neff, CP, Liu, X, Zhang, J, Li, H, Smith, DD, *et al.* (2011). *Mol Ther* **19**: 2228-2238

si1498 (si1498-25) and si1499 (si1499-25) were able to inhibit HIV-1 production (FIG. 14A). Similarly, the 27 bp version of si1498 also inhibits HIV-1 production (FIG. 14B).

5

Example 10: Other shRNAs directed against the identified target site in the gag ORF

The shRNA depicted in Tables 7 and 8 were prepared and tested.

Table 7: 20 bp shRNA sequences targeting HIV-1 NL4-3 (Genbank M19921) from starting positions 1495 to 1501 (sh1498 disclosed above shown in bold).

Target claimed:		ATAGCAGGA ACTACTAGTACCCTTCA	
		NL4-3: 1495-1520	
shRNA	Vector	Stem	Loop
1495-20	<u>ACCTC</u> GTTT	5'-ATAGCAGGA ACTACTAGTAC -3' (SEQ ID NO: 87) 3'-TTG TATCGTCCTTGATGATCATG -5' (SEQ ID NO: 88)	GCTCGAGG
1496-20	<u>ACCTC</u> GTTT	5'-TAGCAGGA ACTACTAGTACC -3' (SEQ ID NO: 89) 3'-TTATCGTCCTTGATGATCATGG-5' (SEQ ID NO: 90)	GCTCGAGG
1497-20	<u>ACCTC</u> GTTT	5'-AGCAGGA ACTACTAGTACCC -3' (SEQ ID NO: 91) 3'-TTATCGTCCTTGATGATCATGGG-5' (SEQ ID NO: 92)	ACTCGAGA
1498-20	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCT -3' (SEQ ID NO: 93) 3'-TTCGTCCTTGATGATCATGGGA-5' (SEQ ID NO: 94)	ACTCGAGA
1499-20	<u>ACCTC</u> GTTT	5'-CAGGA ACTACTAGTACCCTT -3' (SEQ ID NO: 95) 3'-TTGTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 96)	GCTCGAGG
1500-20	<u>ACCTC</u> GTTT	5'-AGGA ACTACTAGTACCCTTC -3' (SEQ ID NO: 97) 3'-TTGTCCTTGATGATCATGGGAAG-5' (SEQ ID NO: 98)	TCTCGAGT
1501-20	<u>ACCTC</u> GTTT	5'-GGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	CCTCGAGC

10

Table 8: shRNAs targeting HIV NL4-3 at starting position 1498 with 17 to 29 bp hairpins (20 bp hairpin sh1498 disclosed above shown in bold)

Target claimed:		TAGCAGGA ACTACTAGTACCCTTCAGGAACA		NL4-3: 1496-1526	
shRNA	Vector	Stem		Loop	
1498-17	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTAC -3' (SEQ ID NO: 101) 3'-TTCGTCCTTGATGATCATG-5' (SEQ ID NO: 102)	G	CTCGAG	
1498-18	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACC -3' (SEQ ID NO: 103) 3'-TTCGTCCTTGATGATCATGG-5' (SEQ ID NO: 104)	G	CTCGAG	
1498-19	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCC -3' (SEQ ID NO: 105) 3'-TTCGTCCTTGATGATCATGGG-5' (SEQ ID NO: 106)	A	CTCGAG	
1498-20	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCT -3' (SEQ ID NO: 93) 3'-TTCGTCCTTGATGATCATGGGA-5' (SEQ ID NO: 94)	A	CTCGAG	
1498-21	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTT -3' (SEQ ID NO: 95) 3'-TTGTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 96)	A	CTCGAG	
1498-22	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTC -3' (SEQ ID NO: 97) 3'-TTGTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 96)	A	CTCGAG	
1498-23	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	A	CTCGAG	
1498-24	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	A	CTCGAG	
1498-25	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	A	CTCGAG	
1498-26	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	A	CTCGAG	
1498-27	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	A	CTCGAG	
1498-28	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	A	CTCGAG	
1498-29	<u>ACCTC</u> GTTT	5'-GCAGGA ACTACTAGTACCCTTCA -3' (SEQ ID NO: 99) 3'-TTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 100)	A	CTCGAG	

21		107)		
	GTTT	3'-TTCGTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 107)	G	
1498-23	<u>ACCTC</u>	5'-GCAGGAACTACTAGTACCCTTCA-3' (SEQ ID NO: 108)	C	CTCGAG
	GTTT	3'-TTCGTCCTTGATGATCATGGGAAAGT-5' (SEQ ID NO: 109)	C	
1498-25	<u>ACCTC</u>	5'-GCAGGAACTACTAGTACCCTTCAGG-3' (SEQ ID NO: 110)	T	CTCGAG
	GTTT	3'-TTCGTCCTTGATGATCATGGGAAAGTCC-5' (SEQ ID NO: 111)	T	
1498-27	<u>ACCTC</u>	5'-GCAGGAACTACTAGTACCCTTCAGGAA-3' (SEQ ID NO: 112)	G	CTCGAG
	GTTT	3'-TTCGTCCTTGATGATCATGGGAAAGTCCTT-5' (SEQ ID NO: 113)	G	
1498-29	<u>ACCTC</u>	5'-GCAGGAACTACTAGTACCCTTCAGGAACA-3' (SEQ ID NO: 114)	T	CTCGAG
	GTTT	3'-TTCGTCCTTGATGATCATGGGAAAGTCCTTGT-5' (SEQ ID NO: 115)	T	
		(SEQ ID NO: 116)		

The shRNA inserts were generated by annealing complementary sense (S) and antisense (AS) oligonucleotides corresponding to the above-noted sequences (vector + stem + loop) using the methods described in Example 1.

5 FIG. 16A shows that sh1495, sh1496, sh1497, sh1498 and sh1501 exhibit HIV-1 inhibitory activity at 10 and 100 ng, and sh1499 exhibits HIV-1 inhibitory activity at 100 ng, confirming that shRNAs directed against different starting positions within the targeted site may be used to inhibit HIV-1. Also, the results depicted in FIG. 16B demonstrate that shRNAs targeting HIV NL4-3 at starting position 1498 with 19 to 29 bp hairpins all exhibit HIV-1 inhibitory activity at 100 ng, with some shRNA being also active at 10 ng (sh1498-20, sh1498-21 and sh1498-23). This data provide evidence that shRNAs directed against the targeted site and having hairpins of different lengths are able to block HIV-1 viral production.

15 Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to". The singular forms "a", "an" and "the" include corresponding plural references unless the context clearly dictates otherwise.

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WHAT IS CLAIMED IS:

1. An antisense nucleic acid molecule directed against a sequence corresponding to about nucleotide 1495 to about nucleotide 1526 of HIV-1 clone pNL4-3 (GenBank accession No. M19921.2), or the complement thereof.
- 5 2. The antisense nucleic acid molecule of claim 1, which is directed against a sequence corresponding to about nucleotide 1497 to about nucleotide 1521 of HIV-1 clone pNL4-3 (GenBank accession No. M19921.2), or the complement thereof.
3. The antisense nucleic acid molecule of claim 1, which is a ribozyme.
4. The antisense nucleic acid molecule of claim 3, wherein said ribozyme is a Specific
10 On/Off Adaptor (SOFA) Hepatitis Delta Virus (HDV) ribozyme.
5. The antisense nucleic acid molecule of claim 4, wherein said ribozyme comprises: a recognition domain (RD) comprising the sequence TTCCTGT, a biosensor (Bs) domain comprising the sequence AAGGGTACTA, and a blocker (BI) domain comprising the sequence GGAA.
- 15 6. The antisense nucleic acid molecule of claim 4 or 5, wherein said ribozyme comprises the sequence of SEQ ID NO:142.
7. The antisense nucleic acid molecule of claim 1 or 2, which is a short hairpin RNA (shRNA).
8. The antisense nucleic acid molecule of claim 7, wherein the shRNA is encoded by a
20 nucleic acid comprising one of the following stem sequences (i) to (xii):
 - (i) 5'-GCAGGAACTACTAGTACCCT-3' (SEQ ID NO: 93)
3'-CGTCCTTGATGATCATGGGA-5' (SEQ ID NO: 118);
 - (ii) 5'-ATAGCAGGAACTACTAGTAC-3' (SEQ ID NO: 87)
3'-TATCGTCCTTGATGATCATG-5' (SEQ ID NO: 119);
 - 25 (iii) 5'-TAGCAGGAACTACTAGTACC-3' (SEQ ID NO: 89)
3'-ATCGTCCTTGATGATCATGG-5' (SEQ ID NO: 120);
 - (iv) 5'-AGCAGGAACTACTAGTACCC-3' (SEQ ID NO: 91)
3'-TCGTCCTTGATGATCATGGG-5' (SEQ ID NO: 121);
 - (v) 5'-CAGGAACTACTAGTACCCTT-3' (SEQ ID NO: 95)
30 3'-GTCCTTGATGATCATGGGAA-5' (SEQ ID NO: 122);
 - (vi) 5'-GGAAGTACTAGTACCCTTCA-3' (SEQ ID NO: 99)

- 3'-CCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 123);
 (vii) 5'-GCAGGAACTACTAGTACCC-3' (SEQ ID NO: 105)
 3'-CGTCCTTGATGATCATGGG-5' (SEQ ID NO: 124);
 (viii) 5'-GCAGGAACTACTAGTACCCTT-3' (SEQ ID NO: 107)
 5 3'-CGTCCTTGATGATCATGGGA-5' (SEQ ID NO: 125);
 (ix) 5'-GCAGGAACTACTAGTACCCTTCA-3' (SEQ ID NO: 109)
 3'-CGTCCTTGATGATCATGGGAAGT-5' (SEQ ID NO: 126);
 (x) 5'-GCAGGAACTACTAGTACCCTTCAGG-3' (SEQ ID NO: 111)
 3'-CGTCCTTGATGATCATGGGAAGTCC-5' (SEQ ID NO: 127);
 10 (xi) 5'-GCAGGAACTACTAGTACCCTTCAGGAA-3' (SEQ ID NO: 113)
 3'-CGTCCTTGATGATCATGGGAAGTCCTT-5' (SEQ ID NO: 128); or
 (xii) 5'-GCAGGAACTACTAGTACCCTTCAGGAACA-3' (SEQ ID NO: 115)
 3'-CGTCCTTGATGATCATGGGAAGTCCTTGT-5' (SEQ ID NO: 129);
 or an analog thereof.

15 9. The antisense nucleic acid molecule of claim 8, wherein the shRNA comprises a 3'-overhang.

10. The antisense nucleic acid molecule of claim 9, wherein the shRNA is encoded by a nucleic acid comprising one of the following sequences (i) to (xii):

- (i) GCAGGAACTACTAGTACCCTACTCGAGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID
 20 NO: 130);
 (ii) ATAGCAGGAACTACTAGTACGCTCGAGGGTACTAGTAGTTCCTGCTATTT (SEQ ID
 NO: 131);
 (iii) TAGCAGGAACTACTAGTACCGCTCGAGGGGTACTAGTAGTTCCTGCTATT (SEQ ID
 NO: 132);
 25 (iv) AGCAGGAACTACTAGTACCCACTCGAGAGGGTACTAGTAGTTCCTGCTTT (SEQ ID
 NO: 133);
 (v) CAGGAACTACTAGTACCCTTGCTCGAGGAAGGGTACTAGTAGTTCCTGTT (SEQ ID
 NO: 134);
 (vi) GGA ACTACTAGTACCCTTCACCTCGAGCTGAAGGGTACTAGTAGTTCCTT (SEQ ID
 30 NO: 135);
 (vii) GCAGGAACTACTAGTACCCACTCGAGAGGGTACTAGTAGTTCCTGCTT (SEQ ID
 NO: 136);
 (viii) GCAGGAACTACTAGTACCCTTGCTCGAGGAAGGGTACTAGTAGTTCCTGCTT (SEQ
 ID NO: 137);

(ix) GCAGGAACTACTAGTACCCTTCACCTCGAGCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 138);

(x) GCAGGAACTACTAGTACCCTTCAGGTCTCGAGTCCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 139);

5 (xi) GCAGGAACTACTAGTACCCTTCAGGAAGCTCGAGGTTCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 140); or

(xii) GCAGGAACTACTAGTACCCTTCAGGAACATCTCGAGTTGTTCTGAAGGGTACTAGTAGTTCCTGCTT (SEQ ID NO: 141);

or an analog thereof.

10 11. The antisense nucleic acid molecule of claim 1 or 2, which is a small interfering RNA (siRNA).

12. The antisense nucleic acid molecule of claim 11, wherein the siRNA comprises one of the following pair of sequences (i) to (iv):

(i) si1497 sense: AGCAGGAACUACUAGUACCCUUCdAdG (SEQ ID NO: 75)

15 si1497 antisense: UAUCGUCCUUGAUGAUGAUGGGAAGUC (SEQ ID NO: 76);

(ii) si1498 sense: GCAGGAACUACUAGUACCCUUCAdGdG (SEQ ID NO: 77)

si1498 antisense: AUCGUCCUUGAUGAUGAUGGGAAGUCC (SEQ ID NO: 78);

(iii) si1499 sense: CAGGAACUACUAGUACCCUUCAGdGdA (SEQ ID NO: 81)

si1499 antisense: UCGUCCUUGAUGAUGAUGGGAAGUCCU (SEQ ID NO: 82); or

20 (iv) si1498-29 sense: GCAGGAACUACUAGUACCCUUCAGGAA (SEQ ID NO: 79)

si1498-29 antisense: dAdTCGUCCUUGAUGAUGAUGGGAAGUCCUU (SEQ ID NO: 80);

or an analog thereof.

25 13. A vector comprising a nucleic acid encoding the antisense nucleic acid molecule of any one of claims 1 to 12.

14. A cell comprising the antisense nucleic acid molecule of any one of claims 1 to 11 and/or the vector of claim 13.

15. A composition comprising (a) the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13 and/or the cell of claim 14; and (b) an excipient.

16. A method for inhibiting HIV-1 replication in a cell, the method comprising contacting said cell with an effective amount of the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15.

17. A method for treating HIV-1 infection in a subject, the method comprising administering to said subject an effective amount of the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15.

18. Use of the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15, for inhibiting HIV-1 replication in a cell.

19. Use of the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15, for the manufacture of a medicament for inhibiting HIV-1 replication in a cell.

20. Use of the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15, for treating HIV-1 infection in a subject.

21. Use of the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15, for the manufacture of a medicament for treating HIV-1 infection in a subject.

22. The antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15, for inhibiting HIV-1 replication in a cell and/or treating HIV-1 infection in a subject.

23. The antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15, for the manufacture of a medicament for inhibiting HIV-1 replication in a cell and/or treating HIV-1 infection in a subject.

24. Use of the antisense nucleic acid molecule of any one of claims 1 to 12, the vector of claim 13, the cell of claim 14 and/or the composition of claim 15, as a medicament.

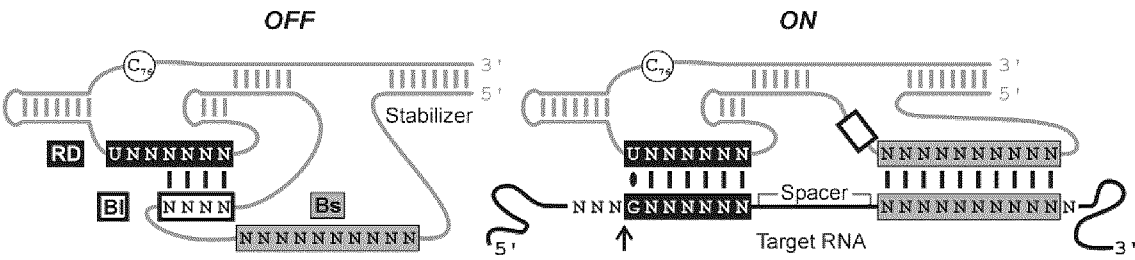


FIG. 1A

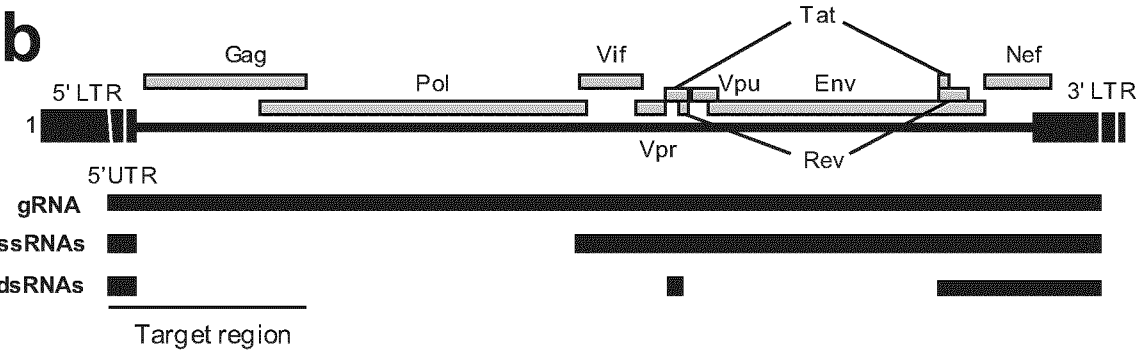


FIG. 1B

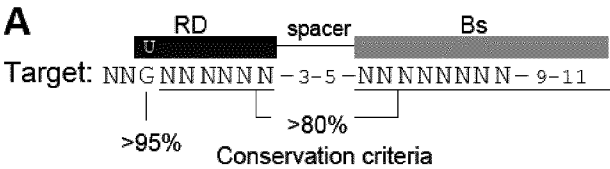


FIG. 2A

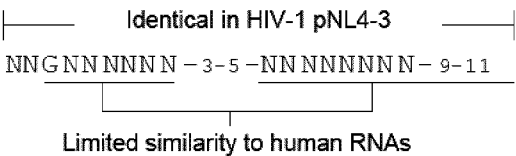


FIG. 2B

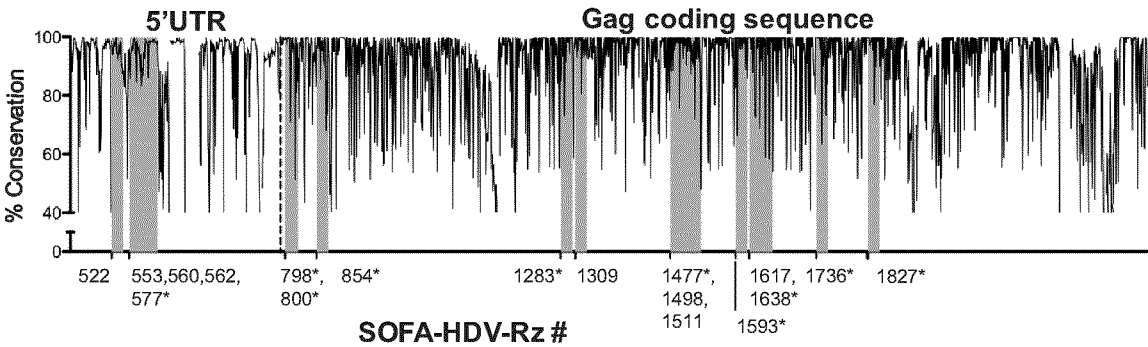


FIG. 2C

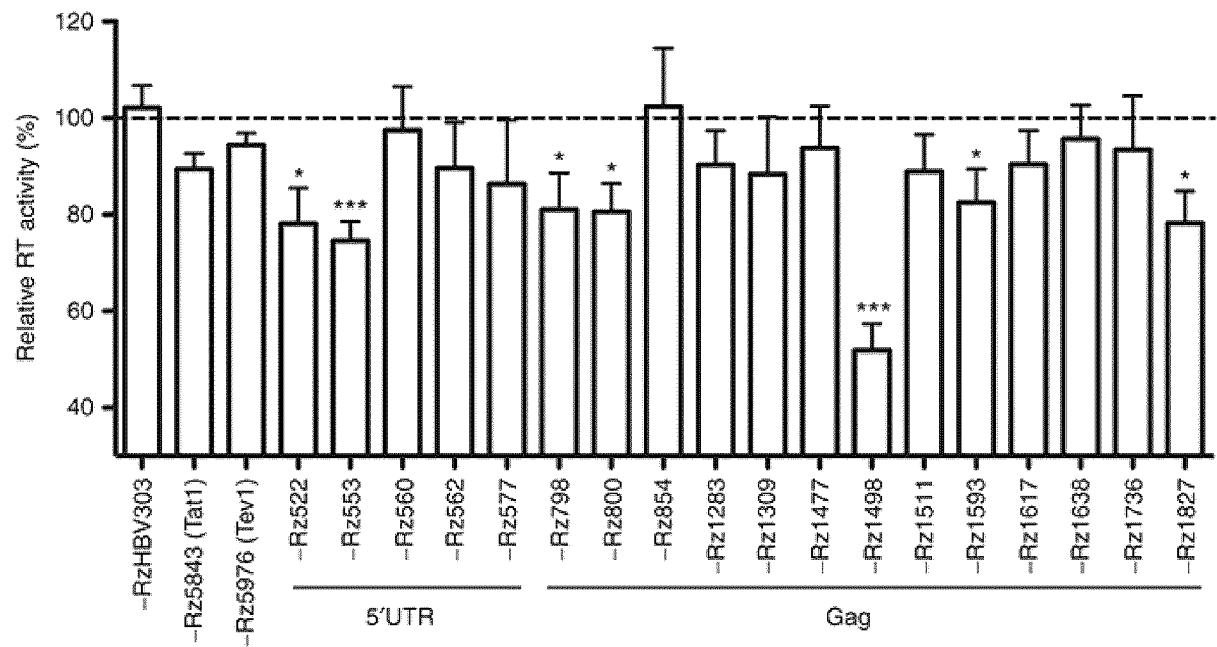


FIG. 3

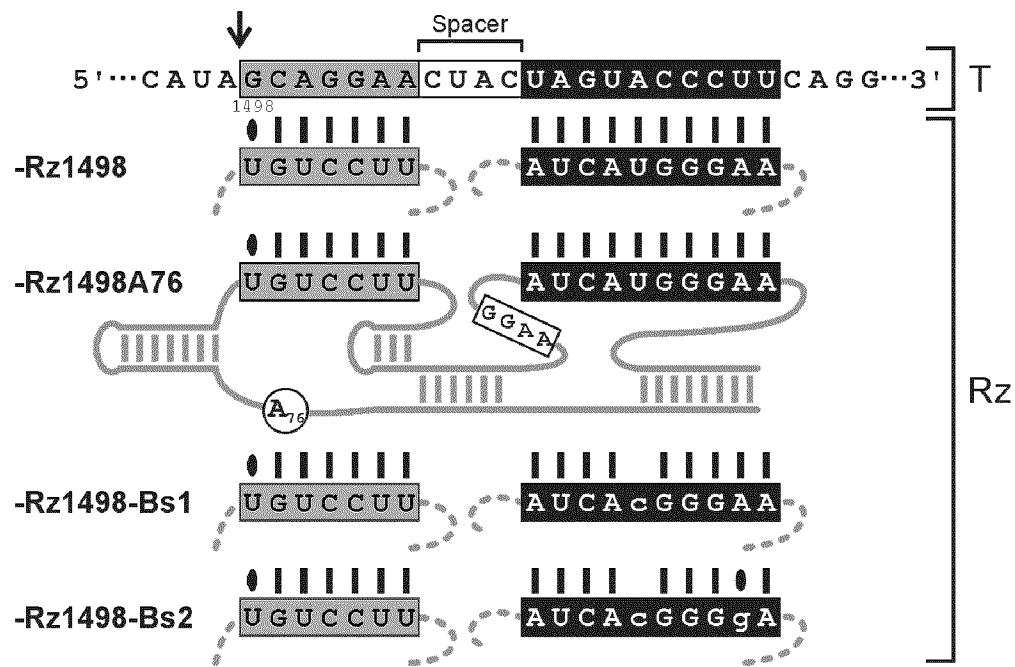


FIG. 4A

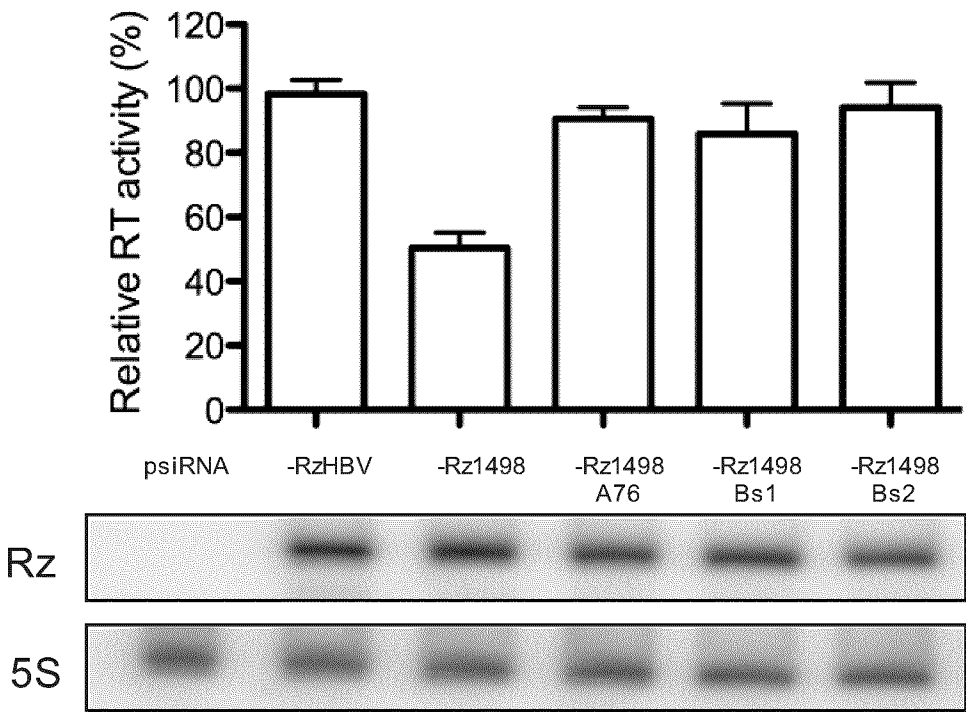
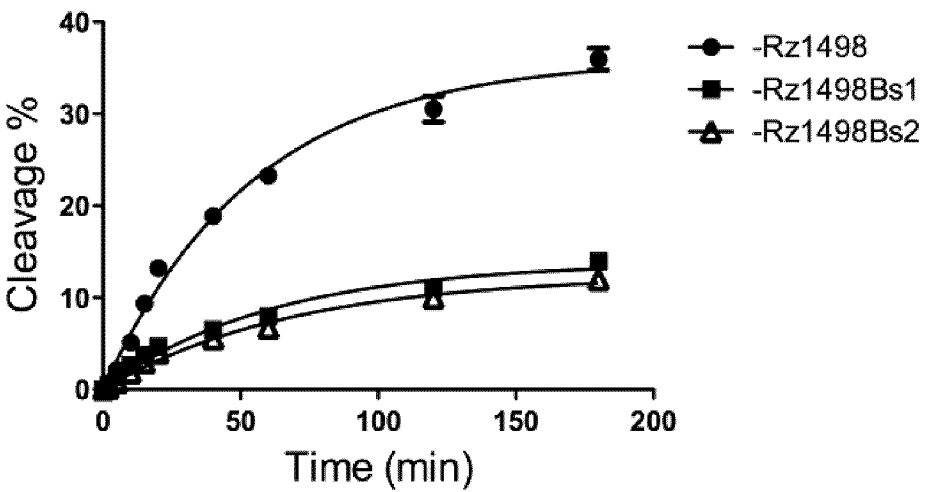


FIG. 4B



	-Rz 1498	-Rz 1498Bs 1	-Rz 1498Bs 2
Fmax +/- SE	36.0 +/- 1.0	13.8 +/- 0.6	12.5 +/- 0.5
Kobs +/- SE	0.018 +/- 0.001	0.016 +/- 0.002	0.015 +/- 0.001

FIG. 4C

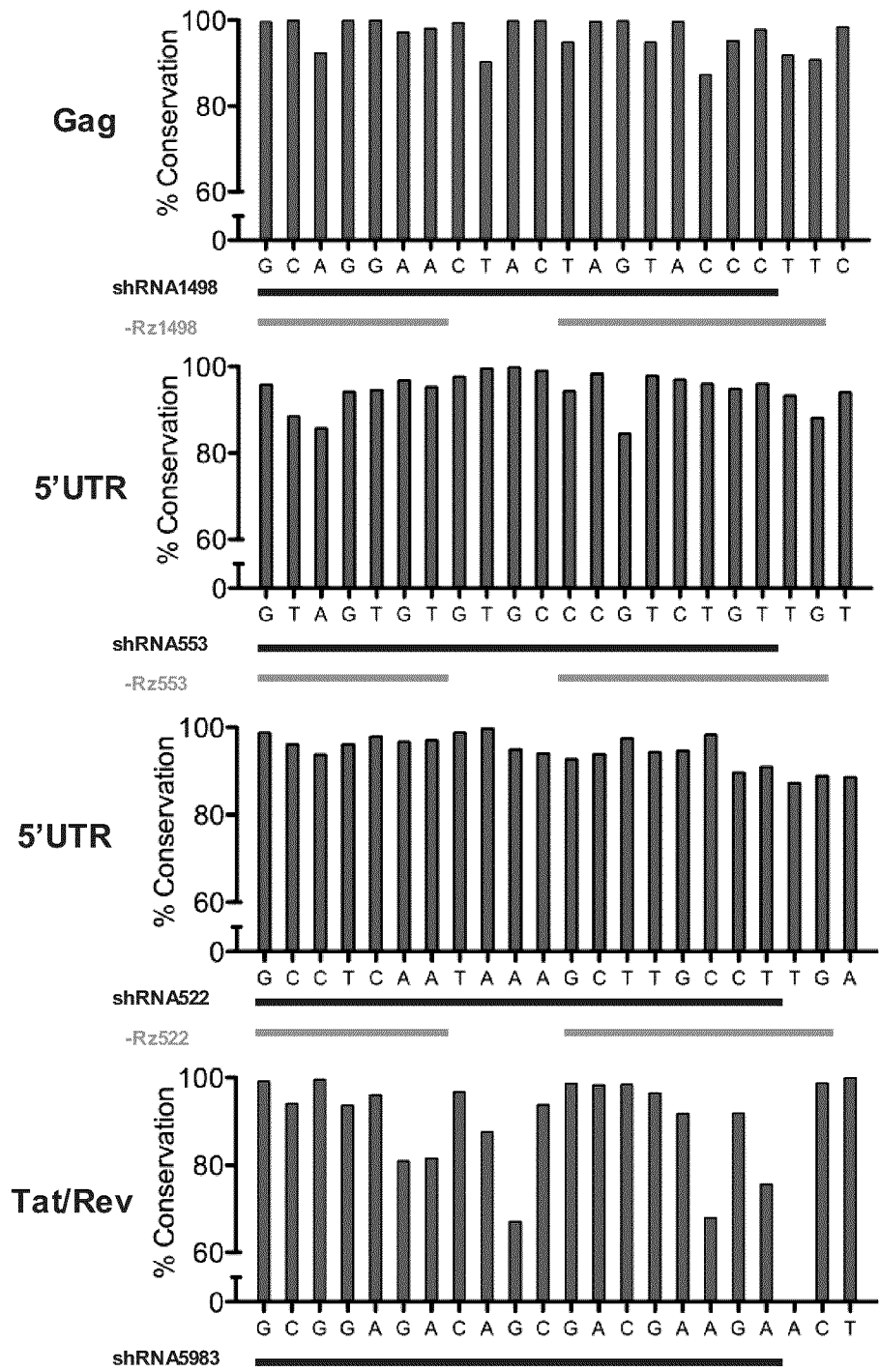


FIG. 5A

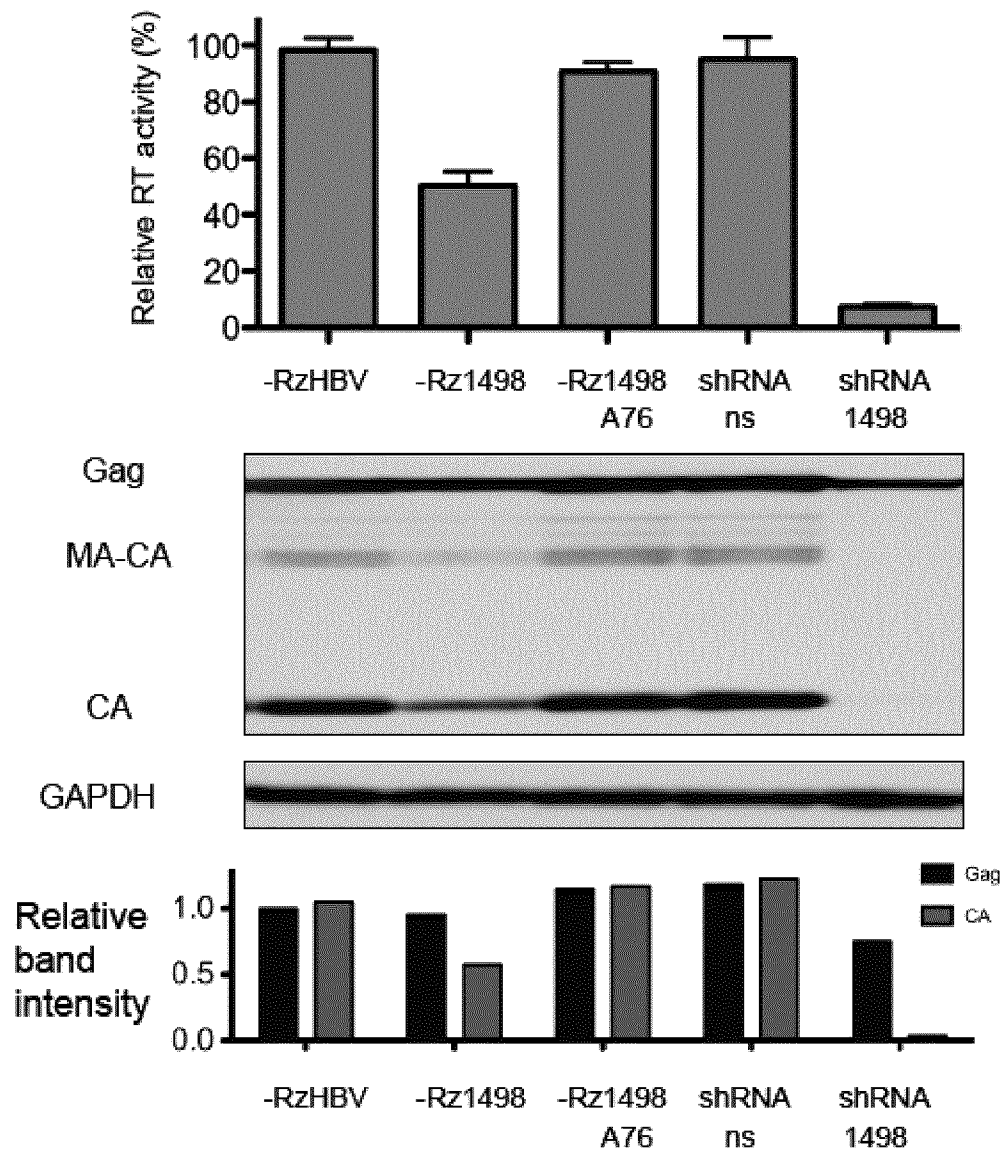


FIG. 5B

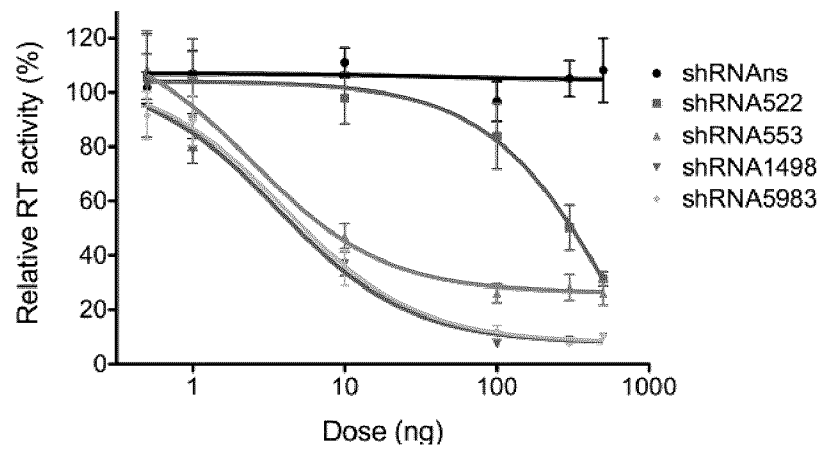


FIG. 5C

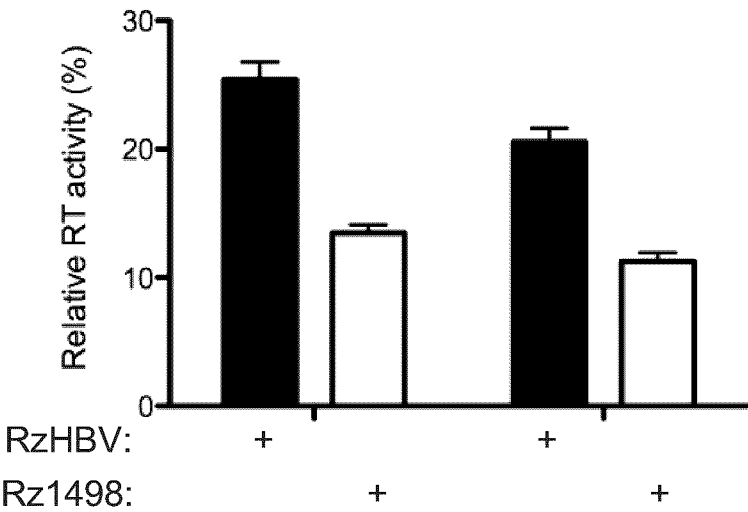


FIG. 5D

HIV-1 strain	Rz/shRNA 1498 target site	HIV-1 subtype
NL4-3: 1470 - GATGAGAGAACCAAGGGGAAGTGACATAGCAGGAAC TACTAGTACCCTTCAGGAAC - 1525	<u>GATGAGAGAACCAAGGGGAAGTGACATAGCAGGAAC TACTAGTACCCTTCAGGAAC</u>	Subtype B
Mal: 1048 - GATGAGAGAACCAAGAGGAAGTGACATAGCAGGAAC TACTAGTACCCTTCAAGAAC - 1103		
AD8: 1470 - GATGAGAGAACCAAGGGGAAGTGACATAGCAGGAAC TACTAGTACCCTTCAAGAAC - 1525		
Indie-C1: 1461 - AATGAGAGAACCAAGGGGAAGTGACATAGCAGGAAC TACTAGTAGCCTTCAGGAAC - 1516	<u>AATGAGAGAACCAAGGGGAAGTGACATAGCAGGAAC TACTAGTAGCCTTCAGGAAC</u>	Subtype C
MJ4: 1465 - AATGAGAGACCCAAGGGGAAGTGACATAGCAGGAAC TACTAGTACCCTTCAGGAAC - 1519		
p94UG114: 819 - ATTGAGAGAACCAAGGGGAAGTGATATAGCAGGAAC TACTAGTAACCTTCAGGAAC - 874	<u>ATTGAGAGAACCAAGGGGAAGTGATATAGCAGGAAC TACTAGTAACCTTCAGGAAC</u>	Subtype D
97GH-AG1: 1472 - GATGAGGGAACCAAGGGGAAGTGACATAGCAGGAAC TACTAGTACCCTTCAAGAAC - 1527	<u>GATGAGGGAACCAAGGGGAAGTGACATAGCAGGAAC TACTAGTACCCTTCAAGAAC</u>	CRF AG

FIG. 6A

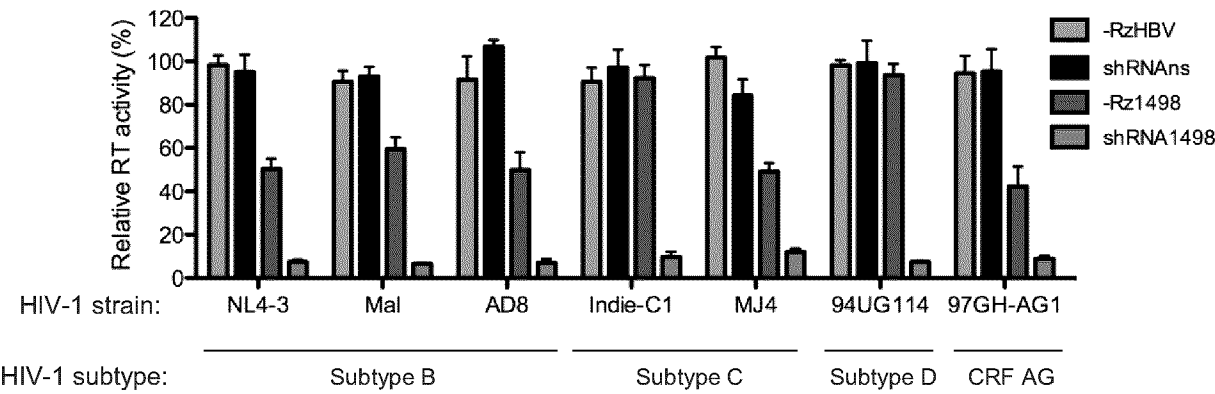


FIG. 6B

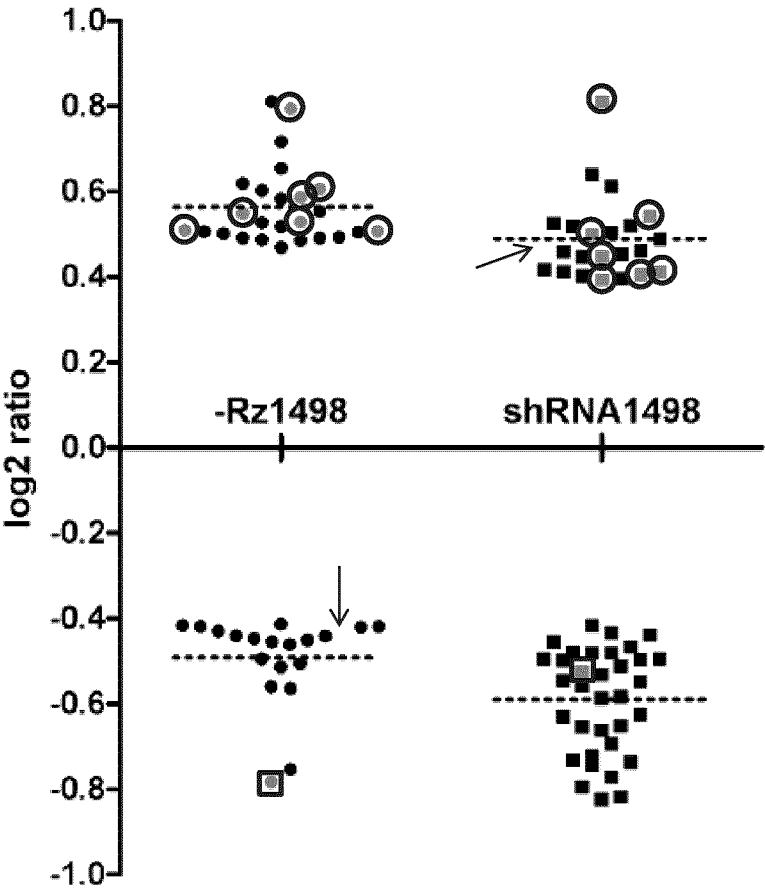


FIG. 7

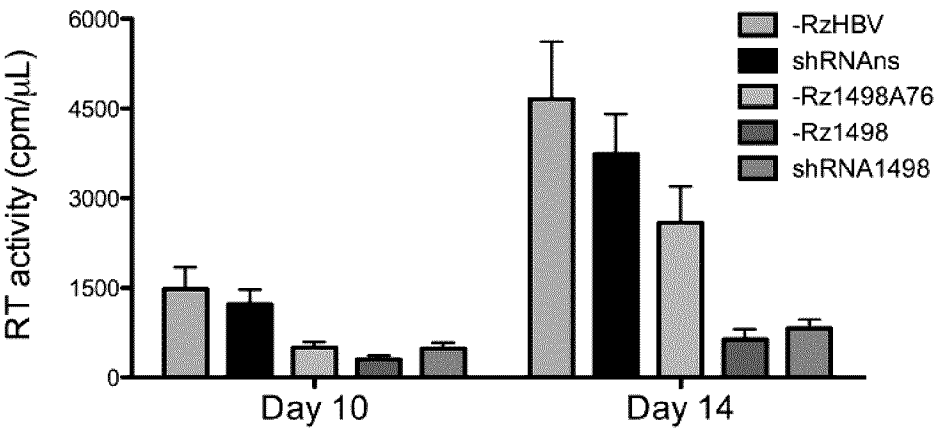


FIG. 8A

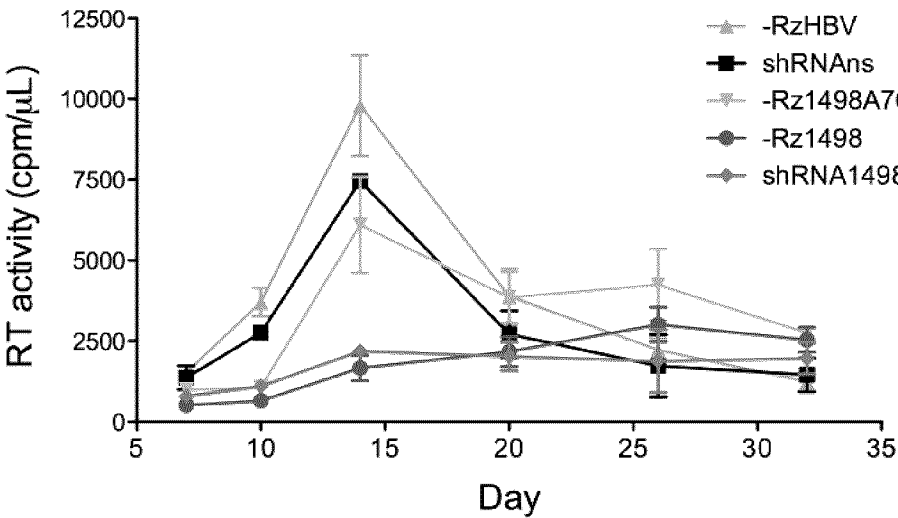


FIG. 8B

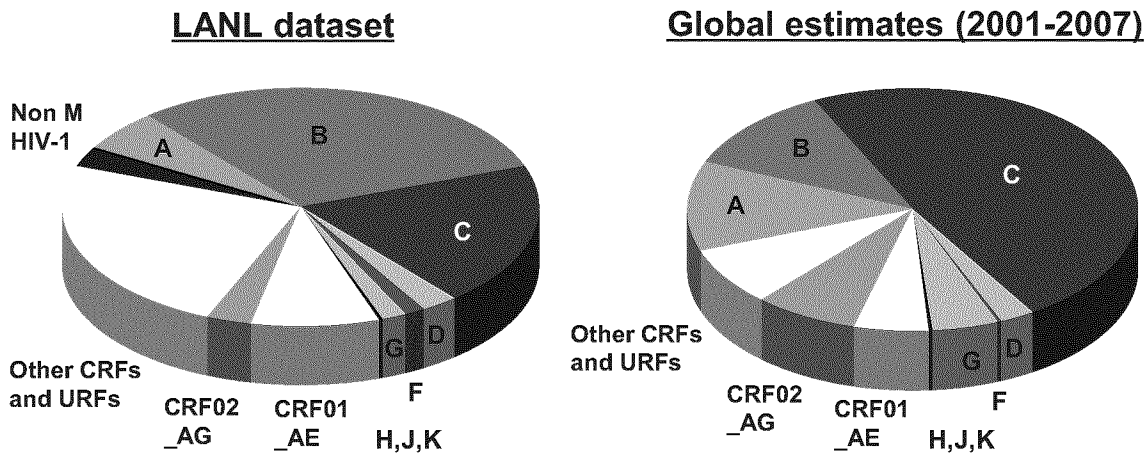


FIG. 9

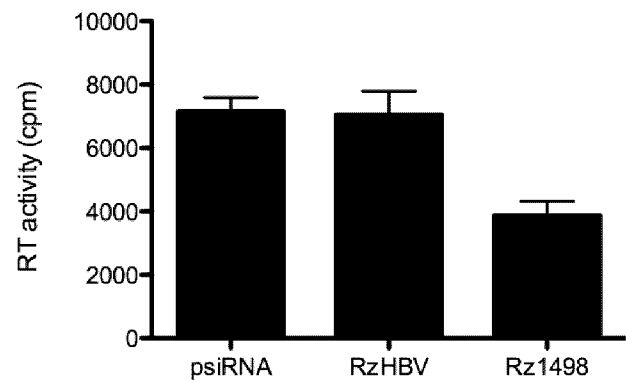


FIG. 10A

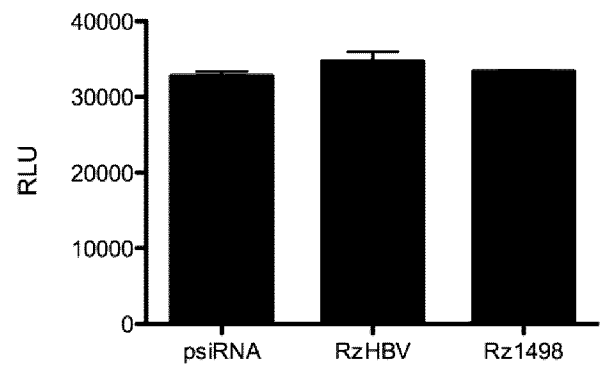


FIG. 10B

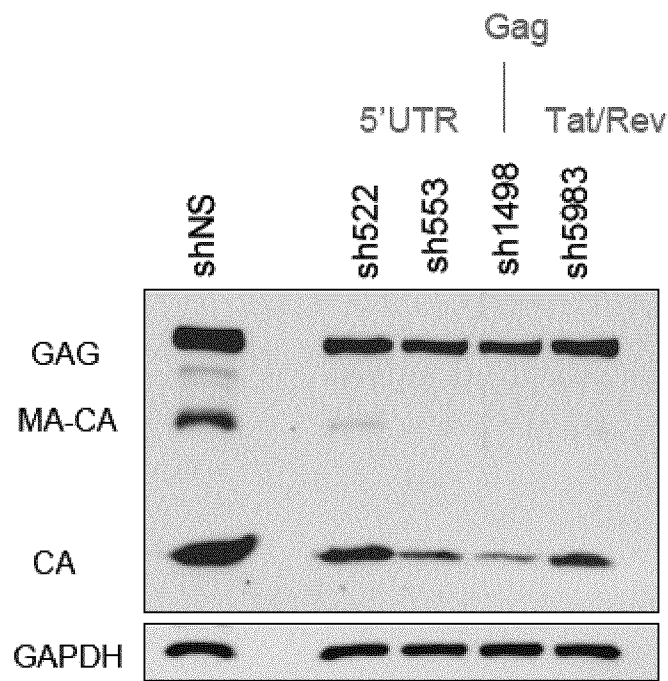


FIG. 11A

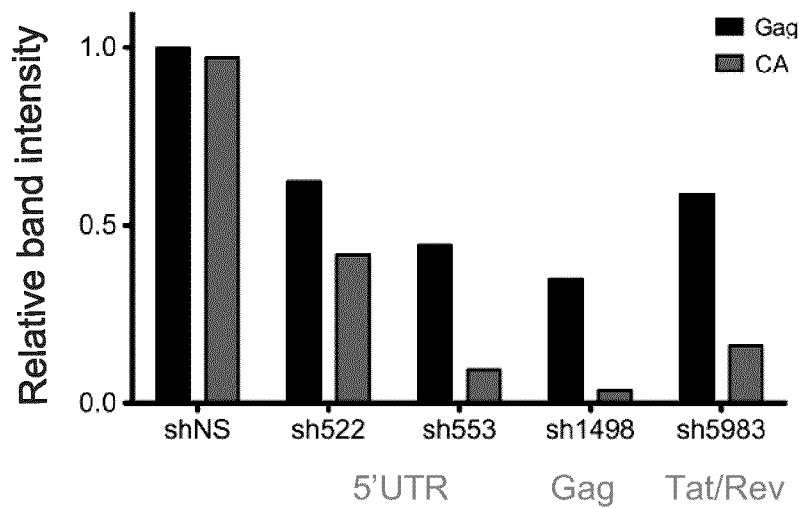
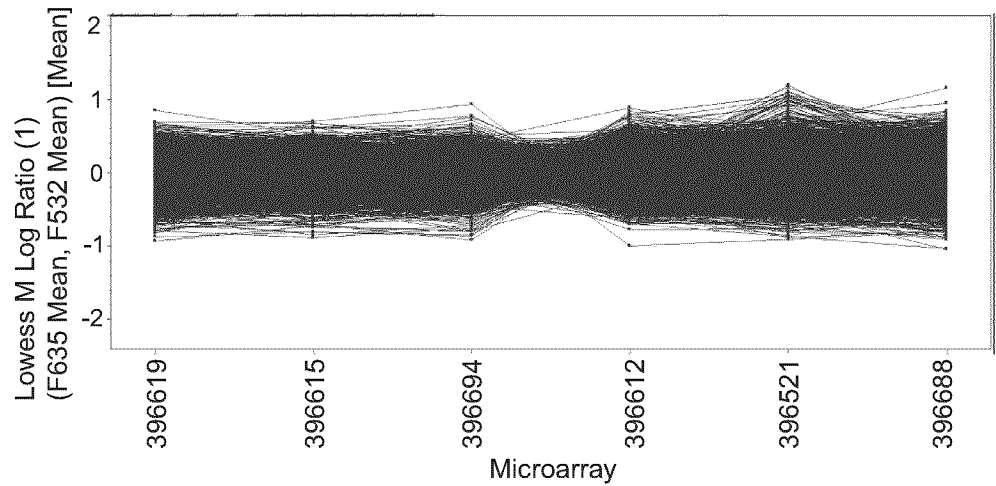


FIG. 11B

SOFA-HDV-Rz1498 : psiRNA



shRNA1498 : psiRNA

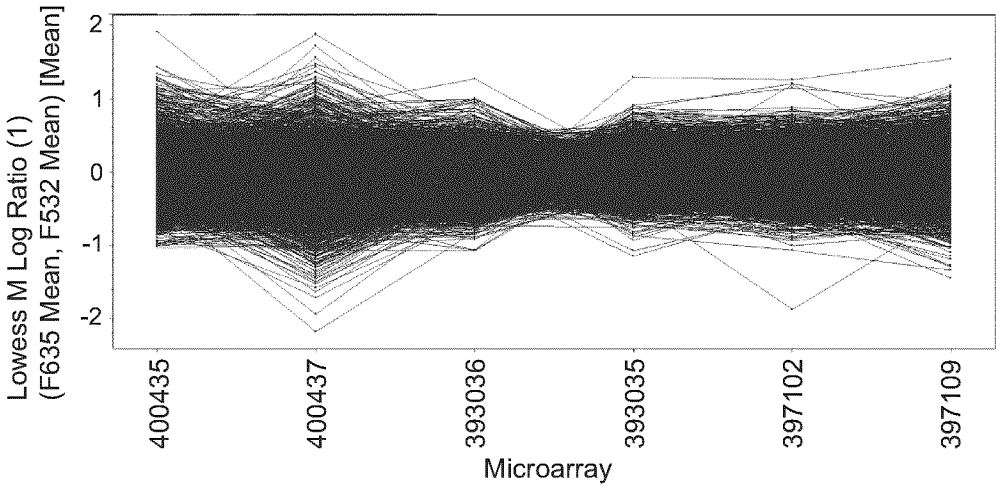


FIG. 12

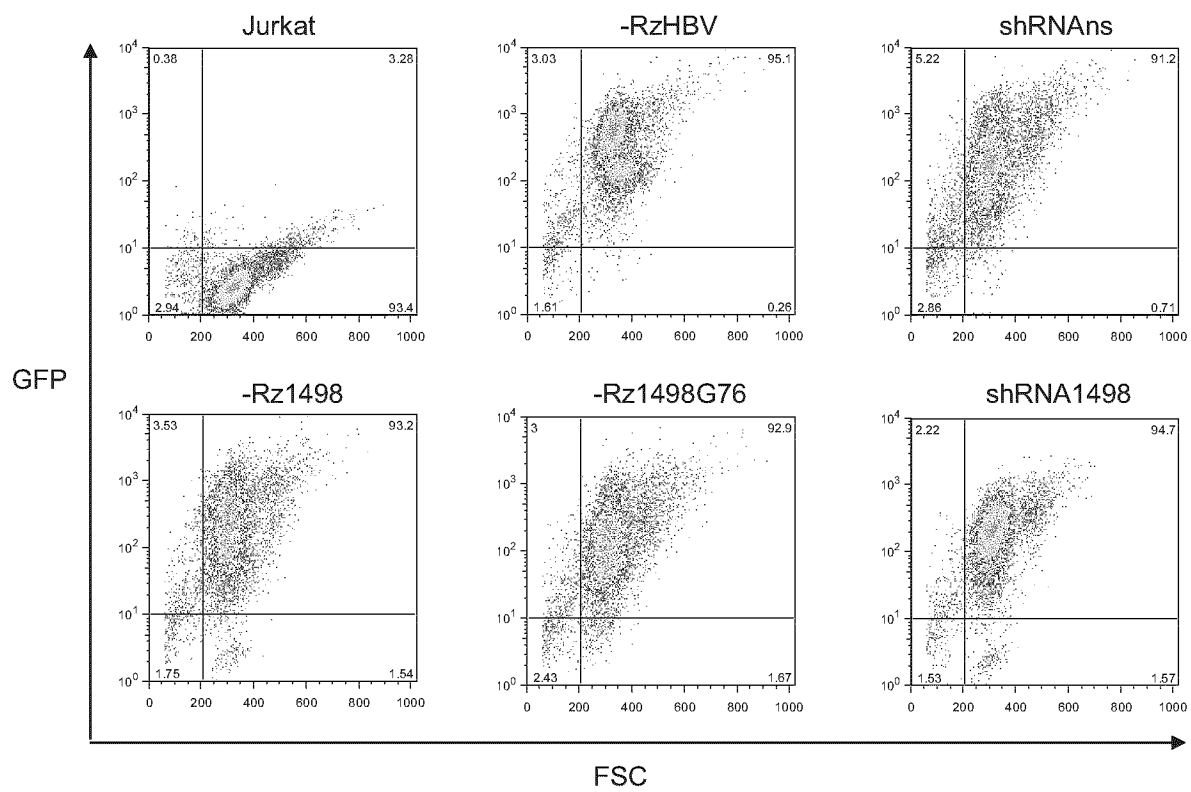


FIG. 13A

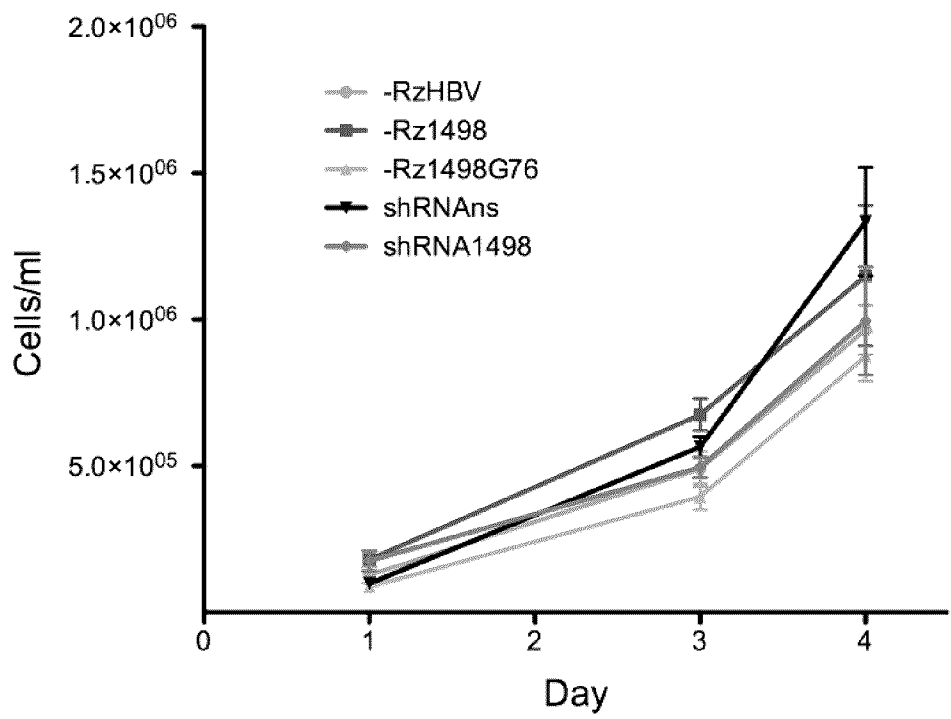


FIG. 13B

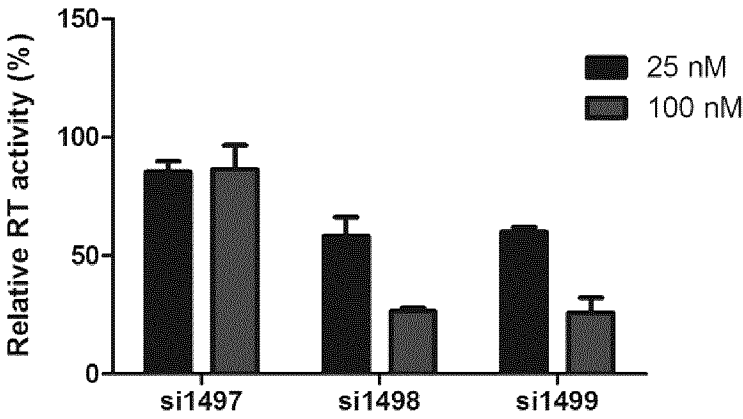


FIG. 14A

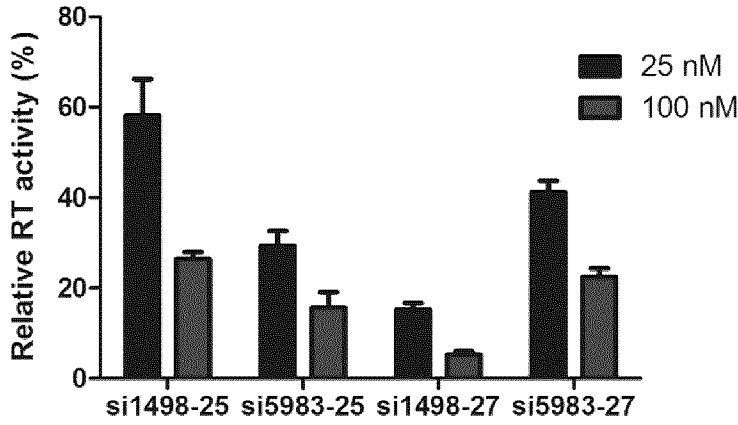


FIG. 14B

ACRI Human2 slides: 396619, 396612, 396615, 396521, 396694, 396688																			
Slides are a triplicate dye-swap (Green/Red) of Rz1498 vs psiRNA																			
Up-regulated in Rz1498 vs psiRNA																			
Total 25																			
Log2 ratio																			
Log2 ratio adjusted (±)																			
id	396619	396615	396694	396612	396521	396688	Gene_Symbol	Target_NM	396619	396615	396694	396612	396521	396688	Mean (g)	SD	Mean (r)	SD	
17393	-0.931	-0.727	-0.801	0.662	0.920	0.721	ATXN2L	NM_148416	0.931	0.727	0.801	0.662	0.920	0.721	0.794	0.111	0.820	0.103	
14991	-0.873	-0.807	-0.843	0.692	1.023	0.620	MID1P1	NM_021242	0.873	0.807	0.843	0.692	1.023	0.620	0.810	0.142	0.841	0.033	
14906	-0.819	-0.439	-0.556	0.398	0.584	0.150	ATP11C	NM_173694	0.819	0.439	0.556	0.398	0.584	0.150	0.491	0.223	0.605	0.195	
11719	-0.789	-0.885	-0.746	0.307	0.572	0.216	EI24	JM_00100727	0.789	0.885	0.746	0.307	0.572	0.216	0.586	0.272	0.807	0.071	
11504	-0.747	-0.708	-0.919	0.505	0.411	0.634	ACTB	NM_001101	0.747	0.708	0.919	0.505	0.411	0.634	0.654	0.181	0.791	0.112	
11636	-0.713	-0.390	-0.539	0.733	0.443	0.468	IRS4	NM_003604	0.713	0.390	0.539	0.733	0.443	0.468	0.548	0.144	0.547	0.162	
15757	-0.705	-0.814	-0.864	0.551	0.450	0.330	CS	NM_004077	0.705	0.814	0.864	0.551	0.450	0.330	0.619	0.211	0.794	0.081	
13340	-0.685	-0.705	-0.553	0.251	0.625	0.287	OAF	NM_178507	0.685	0.705	0.553	0.251	0.625	0.287	0.518	0.200	0.648	0.083	
13767	-0.680	-0.401	-0.451	0.384	0.636	0.619	PABPC4	NM_003819	0.680	0.401	0.451	0.384	0.636	0.619	0.529	0.131	0.511	0.149	
12023	-0.657	-0.448	-0.307	0.638	0.483	0.498	none	NM_138471	0.657	0.448	0.307	0.638	0.483	0.498	0.505	0.129	0.471	0.176	
14084	-0.610	-0.444	-0.558	0.313	0.468	0.423	none	NM_006857	0.610	0.444	0.558	0.313	0.468	0.423	0.469	0.105	0.537	0.085	
3922	-0.609	-0.348	-0.705	0.841	0.635	1.156	PPP2R1A	NM_014225	0.609	0.348	0.705	0.841	0.635	1.156	0.716	0.269	0.554	0.185	
5947	-0.600	-0.342	-0.268	0.580	0.685	0.560	PLAGL1	NM_002656	0.600	0.342	0.268	0.580	0.685	0.560	0.506	0.163	0.403	0.174	
3235	-0.593	-0.353	-0.324	0.689	0.555	0.528	FYN	NM_002037	0.593	0.353	0.324	0.689	0.555	0.528	0.507	0.142	0.423	0.148	
1893	-0.562	-0.655	-0.602	0.513	0.458	0.533	SFRS6	NM_006275	0.562	0.655	0.602	0.513	0.458	0.533	0.554	0.069	0.606	0.047	
16368	-0.560	-0.610	-0.533	0.385	0.305	0.567	UBE2R2	NM_017811	0.560	0.610	0.533	0.385	0.305	0.567	0.493	0.120	0.568	0.039	
6374	-0.522	-0.422	-0.604	0.524	0.528	0.561	SCAMP4	NM_079834	0.522	0.422	0.604	0.524	0.528	0.561	0.527	0.060	0.516	0.091	
727	-0.522	-0.108	-0.506	0.791	1.065	0.621	UNC5B	NM_170744	0.522	0.108	0.506	0.791	1.065	0.621	0.602	0.319	0.379	0.235	
10516	-0.470	-0.421	-0.413	0.480	0.583	0.544	HSPA9	NM_004134	0.470	0.421	0.413	0.480	0.583	0.544	0.485	0.067	0.435	0.031	
15923	-0.396	-0.477	-0.506	0.311	0.534	0.698	SUMF2	NM_015411	0.396	0.477	0.506	0.311	0.534	0.698	0.487	0.131	0.460	0.057	
6219	-0.376	-0.647	-0.427	0.502	0.508	0.599	CFL1	NM_005507	0.376	0.647	0.427	0.502	0.508	0.599	0.510	0.102	0.483	0.144	
2388	-0.352	-0.733	-0.479	0.810	0.500	0.757	DDR2	NM_006182	0.352	0.733	0.479	0.810	0.500	0.757	0.605	0.186	0.521	0.194	
4283	-0.335	-0.437	-0.505	0.483	0.507	0.677	EIF5A	NM_001970	0.335	0.437	0.505	0.483	0.507	0.677	0.491	0.112	0.426	0.086	
11318	-0.304	-0.423	-0.429	0.627	0.932	0.776	C5	NM_001735	0.304	0.423	0.429	0.627	0.932	0.776	0.582	0.240	0.385	0.071	
7702	-0.259	-0.363	-0.526	0.482	0.728	0.648	H2AFX	NM_002105	0.259	0.363	0.526	0.482	0.728	0.648	0.501	0.174	0.383	0.135	
															Max	0.810			
															Min	0.469			
															Mean	0.563			
															SD	0.094			

FIG. 15A

Down-regulated in Rz1498 vs psiRNA										Total 20									
log2 ratio										Log2 ratio adjusted (±)									
id	396619	396615	396694	396612	396521	396688	Gene_Symbol	Target_NM	396619	396615	396694	396612	396521	396688	Mean (g)	SD	Mean (R)	SD	
15523	0.854	0.526	0.609	-0.999	-0.909	-0.800	TMEPAI	NM_020182	-0.854	-0.526	-0.609	-0.999	-0.909	-0.800	-0.783	0.181	-0.903	0.100	
15477	0.698	0.681	0.467	-0.549	-0.292	-0.405	C4orf35	NM_033122	-0.698	-0.681	-0.467	-0.549	-0.292	-0.405	-0.515	0.159	-0.415	0.129	
6571	0.630	0.701	0.937	-0.704	-0.515	-1.044	DDIT4	NM_019058	-0.630	-0.701	-0.937	-0.704	-0.515	-1.044	-0.755	0.198	-0.754	0.268	
1630	0.617	0.628	0.522	-0.363	-0.282	-0.108	HIST2H2BE	NM_003528	-0.617	-0.628	-0.522	-0.363	-0.282	-0.108	-0.420	0.206	-0.589	0.058	
6216	0.607	0.483	0.611	-0.678	-0.567	-0.441	C1orf91	NM_019118	-0.607	-0.483	-0.611	-0.678	-0.567	-0.441	-0.565	0.088	-0.567	0.073	
11114	0.588	0.399	0.278	-0.644	-0.665	-0.783	BRUNOL6	NM_052840	-0.588	-0.399	-0.278	-0.644	-0.665	-0.783	-0.560	0.187	-0.422	0.156	
11185	0.534	0.378	0.628	-0.423	-0.207	-0.476	TUBD1	NM_016261	-0.534	-0.378	-0.628	-0.423	-0.207	-0.476	-0.441	0.144	-0.513	0.126	
6117	0.467	-0.004	0.174	-0.676	-0.712	-0.479	CD180	NM_005582	-0.467	0.004	-0.174	-0.676	-0.712	-0.479	-0.417	0.282	-0.212	0.238	
1541	0.458	0.336	0.719	-0.268	-0.198	-0.598	IER3	NM_003897	-0.458	-0.336	-0.719	-0.268	-0.198	-0.598	-0.430	0.201	-0.504	0.196	
4200	0.442	0.204	0.104	-0.611	-0.849	-0.518	APCS	NM_001639	-0.442	-0.204	-0.104	-0.611	-0.849	-0.518	-0.455	0.272	-0.250	0.174	
5995	0.426	0.473	0.628	-0.385	-0.540	-0.581	ID4	NM_001546	-0.426	-0.473	-0.628	-0.385	-0.540	-0.581	-0.506	0.094	-0.509	0.106	
1082	0.423	0.605	0.600	-0.271	-0.449	-0.306	HSPA1B	NM_005346	-0.423	-0.605	-0.600	-0.271	-0.449	-0.306	-0.442	0.141	-0.543	0.104	
7833	0.379	0.139	0.355	-0.526	-0.648	-0.721	SHANK1	NM_016148	-0.379	-0.139	-0.355	-0.526	-0.648	-0.721	-0.461	0.214	-0.291	0.132	
5599	0.376	0.615	0.661	-0.077	-0.302	-0.603	RN7SK	NR_001445	-0.376	-0.615	-0.661	-0.077	-0.302	-0.603	-0.439	0.228	-0.551	0.153	
16736	0.364	0.443	0.093	-0.692	-0.520	-0.576	RPP30	NM_006413	-0.364	-0.443	-0.093	-0.692	-0.520	-0.576	-0.448	0.207	-0.300	0.184	
1423	0.304	0.682	0.422	-0.368	-0.340	-0.407	SIX1	NM_005982	-0.304	-0.682	-0.422	-0.368	-0.340	-0.407	-0.421	0.135	-0.469	0.193	
12510	0.228	0.343	0.094	-0.682	-0.677	-0.683	PPFIBP1	NM_003622	-0.228	-0.343	-0.094	-0.682	-0.677	-0.683	-0.451	0.263	-0.222	0.125	
14332	0.210	0.492	0.560	-0.307	-0.720	-0.678	FOXO3	NM_001455	-0.210	-0.492	-0.560	-0.307	-0.720	-0.678	-0.495	0.202	-0.421	0.186	
13245	0.118	0.193	-0.041	-0.556	-0.811	-0.847	CD302	NM_014880	-0.118	-0.193	0.041	-0.556	-0.811	-0.847	-0.414	0.376	-0.090	0.119	
11160	0.048	-0.003	-0.119	-0.690	-0.868	-1.036	ZNF431	NM_133473	-0.048	0.003	0.119	-0.690	-0.868	-1.036	-0.420	0.502	0.025	0.086	

Up-regulated in sh1498 vs psiRNA										Total 23											
log2 ratio										Log2 ratio adjusted (±)											
id	400442	400444	400436	393040	393041	393034	Gene_Symbol	Target_NM		396619	396615	396694	396612	396521	396688	Mean (g)	SD	Mean (g)	SD	Mean(rd)	SD
11636	-1.013	-0.930	-0.684	0.898	0.561	0.772	IRS4	NM_003604		1.013	0.930	0.684	0.898	0.561	0.772	0.810	0.169	0.876	0.171	0.744	0.170
6317	-0.764	-0.795	-0.543	0.276	0.287	0.086	ARS2	NM_015908		0.764	0.795	0.543	0.276	0.287	0.086	0.459	0.288	0.701	0.137	0.216	0.113
14687	-0.755	-0.612	-0.511	0.622	0.607	0.567	SLC7A1	NM_003045		0.755	0.612	0.511	0.622	0.607	0.567	0.612	0.081	0.626	0.123	0.599	0.028
9386	-0.732	-0.597	-0.501	0.693	0.205	0.387	CENTB5	NM_030649		0.732	0.597	0.501	0.693	0.205	0.387	0.519	0.199	0.610	0.116	0.428	0.247
14710	-0.726	-0.986	-0.898	0.127	0.079	-0.098	TMEM43	NM_024334		0.726	0.986	0.898	0.127	0.079	-0.098	0.453	0.470	0.870	0.132	0.036	0.119
7630	-0.707	-0.441	-0.606	0.759	0.712	0.612	HTR5B	NR_003947		0.707	0.441	0.606	0.759	0.712	0.612	0.640	0.114	0.585	0.134	0.694	0.075
2072	-0.679	-0.579	-0.580	0.619	0.362	0.330	MOBK11B	NM_018221		0.679	0.579	0.580	0.619	0.362	0.330	0.525	0.144	0.613	0.057	0.437	0.158
6219	-0.647	-0.638	-0.583	0.307	0.370	0.128	CFL1	NM_005507		0.647	0.638	0.583	0.307	0.370	0.128	0.446	0.211	0.623	0.035	0.268	0.126
2698	-0.614	-0.716	-0.530	0.103	0.518	0.197	C8orf53	NM_032334		0.614	0.716	0.530	0.103	0.518	0.197	0.446	0.242	0.620	0.093	0.273	0.218
14975	-0.560	-0.686	-0.716	0.368	0.396	0.297	DAZAP2	NM_014764		0.560	0.686	0.716	0.368	0.396	0.297	0.504	0.176	0.654	0.083	0.354	0.051
13767	-0.555	-0.551	-0.613	0.584	0.390	0.550	PABPC4	NM_003819		0.555	0.551	0.613	0.584	0.390	0.550	0.541	0.078	0.573	0.035	0.508	0.104
12850	-0.546	-0.874	-0.931	0.014	0.148	-0.013	GTF2I	NM_032999		0.546	0.874	0.931	0.014	0.148	-0.013	0.417	0.426	0.784	0.208	0.050	0.086
11388	-0.510	-0.760	-0.756	0.343	0.477	0.276	TMEM87A	NM_015497		0.510	0.760	0.756	0.343	0.477	0.276	0.520	0.203	0.675	0.143	0.365	0.102
2388	-0.492	-0.376	-0.432	0.775	0.438	0.474	DDR2	NM_006182		0.492	0.376	0.432	0.775	0.438	0.474	0.498	0.142	0.433	0.058	0.562	0.185
1151	-0.411	-0.441	-0.615	0.390	0.495	0.418	SNRPD3	NM_004175		0.411	0.441	0.615	0.390	0.495	0.418	0.462	0.083	0.489	0.110	0.434	0.054
10276	-0.410	-0.584	-0.553	0.794	0.273	0.317	PSIP1	NM_021144		0.410	0.584	0.553	0.794	0.273	0.317	0.489	0.194	0.516	0.093	0.461	0.289
11719	-0.402	-0.664	-0.548	0.259	0.193	0.295	EI24	NM_0010072		0.402	0.664	0.548	0.259	0.193	0.295	0.394	0.182	0.538	0.131	0.249	0.052
3235	-0.373	-0.344	-0.284	0.468	0.600	0.402	FYN	NM_002037		0.373	0.344	0.284	0.468	0.600	0.402	0.412	0.111	0.334	0.045	0.490	0.101
5599	-0.371	-0.272	-0.190	0.817	0.633	0.610	RN7SK	NR_001445		0.371	0.272	0.190	0.817	0.633	0.610	0.482	0.242	0.278	0.091	0.687	0.113
11955	-0.352	-0.482	-0.546	0.286	0.400	0.316	TBC1D23	NM_018309		0.352	0.482	0.546	0.286	0.400	0.316	0.397	0.100	0.460	0.099	0.334	0.059
14548	-0.254	-0.335	-0.500	0.524	0.516	0.275	CCDC50	NM_178335		0.254	0.335	0.500	0.524	0.516	0.275	0.401	0.126	0.363	0.125	0.438	0.142
10560	-0.239	-0.025	-0.099	0.723	0.685	0.694	HIST2H2AC	NM_003517		0.239	0.025	0.099	0.723	0.685	0.694	0.411	0.325	0.121	0.109	0.701	0.020
17393	-0.207	-0.213	-0.483	0.630	0.437	0.458	ATXN2L	NM_148416		0.207	0.213	0.483	0.630	0.437	0.458	0.405	0.165	0.301	0.158	0.508	0.106
										Max										0.810	
										Min										0.394	
										Mean										0.489	
										SD										0.096	

FIG. 15C

Down-regulated in sh1498 vs psiRNA																	Total 34				
log2 ratio																					
id	400442	400444	400436	393040	393041	393034	Gene_Symbol	Target	NM	396619	396615	396694	396612	396521	396688	Mean (g) SD	Mean (rd) SD				
11685	1.494	0.959	1.377	-0.472	-0.402	0.073	SOX3	NM_005634		-1.494	-0.959	-1.377	-0.472	-0.402	0.073	-0.772	0.610	-1.277	0.281	-0.267	0.297
532	1.436	1.041	1.388	-0.274	-0.645	-0.166	GFR1	NM_005264		-1.436	-1.041	-1.388	-0.274	-0.645	-0.166	-0.825	0.549	-1.288	0.216	-0.362	0.251
1227	1.423	0.939	1.150	-0.359	-0.352	-0.110	TIGD4	NM_145720		-1.423	-0.939	-1.150	-0.359	-0.352	-0.110	-0.722	0.522	-1.171	0.243	-0.274	0.142
13599	1.406	1.105	1.402	-0.593	-0.320	-0.091	MTERFD1	NM_015942		-1.406	-1.105	-1.402	-0.593	-0.320	-0.091	-0.820	0.565	-1.304	0.173	-0.335	0.251
16774	1.331	1.050	1.205	-0.177	-0.353	0.139	TASIR2	NM_152232		-1.331	-1.050	-1.205	-0.177	-0.353	0.139	-0.663	0.611	-1.195	0.141	-0.130	0.249
8777	1.293	1.060	1.387	-0.225	-0.428	0.001	ADH16A1	NM_153329		-1.293	-1.060	-1.387	-0.225	-0.428	0.001	-0.732	0.590	-1.247	0.168	-0.217	0.215
17349	1.244	0.845	1.292	-0.165	-0.375	0.164	MYO9B	NM_004145		-1.244	-0.845	-1.292	-0.165	-0.375	0.164	-0.626	0.595	-1.127	0.245	-0.125	0.272
498	1.230	1.035	1.418	-0.202	-0.530	-0.008	CREBBP	NM_004380		-1.230	-1.035	-1.418	-0.202	-0.530	-0.008	-0.737	0.576	-1.228	0.192	-0.247	0.264
7716	1.219	0.768	0.821	-0.251	-0.049	0.136	ATP10D	NM_020453		-1.219	-0.768	-0.821	-0.251	-0.049	0.136	-0.495	0.522	-0.936	0.247	-0.055	0.194
2546	1.165	0.898	1.411	-0.415	-0.807	-0.071	NOXO1	NM_144603		-1.165	-0.898	-1.411	-0.415	-0.807	-0.071	-0.795	0.489	-1.158	0.257	-0.431	0.368
1729	1.127	0.811	1.401	-0.042	-0.413	0.264	PPGS	NM_004957		-1.127	-0.811	-1.401	-0.042	-0.413	0.264	-0.588	0.641	-1.113	0.295	-0.064	0.339
16434	1.072	0.868	1.540	-0.138	-0.492	0.179	HPS4	NM_022081		-1.072	-0.868	-1.540	-0.138	-0.492	0.179	-0.655	0.631	-1.160	0.345	-0.150	0.336
5741	1.067	0.807	0.934	-0.039	-0.606	0.114	none	XM_065899		-1.067	-0.807	-0.934	-0.039	-0.606	0.114	-0.557	0.487	-0.936	0.130	-0.177	0.379
12182	1.066	0.602	1.062	-0.184	-0.165	0.102	NDRG2	NM_201536		-1.066	-0.602	-1.062	-0.184	-0.165	0.102	-0.496	0.494	-0.910	0.267	-0.082	0.160
11305	1.064	0.824	1.393	-0.451	-0.649	0.214	ACPT	NM_033068		-1.064	-0.824	-1.393	-0.451	-0.649	0.214	-0.695	0.553	-1.094	0.286	-0.295	0.452
6965	1.056	0.808	0.961	-0.232	-0.333	0.121	SERPIND1	NM_000185		-1.056	-0.808	-0.961	-0.232	-0.333	0.121	-0.545	0.467	-0.942	0.125	-0.148	0.238
14553	1.017	0.539	0.666	-0.176	-0.459	-0.029	SIC11A1	NM_000578		-1.017	-0.539	-0.666	-0.176	-0.459	-0.029	-0.481	0.353	-0.741	0.248	-0.221	0.219
16079	0.998	0.781	1.324	-0.323	-0.510	0.151	MAGEB4	NM_002367		-0.998	-0.781	-1.324	-0.323	-0.510	0.151	-0.631	0.521	-1.034	0.273	-0.227	0.341
5290	0.955	0.635	0.832	-0.180	-0.024	-0.014	PPM1F	NM_014634		-0.955	-0.635	-0.832	-0.180	-0.024	-0.014	-0.440	0.419	-0.807	0.161	-0.073	0.093
7002	0.949	0.757	1.135	-0.194	-0.627	0.150	DUOX2	NM_014080		-0.949	-0.757	-1.135	-0.194	-0.627	0.150	-0.585	0.482	-0.947	0.189	-0.224	0.389
8224	0.941	0.696	1.130	-0.518	-0.932	-0.257	SEPT5	NM_002688		-0.941	-0.696	-1.130	-0.518	-0.932	-0.257	-0.746	0.321	-0.922	0.218	-0.569	0.340
8479	0.928	0.472	1.143	-0.407	-0.827	-0.138	C1orf198	NM_032800		-0.928	-0.472	-1.143	-0.407	-0.827	-0.138	-0.653	0.375	-0.848	0.343	-0.457	0.347
16506	0.917	0.665	0.642	-0.063	-0.260	-0.255	ZNF577	NM_032679		-0.917	-0.665	-0.642	-0.063	-0.260	-0.255	-0.467	0.324	-0.741	0.153	-0.193	0.112
2080	0.900	0.628	1.106	-0.140	-0.333	-0.186	GJB1	NM_000166		-0.900	-0.628	-1.106	-0.140	-0.333	-0.186	-0.549	0.396	-0.878	0.240	-0.220	0.101
894	0.757	0.527	0.988	-0.203	-0.539	-0.066	NFATC1	NM_006162		-0.757	-0.527	-0.988	-0.203	-0.539	-0.066	-0.513	0.341	-0.757	0.231	-0.269	0.243
12241	0.746	0.487	0.584	-0.336	-0.346	-0.107	EIF4A3	NM_014740		-0.746	-0.487	-0.584	-0.336	-0.346	-0.107	-0.434	0.222	-0.606	0.131	-0.263	0.135
16262	0.610	0.485	0.585	-0.371	-0.438	-0.504	GDF15	NM_004864		-0.610	-0.485	-0.585	-0.371	-0.438	-0.504	-0.499	0.089	-0.560	0.066	-0.438	0.067
12951	0.600	0.624	0.535	-0.380	-0.559	-0.491	RBM34	NM_015014		-0.600	-0.624	-0.535	-0.380	-0.559	-0.491	-0.532	0.088	-0.586	0.046	-0.477	0.090
11980	0.589	0.857	0.592	-0.184	-0.364	-0.394	LMNB1	NM_005573		-0.589	-0.857	-0.592	-0.184	-0.364	-0.394	-0.497	0.234	-0.679	0.154	-0.314	0.114
15523	0.559	0.365	0.390	-0.625	-0.563	-0.640	TMEPA1	NM_020182		-0.559	-0.365	-0.390	-0.625	-0.563	-0.640	-0.524	0.118	-0.438	0.106	-0.609	0.041
5735	0.540	0.324	0.499	-0.528	-0.482	-0.501	LYPLA3	NM_012320		-0.540	-0.324	-0.499	-0.528	-0.482	-0.501	-0.479	0.079	-0.454	0.115	-0.504	0.023
2094	0.507	0.389	0.209	-0.604	-0.557	-0.621	ARL6IP6	NM_152522		-0.507	-0.389	-0.209	-0.604	-0.557	-0.621	-0.481	0.157	-0.368	0.150	-0.594	0.033
1849	0.303	0.406	0.297	-0.629	-0.606	-0.490	GJA1	NM_000165		-0.303	-0.406	-0.297	-0.629	-0.606	-0.490	-0.455	0.145	-0.335	0.061	-0.575	0.075
8036	0.214	0.205	0.166	-0.702	-0.592	-0.620	UBE2W	NM_018299		-0.214	-0.205	-0.166	-0.702	-0.592	-0.620	-0.417	0.246	-0.195	0.026	-0.638	0.057
															Max	-0.417					
															Min	-0.825					
															Mean	-0.591					
															SD	0.122					

FIG. 15D

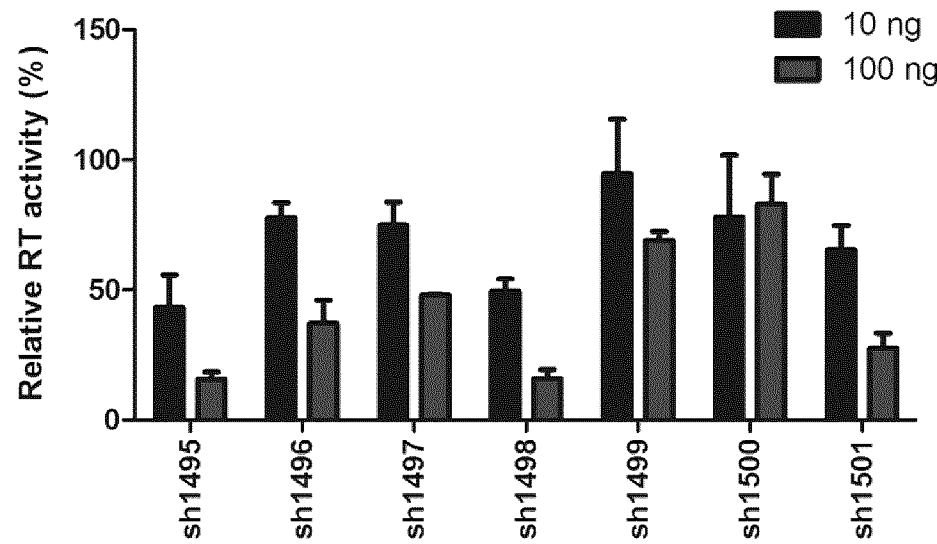


FIG. 16A

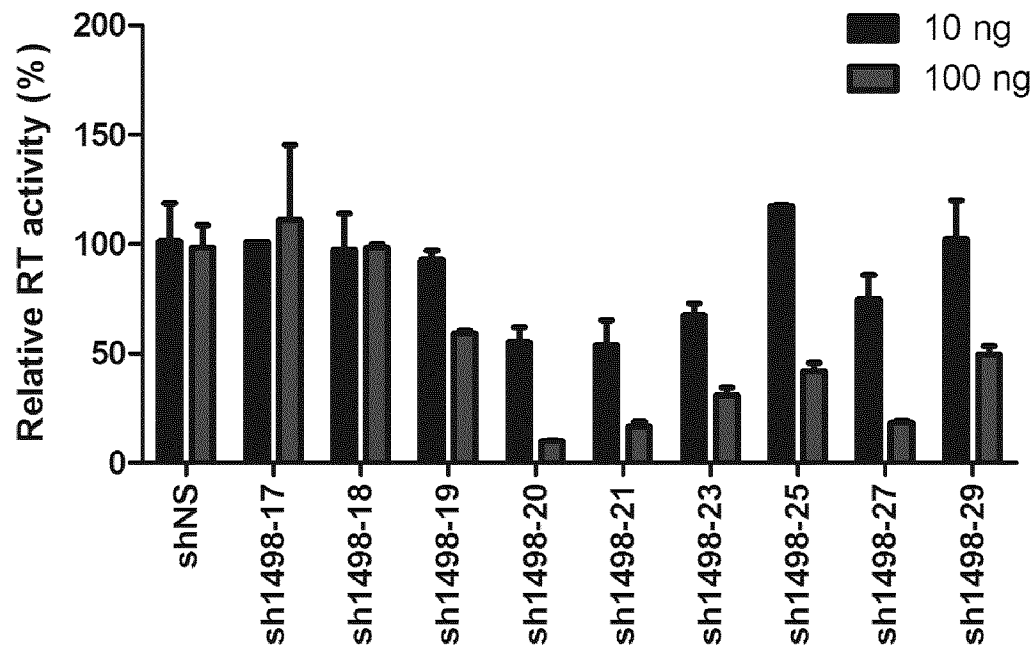


FIG. 16B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2014/050814

A. CLASSIFICATION OF SUBJECT MATTER IPC: <i>C12N 15/113</i> (2010.01), <i>A61K 31/7088</i> (2006.01), <i>A61K 31/7105</i> (2006.01), <i>A61K 31/713</i> (2006.01), <i>A61P 31/18</i> (2006.01), <i>C07H 21/02</i> (2006.01), <i>C12N 15/49</i> (2006.01) 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) IPC: <i>C12N 15/113</i> (2010.01), <i>A61K 31/7088</i> (2006.01), <i>A61K 31/7105</i> (2006.01), <i>A61K 31/713</i> (2006.01), <i>A61P 31/18</i> (2006.01), <i>C07H 21/02</i> (2006.01), <i>C12N 15/49</i> (2006.01) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) FAMPAT, Biosis, CAPLUS, Medline, Canadian Patent Database, GenomeQuest (SEQ ID NO: 117 and 20 nucleotide long fragments thereof, SEQ ID NO: 130-142, using all available databases); Keywords: HIV, human immunodeficiency virus, antisense, gag, orf, open reading frame, ribozyme, siRNA, shRNA		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	McINTYRE, G.J. et al. 96 shRNAs designed for maximal coverage of HIV-I variants. Retrovirology [online], issue of 4 June 2009 (04-06-2009) [retrieved on 19-11-2014], Volume 6, page 55 (1-15) and Additional Files 1 and 2, ISSN 1742-4690. Retrieved from the Internet: <URL: http://www.retrovirology.com/content/6/1/55 >.	1, 2, 7, 11, 13-24
A	- cited in the description; Figure 3, Additional File 2	3-6, 8-10, 12
P, X	SCARBOROUGH, R. J. et al. A conserved target site in HIV-I Gag RNA is accessible to inhibition by both an HDV ribozyme and a short hairpin RNA. Molecular Therapy – Nucleic Acids [online], issue of 29 July 2014 (29-07-2014) [retrieved on 19-11-2014], Volume 3, page e178 (1-11), Supplementary Figures S1-S5, Supplementary Table S1 and Supplementary Files S1 and S2, ISSN 2162-2531. Retrieved from the Internet: <URL: http://www.nature.com/mtna/journal/v3/n7/full/mtna201431a.html >.	1-24
☒ 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	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 19 November 2014 (19-11-2014)		Date of mailing of the international search report 26 November 2014 (26-11-2014)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Michael W. De Vouge (819) 997-2952

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2014/050814

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN102352360 A (CHANG, L. et al) 15 Feb 2012 (15-02-2012)	
A	WO2006023491 A2 (LIEBERMAN, J. and SONG, E.) 2 March 2006 (02-03-2006)	
A	WO9746673 A2 (BOEHNLEIN, E. et al) 11 December 1997 (11-12-1997)	
A	WO94/08004 A1 (AGRAWAL, S. and TANG, J.-Y.) 14 April 1994 (14-04-1994)	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2014/050814**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. ☒ Claim Nos.: 16, 17
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 16 and 17 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to examine under Rule 67.1(iv) of the PCT. Moreover, this Authority has established a written opinion based on the alleged use of the antisense nucleic acid molecule, vector, cell or composition of claims 1-15. Applicant should note that the method of claim 16 is considered to be a method of therapy unless the inhibition of HIV-1 replication in a cell is explicitly stated as being *in vitro*.

2. ☐ Claim 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. ☐ Claim 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 additional fees, this Authority did not invite payment of 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 claim 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 claim 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
Information on patent family members

International application No.

PCT/CA2014/050814

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
CN102352360A	15 February 2012 (15-02-2012)	CN102352360B	10 October 2012 (10-10-2012)
WO2006023491A2	02 March 2006 (02-03-2006)	WO2006023491A3 AT555202T AU2005277547A1 AU2005277547B2 CA2576925A1 CA2576925C EP1789447A2 EP1789447A4 EP1789447B1 JP2008509705A JP4903146B2 US2008153737A1 US8168601B2 US2013302331A1 US8785618B2 US2012258534A1	27 July 2006 (27-07-2006) 15 May 2012 (15-05-2012) 02 March 2006 (02-03-2006) 25 August 2011 (25-08-2011) 02 March 2006 (02-03-2006) 10 December 2013 (10-12-2013) 30 May 2007 (30-05-2007) 21 April 2010 (21-04-2010) 25 April 2012 (25-04-2012) 03 April 2008 (03-04-2008) 28 March 2012 (28-03-2012) 26 June 2008 (26-06-2008) 01 May 2012 (01-05-2012) 14 November 2013 (14-11-2013) 22 July 2014 (22-07-2014) 11 October 2012 (11-10-2012)
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WO9408004A1	14 April 1994 (14-04-1994)	AT146819T AU5402894A AU678415B2 BR9307191A CA2146364A1 CZ9500854A3 DE69306969D1 DE69306969T2 DK0664833T3 EP0664833A1 EP0664833B1 ES2096343T3 FI951600A GR3022315T3 HU9500995D0 HUT72400A JPH08504570A NO951307D0 NO951307A NZ257434A PL308261A1 US5684147A	15 January 1997 (15-01-1997) 26 April 1994 (26-04-1994) 29 May 1997 (29-05-1997) 30 March 1999 (30-03-1999) 14 April 1994 (14-04-1994) 13 March 1996 (13-03-1996) 06 February 1997 (06-02-1997) 07 May 1997 (07-05-1997) 14 April 1997 (14-04-1997) 02 August 1995 (02-08-1995) 27 December 1996 (27-12-1996) 01 March 1997 (01-03-1997) 10 May 1995 (10-05-1995) 30 April 1997 (30-04-1997) 28 June 1995 (28-06-1995) 29 April 1996 (29-04-1996) 21 May 1996 (21-05-1996) 04 April 1995 (04-04-1995) 01 June 1995 (01-06-1995) 29 January 1997 (29-01-1997) 24 July 1995 (24-07-1995) 04 November 1997 (04-11-1997)