Video Article

Evaluation of the Efficacy And Toxicity of RNAs Targeting HIV-1 Production for Use in Gene or Drug Therapy

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Abstract

Small RNA therapies targeting post-integration steps in the HIV-1 replication cycle are among the top candidates for gene therapy and have the potential to be used as drug therapies for HIV-1 infection. Post-integration inhibitors include ribozymes, short hairpin (sh) RNAs, small interfering (si) RNAs, U1 interference (U1i) RNAs and RNA aptamers. Many of these have been identified using transient co-transfection assays with an HIV-1 expression plasmid and some have advanced to clinical trials. In addition to measures of efficacy, small RNAs have been evaluated for their potential to affect the expression of human RNAs, alter cell growth and/or differentiation, and elicit innate immune responses. In the protocols described here, a set of transient transfection assays designed to evaluate the efficacy and toxicity of RNA molecules targeting post-integration steps in the HIV-1 replication cycle are described. We have used these assays to identify new ribozymes and optimize the format of shRNAs and siRNAs targeting HIV-1 RNA. The methods provide a quick set of assays that are useful for screening new anti-HIV-1 RNAs and could be adapted to screen other post-integration inhibitors of HIV-1 replication.

Video Link

The video component of this article can be found at https://www.jove.com/video/54486/

Introduction

A limitation of current HIV-1 treatments is that they must be chronically administered to prevent disease progression. Transplant of HIV-1 resistant T lymphocyte, or hematopoietic stem cells, has the potential to provide long term control of HIV-1 replication in the absence of drug therapy[1,2] and may also be an effective approach to attain an HIV-1 cure[3]. One way to render cells resistant to HIV-1 replication is to insert one or more genes coding for anti-HIV-1 RNAs or peptides into an infected individual's cells during an autologous transplant[4]. Several candidate anti-HIV-1 genes have been designed with some entering clinical trials in combinations of two[5] or three[6], to prevent the development of HIV-1 resistance to any single gene.

Anti-HIV-1 RNAs are among the top candidates for combination gene therapy due to their low potential to elicit immune responses and because they are transcribed from very short gene sequences. Some anti-HIV-1 RNAs have been designed to target viral entry and integration. However, most anti-HIV-1 RNAs target post-integration steps in the viral life cycle (**Figure 1**). Post-integration inhibitors include decoy RNAs, targeting the HIV-1 regulatory proteins Tat or Rev[1], and antisense-based RNAs, targeting different sites in HIV-1 RNA, such as ribozymes[7], shRNAs[8] and U1i RNAs[9]. Methods that have been used to compare the efficacy of anti-HIV-1 RNAs include monitoring viral replication in cells transduced with genes coding for candidate RNAs and measuring viral production in cells transiently transfected with plasmids expressing candidate RNAs and an HIV-1 expression plasmid[10-13]. We have previously used an HIV-1 production assay to screen HIV-1 RNA for new ribozyme target sites[13-15]. These methods have since been refined to optimize the format of an RNA interference molecule expressed from plasmid DNA as an shRNA or delivered as a synthetic siRNA[16]. The assay measures the production of mature viruses from human embryonic kidney (HEK) 293T cells, and can be used to compare the effects of inhibitors that target post-integration steps in the HIV-1 replication cycle (**Figure 1**). For inhibitors that target pre-integration steps, alternative assays such as a TZM-bl cell infectivity assay[17] are needed to evaluate antiviral efficacy.

Major safety concerns for the delivery of anti-HIV-1 RNAs in the clinic include potential off-target effects on human RNAs or proteins, and activation of innate immune sensors. To evaluate the toxicity of anti-HIV-1 siRNAs, we have used a cell viability assay in different cell lines[16]. We also measured activation of the double stranded RNA immune sensors, RNA activated protein kinase R (PKR) and Toll like receptor 3 (TLR3), as well as expression of the interferon stimulated gene, ADAR1 p150. These assays can be used to confirm that the efficacy of anti-HIV-1 RNAs is not due to indirect effects on cell viability or immune sensor activation. They are also useful in excluding candidate RNAs with potential toxicities from further development.

In the following protocols, procedures to identify new therapeutic RNAs and optimize the format of existing ones are described. The methods are useful for screening RNA based post-integration inhibitors of HIV-1 replication and could be adapted to screen other post-integration inhibitors, such as small molecules targeting Rev mediated export of viral RNA[18] or CRISPR/Cas systems designed to target integrated HIV-1 DNA[19].

Protocol

1. Cells and Transfections

1. Culture HEK 293T cells in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Prepare a 2 x 10^5 cells/ml suspension in the cell culture medium. Add 500, 100 and 1,000 µl of the cell suspension to each well of 24-well, 96-well and 12-well plates, for viral production, cell viability and immune activation assays, respectively (**Figure 2A**).
2. Gently swirl the plates and incubate them O/N at 37 °C with 5% CO_2. Grow cells to 50-70% confluency.
3. According to a transfection plan, prepare dilutions of test RNAs and their controls in 1.5 ml micro tubes (Example, **Figure 2B**).
    1. For the viral production assay, prepare a 10 ng/µl dilution of an HIV-1 expression plasmid and add 10 µl to each tube. Next prepare 5 µM dilutions of test RNAs and a negative control RNA. Add 2.5 or 10 µl of each test RNA and negative control dilution to the corresponding tubes for 25 or 100 nM final concentrations.
    2. For the cell viability assay, prepare 5 µM dilutions of test RNAs and a 10 mg/ml dilution of the positive control RNA, low molecular weight Poly I:C. Add 2 µl of test RNA or positive control RNA dilutions to the corresponding tubes for 100 nM or 200 µg/ml final concentrations, respectively.
    3. For the immune activation assay, add 20 µl of test RNA or positive control RNA dilutions prepared in step 1.3.2. to the corresponding tubes for 100 nM or 200 µg/ml final concentrations, respectively.
    Note: For testing RNA expression plasmids, prepare 10 ng/µl dilutions in place of the 5 µM dilutions of test RNAs, giving final amounts of 25 and 100 ng for step 1.3.1. and 100 ng for steps 1.3.2. and 1.3.3.
4. Add 50, 25 or 75 µl of DMEM to each transfection tube for viral production, cell viability and immune activation assays, respectively. For viral production assays, bring cells and prepared transfection tubes to a bio-safety level 3 (BSL3) laboratory before the next step.
5. Add 2 µl of the transfection reagent sequentially to the transfection tubes and incubate 15 to 20 min to allow complexes to form. See the Table of Materials for the specific transfection reagent to be used.
6. Add the entire transfection mixture from each micro tube drop-wise to the corresponding positions in the cell culture plates. Gently swirl and incubate the plates for 48 hr at 37 °C with 5% CO_2.
7. Measure HIV-1 production (section 2), cell viability (section 3) and immune activation (section 4) in the cell culture plates (**Figure 2C**).

2. Viral Production Assay

1. Remove the 24-well cell culture plates from the incubator and gently swirl the plates inside the BSL3 cell culture hood. Transfer 150 µl of supernatant from each well to a corresponding well in a 96-well flat bottom plate, which will be used to quantify HIV-1 production.
Note: Common viral quantification assays include measuring the expression of the HIV-1 capsid protein (p24) by enzyme-linked immunosorbent assay (ELISA)[20], quantifying viral RNA by reverse transcription polymerase chain reaction (RT-PCR)[21] and measuring the activity of the HIV-1 RT enzyme. Steps 2.2 to 2.5 explain methods to quantify HIV-1 RT activity[13,15,16].
2. Transfer 5 µl of supernatant to corresponding wells in a 96-well plate, containing 25 µl of a viral disruption cocktail[15] (**Table 1**). Incubate the mixture for 5 min at RT and transfer the plate to a radioactivity workstation.
Note: Step 2.2. is only necessary if a radioactivity workstation is not available in the BSL3 laboratory. If the plates do not need to be removed from the BSL3 laboratory, proceed to step 2.3. and add 50 µl of radioactive/viral disruption cocktail (**Table 1**) in place of the 25 µl of radioactive cocktail.
3. Prepare a radioactive cocktail[15] (**Table 1**), and add 25 µl to each well of viral supernatant and disruption cocktail. Incubate the plates at 37 °C for 2 hr.
4. Spot 5 µl of the reaction mixture onto corresponding squares in a glass fiber Di Ethyl Amino Ethyl (DEAE) filtermat paper and allow the spots to dry for 10 min. Spot the reaction mixture on every other square so that no two samples directly boarder one another. This helps to avoid cross-over between samples when determining counts per minute (cpm) in step 2.6.
5. Wash the papers 5x for 5 min with 2x saline sodium citrate (SSC) buffer (**Table 1**), followed by two 1 min washes with 95% ethanol. Allow the papers to dry and seal them in sample bags.
6. Clip sample bags containing the filtermat paper into a cassette and insert the cassette into a microplate scintillation counter. Set the counter to read cpm for ^{32}P with the reference date provided for the batch of [^{32}P]dTTP used in the experiment. Select which samples to read using the plate map and start the counter.
7. For any viral quantification method, divide the values obtained for each test RNA by the adjacent negative control and multiply this value by 100 to get the percent inhibition of HIV-1 production for each replicate of the test RNAs. An example of results comparing different test RNAs at different concentrations is provided in **Figure 3**.

3. Cell Viability Assay

1. Remove the 96-well plates from the incubator and add 20 µl of 5 mg/ml MTT (3-[4.5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) diluted in Dulbecco's phosphate buffered saline (DPBS) to each well. Incubate the plates for 3 hr at 37 °C.
2. Add 150 µl of acidified isopropanol with detergent (1% NP-40, 4 mM HCl in isopropanol) to each well and incubate the plates for 2 hr at RT.
3. Determine the absorbance at 570 nm in a microplate spectrophotometer.
4. Calculate the relative MTT metabolism for each positive control and test RNA by dividing the value obtained for each sample by its adjacent transfection control. An example of results comparing different test RNAs and a positive control is provided in **Figure 4**.

4. Immune Activation Assay

1. Remove the 12-well plates from the incubator and aspirate the culture media. Gently wash the cells twice with DPBS and add 70 µl of cold lysis buffer[22] (including protease and phosphatase inhibitors, **Table 1**) to each well. Incubate the plates for 10 min on ice.
2. Transfer cell lysates to micro tubes and fast freeze them by immersing the tubes in liquid nitrogen. Allow the samples to thaw and repeat for 2 more freeze thaw cycles for a total of 3.
3. Centrifuge the lysates for 15 min at 4 °C, 15,700 x g, to pellet cell debris. Transfer the supernatant to new micro tubes and determine the protein concentration using the Coomassie blue (Bradford) method[23,24].
4. Resolve 75 µg of protein from each sample in a 10% denaturing polyacrylamide gel and transfer to a nitrocellulose membrane as previously described[25,26].
5. Following electrophoresis and transfer to a membrane, reveal protein bands by incubating the membrane in Ponceau S (**Table 1**) for 1 min followed by washing in double distilled water. Use the bands and protein ladder as a guide to cut the membrane at 80 and 55 kDa.
   Note: In step 4.4 samples can be run on 16 x 18 cm^2 gels down to 34 kDa, so that it is easy to cut the membrane at the specified positions and not cut through the bands of interest. Alternatively, several gels can be run with the same samples to avoid cutting the membrane.
6. Wash out the Ponceau S staining with Tris-buffered saline containing 0.05% Tween 20 (TBST, **Table 1**). Add TBST with 5% non-fat milk to completely cover the membranes. Incubate the membranes at RT with agitation for 1 hr.
7. Incubate the membranes O/N in TBST with 3% bovine serum albumin (BSA) and antibodies diluted to 1 in 1,000 against ADAR1 (110 and 150 kDa), phospho-PKR (62 kDa), and phospho-IRF3 (47 kDa), for the top, middle and bottom pieces of the membrane, respectively.
8. Wash the membranes 5x for 5 min with TBST and incubate in TBST with 5% non-fat milk and peroxidase-labeled goat anti-rabbit secondary antibodies (diluted to 1 in 5,000) for 1 hr.
9. Wash the membranes 5x for 5 min with TBST and apply electrochemiluminescence (ECL) solution to visualize the bands on films according the manufacturer's instructions.
10. After visualizing the protein bands on films, wash the middle and bottom pieces of the membrane for 10 min with an antibody stripping solution. Incubate the membranes O/N in TBST with 3% BSA and antibodies against total PKR (at 1 in 500) and IRF3 (at 1 in 1,000), for the middle and bottom pieces, respectively. Repeat steps 4.8. and 4.9. using peroxidase-labeled goat anti-mouse in place of the anti-rabbit secondary antibody for the PKR membrane (middle).
11. Wash the bottom piece of the membrane 5x for 5 min with TBST and incubate the membrane in TBST with 3% BSA for 1 hr with an antibody against actin (diluted to 1 in 5,000). Repeat steps 4.8. and 4.9. using peroxidase-labeled goat anti-mouse in place of the anti-rabbit secondary antibody. An example of results comparing different test RNAs and a positive control is provided in **Figure 5**. See the **Table of Materials** for the specific antibodies to be used.

Representative Results

A general schematic of the procedures is shown in **Figure 2** with an example transfection plan for three test RNAs and a control RNA provided in **Figure 2B**. For viral production and cell viability assays, the read-out for each test construct is normalized to a negative control. Replicates are transfected in sets, so that each test RNA is normalized to its adjacent negative control. This is done to avoid inaccurate data related to the time between complexing and transfection, which may result if, for example, all of the negative controls are transfected first. Since HIV-1 can affect immune responses[27,28], the cell viability and immune activation assays are done without the addition of an HIV-1 expressing plasmid.

To identify the optimal length of RNA interference molecules targeting a conserved site in HIV-1 RNA (position 1498-1519 in HIV-1 strain NL4-3)[13], a set of siRNAs were designed (**Figure 3A**). Using the HIV-1 production assay, the percent of RT activity compared to cells transfected with a long Dicer substrate nonsense siRNA (siNS) was calculated for cells transfected with each test siRNA at different amounts of co-transfection with 100 ng of a plasmid expressing HIV-1 strain NL4-3 (**Figure 3B**).

Using the cell viability assay, the percent metabolism of MTT was determined for cells transfected with the most effective siRNAs identified in **Figure 3B**. Data were normalized to MTT metabolism in cells treated with the transfection reagent alone (**Figure 4**). A long double stranded RNA (Poly I:C) reduced cell viability; however, the effect was only significant at the highest dose evaluated. No significant reduction in cell viability was observed for siRNAs targeting the 1498 site in HIV-1 RNA, regardless of their length. Using the immune activation assay, the same set of RNAs were evaluated for their potential to trigger immune responses (**Figure 5**). In conditions where Poly I:C activated the expression of ADAR1 p150 and induced phosphorylation of PKR and IRF3, no significant effect on immune activation markers could be observed for any of the test RNAs.

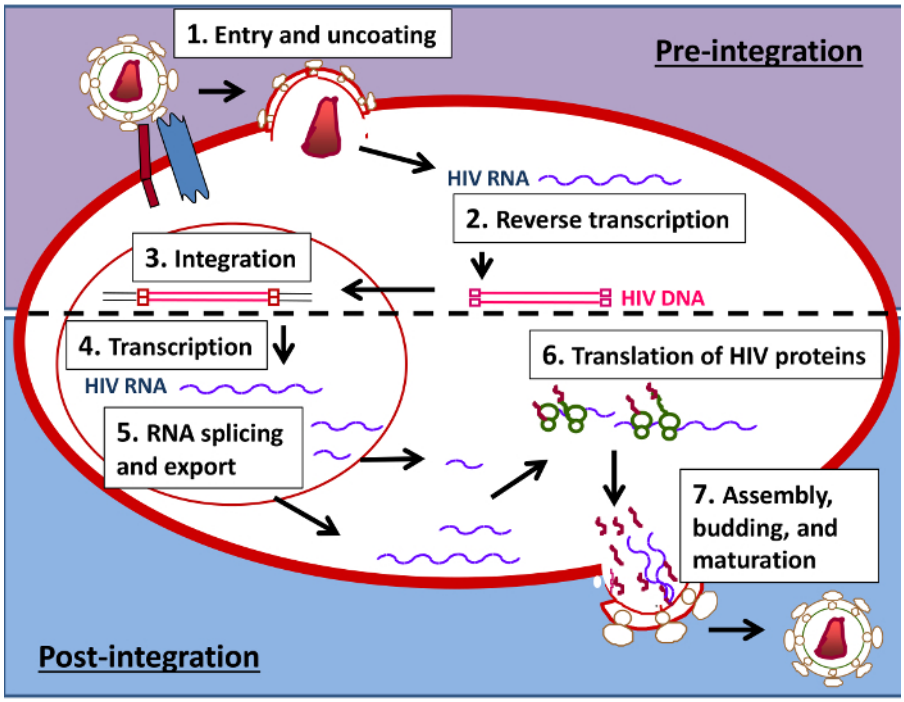
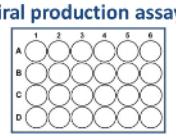


Figure 1. Schematic of pre- and post-integration steps in the HIV-1 replication cycle. Pre-integration (1-3) and post-integration (4-7) steps in the HIV-1 replication cycle are shown in outlined boxes. Please click here to view a larger version of this figure.

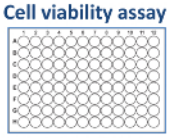
A) Plate adherent cells and culture for 24 hrs

Viral production assay



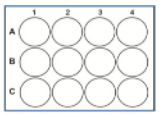
24-well plate, 500 µl/well

Cell viability assay



96-well plate, 100 µl/well

Immune activation assay



12-well plate, 1 ml/well

B) Prepare transfection tubes, transfect cells, and culture for 48 hrs

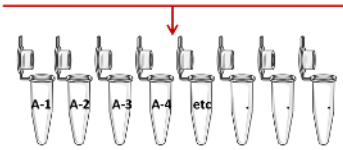
Example transfection plan

Viral production assay			
Tube	RNA	RNA amount	HIV-1 (ng)
A-1	No RNA		100
A-2	[1]Control	25 nM	100
A-3	Test RNA1	25 nM	100
A-4	Test RNA2	25 nM	100
A-5	Test RNA3	25 nM	100
A-6	No RNA		100
B-1	[1]Control	25 nM	100
B-2	Test RNA1	25 nM	100
B-3	Test RNA2	25 nM	100
B-4	Test RNA3	25 nM	100
B-5	No RNA		100
B-6	[1]Control	100 nM	100
etc	...	...	...

Cell Viability assay		
Tube	RNA	RNA amount
A-1	No RNA	
A-2	[2]Control	200 µg/mL
A-3	Test RNA1	100 nM
A-4	Test RNA2	100 nM
A-5	Test RNA3	100 nM
A-6	No RNA	
A-7	[2]Control	100 nM
A-8	Test RNA1	100 nM
A-9	Test RNA2	100 nM
A-10	Test RNA3	100 nM
A-11	No RNA	
A-12	[2]Control	200 µg/mL
etc	...	...

Immune activation assay		
Tube	RNA	RNA amount
A-1	No RNA	
A-2	[2]Control	200 µg/mL
A-3	Test RNA1	100 nM
A-4	Test RNA2	100 nM
B-1	Test RNA3	100 nM
B-2	No RNA	
B-3	[2]Control	100 nM
B-4	Test RNA1	100 nM
C-1	Test RNA2	100 nM
C-2	Test RNA3	100 nM
C-3	No RNA	
C-4	[2]Control	200 µg/mL
etc	...	...

[1]**Control:** an RNA that does not affect HIV-1 production. [2]**Control:** an RNA that affects cell viability and triggers innate immune responses, Ex. poly I:C (500 to 1000 base pairs) at 200 µg/mL (equivalent to approximately 0.5 to 1 µM).



Transfection

1. Sequentially, add cell culture media followed by transfection reagent.
2. Wait 15-20 min from the addition of transfection reagent to tube A-1.
3. Add mixture to corresponding wells in cell culture plates.

C) Read-out

Viral production assay

Quantify HIV-1 virions in cell culture supernatant,
Ex. HIV-1 RT assay

Cell viability assay

Measure metabolism of a cellular NADPH substrate,
Ex. MTT metabolism

Immune activation assay

Measure activation of immune responses in cell culture lysates,
Ex. phosphorylation of PKR and IRF3, expression of ADAR p150

Figure 2. Schematic of viral production, cell viability and immune activation assays. (**A**) Plate adherent cells and culture for 24 hr. Cells are plated in 24-, 96- and 12- well plates in 500, 100 and 1,000 µl of cell culture media for viral production, cell viability and immune activation assays, respectively. (**B**) Prepare transfection tubes, transfect cells, and culture for 48 hr. An example transfection plan for a set of three test RNAs (RNA1-3) is shown with appropriate controls. The transfection procedure is also illustrated. (**C**) Read-out. The read-out for viral production, cell viability and immune activation assays are written and explained in detail in sections 2, 3 and 4, respectively. Please click here to view a larger version of this figure.

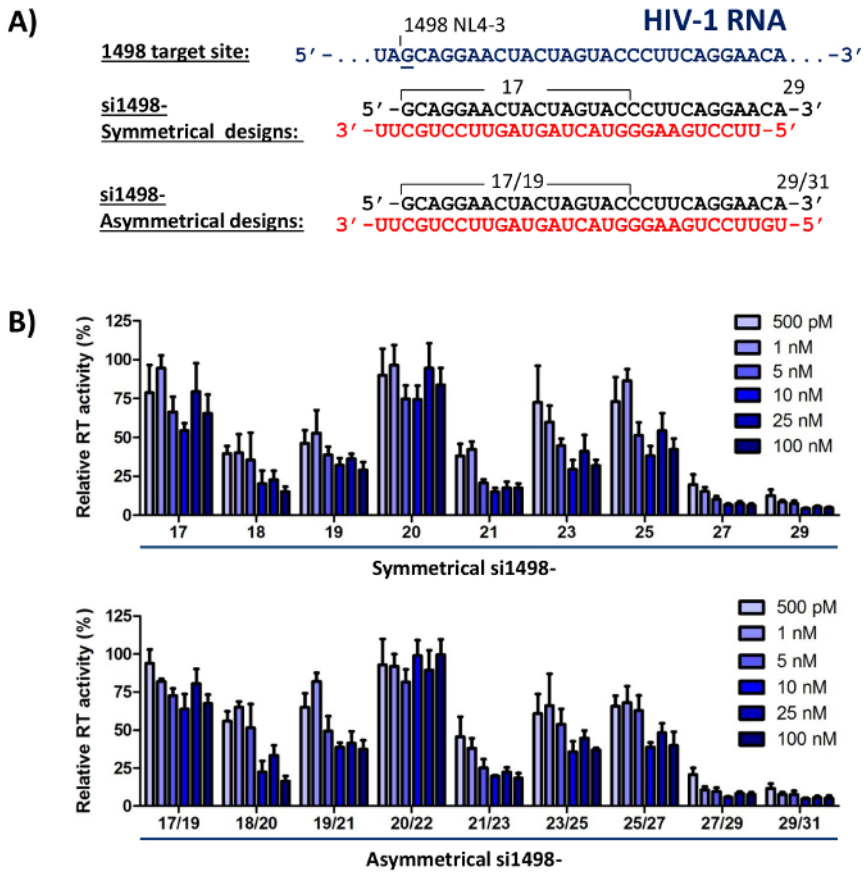


Figure 3. Effect of test siRNAs on HIV-1 production. (**A**) Design of siRNAs with different lengths and symmetries. A conserved sequence in HIV-1 RNA (1498 target site) was used to design symmetrical and asymmetrical siRNAs (si1498-) with different lengths (17 to 29 nucleotide sense strands). The expected sense (top, black) and antisense (bottom, red) strands are indicated. (**B**) Inhibition of HIV-1 production by si1498 length variants. The percent (%) inhibition of HIV-1 RT activity in the supernatant of HEK293T cells transfected with symmetrical or asymmetrical si1498 length variants is shown relative to a long Dicer substrate nonsense siRNA (siNS). Each test RNA was compared to siNS at different doses in at least two independent transfections with two to three replicates. The data are expressed as mean values ± standard error means (SEMs). This figure has been modified from[16], copyright American Society for Microbiology. Please click here to view a larger version of this figure.

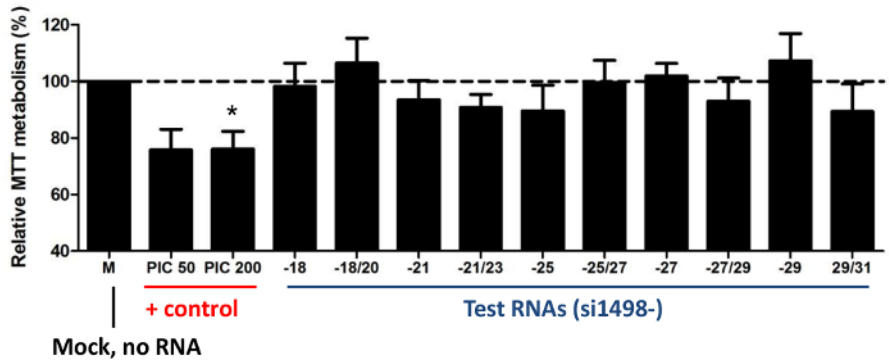


Figure 4. Effect of si1498 length variants on cell viability. HEK293T cells were transfected with poly I:C at 50 and 200 µg/ml (PIC50 and PIC200) and different lengths and formats of si1498 at 100 nM. The % metabolism of MTT was determined relative to cells treated with the transfection reagent alone (Mock, M) in three independent transfections with two to three replicates. The data are expressed as mean values ± SEMs. Unpaired student t-tests were used to determine whether the different transfections significantly (*, P <0.05) reduced cell viability relative to the mock transfected cells. This figure has been modified from[16], copyright American Society for Microbiology. Please click here to view a larger version of this figure.

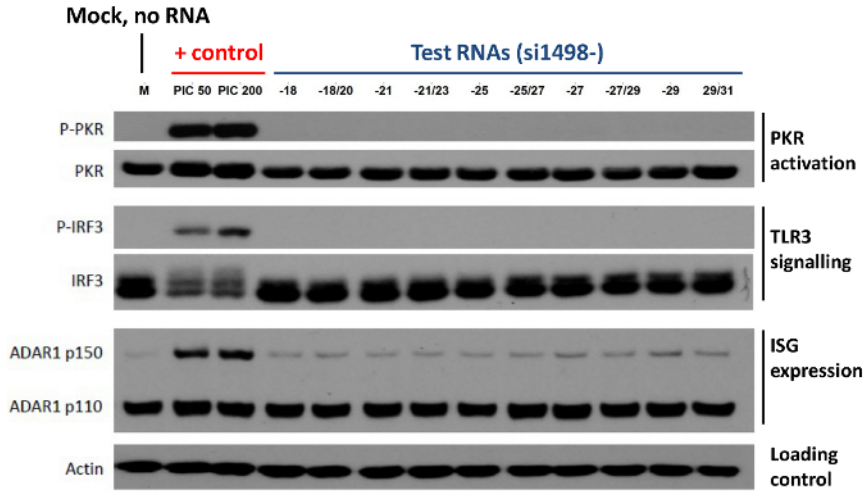


Figure 5. Effect of si1498 length variants on innate immune responses. HEK293T cells were transfected with PIC50, PIC200 and different lengths and formats of si1498 at 100 nM. Activation of PKR and TLR3 were evaluated by measuring phosphorylated PKR and IRF3, respectively. Expression of an interferon stimulated gene (ISG) was evaluated by measuring the ISG ADAR1 p150 levels relative to the constitutively expressed variant, ADAR1 p110. The expression of actin was used as a loading control. This figure has been modified from[16], copyright American Society for Microbiology. Please click here to view a larger version of this figure.

Name of Buffer / Reagent	Composition
Viral disruption cocktail	60 mM Tris-HCl (from 1 M Tris-HCl, pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 1.04 mM EDTA, 1% NP-40.
Radioactive cocktail	60 mM Tris-HCl (from 1 M Tris-HCl, pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 1.04 mM EDTA, 10 µg/ml Poly(A), 0.33 µg/ml oligo dT. Added immediately before use: 8 mM dithiothreitol (DTT, $C_4H_{10}O_2S_2$) and 5 µl [^{32}P] dTTP (3,000 Ci/mmol) for each 500 µl of cocktail.
Radioactive/viral disruption cocktail	60 mM Tris-HCl (from 1 M Tris-HCl, pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 1.04 mM EDTA, 0.1% NP-40, 5 µg/ml Poly(A), 0.16 µg/ml oligo dT. Added immediately before use: 8 mM dithiothreitol (DTT, $C_4H_{10}O_2S_2$) and 5 µl [^{32}P] dTTP (3,000 Ci/mmol) for each 1 ml of cocktail.
2x SSC	17.53 g NaCl and 8.82 g sodium citrate - $2H_2O$ in 1 L H_2O.
Lysis buffer	50 mM Tris-HCl (from 1 M Tris-HCl, pH 7.4), 150 mM NaCl, 5 mM EDTA (from 0.5 M, pH 8.0), 10 % v/v glycerol, 1 % v/v NP-40.
Ponceau S	2.5 g Ponceau S and 5 ml acetic acid in 500 ml H_2O.
TBST	6.05 g Tris, 8.76 g NaCl and 1 ml Tween 20 in 1 L H_2O.

Table 1: Components of non-commercial buffers and reagents. Recipes for all non-commercial buffers and reagents are provided.

Discussion

The HIV-1 production assay described was performed using HEK293T cells (**Figure 2**) and is similar to assays used to screen HIV-1 RNA for effective ribozyme[13], shRNA[10,29], siRNA[30], and U1i RNA[11,31] target sites. Using different methods to quantify HIV-1 production, most studies have measured viral production 48 hr after co-transfection of an HIV-1 expression plasmid with candidate RNAs. Following the production of HIV-1, immature virions undergo proteolytic cleavage of their polyproteins by the HIV-1 protease to become mature virions, capable of infecting new cells. For the HIV-1 RT enzyme activity assay described in steps 2.2. to 2.5., both the production and maturation steps are evaluated, since the RT enzyme is only active in mature virions. In contrast, the capsid protein and viral RNA may be present in both mature and immature virions. Therefore, quantifying HIV-1 production by p24 ELISA or RT-PCR may miss effects of therapeutic RNAs that act on the maturation step of the HIV-1 replication cycle, such as ribozymes that localize to HIV-1 virions[32] and antisense-based molecules that inhibit Gag processing in addition to HIV-1 protein expression[13,31]. A limitation of viral production assays is that the cells used do not express the appropriate receptors for HIV-1 entry and cannot be used to evaluate the effects of therapeutic RNAs on HIV-1 entry or integration.

To evaluate the effects of anti-HIV-1 RNAs on the entire replication cycle, various HIV-1 infection models have been used in different T lymphocyte cell lines or primary blood cells[5,33]. Since these cell lines are difficult to transfect, more labor intensive methods such as lentiviral vector gene insertion or aptamer/peptide conjugation are necessary to deliver sufficient amounts of anti-HIV-1 RNAs to observe effects on HIV-1 replication. This limitation makes it difficult to rapidly compare the structure-activity relationship between variants of a particular anti-HIV-1 RNA or perform large-scale screening to identify the optimal target site for new classes of anti-HIV-1 RNAs. While viral production assays have been

useful for identifying new anti-HIV-1 RNA molecules, alternative cellular models in easily transfected cell lines that support HIV-1 replication, such as TZM-bl cells, would be useful for screening anti-HIV-1 RNAs targeting other steps in the replication cycle, such as entry and integration.

Depending on the class of anti-HIV-1 RNA, several potential mechanisms of toxicity have been described. For example, antisense-based RNAs may have off-target effects on cellular RNAs containing the same or a similar sequence to their intended target site in HIV-1 RNA. Similarly, RNA aptamers, modeled after the trans-activation response element (TAR) or Rev response element (RRE), could affect the function of cellular proteins, such as the TAR RNA binding protein (TRBP)[34]. shRNAs and siRNAs have the added potential to affect the RNA interference pathway by sequestering RNAi proteins[35] and some U1i molecules have been shown to affect the splicing and processing of cellular RNAs by sequestering proteins of the U1 small nuclear RNA-protein complex[36]. While some of these effects can be minimized by careful design, measurements of cellular toxicity in screens for new anti-HIV-1 RNA molecules are useful in excluding molecules with potential off-target effects from further development. Furthermore, off-target effects could indirectly inhibit HIV-1 production, making cellular toxicity an important measurement in validating the efficacy of new anti-HIV-1 molecules identified from HIV-1 production screens.

The cell viability assay described in steps 3.1. to 3.4. is a variation of a standard assay that has been used to screen diverse antiviral molecules[37]. The assay measures the activity of NAD(P)H-dependent cellular enzymes to reduce the MTT reagent to its insoluble purple form, formazan. The protocol is adapted from previously published methods[38] and several kits are available using MTT or other closely related reagents. While it is important to assay cell viability in the cell line used for screening anti-HIV-1 RNAs, it should be noted that different cell lines vary in their sensitivity toward RNA-induced toxicity. For example, Reynolds *et al.* demonstrated that Dicer substrate siRNAs had no effect on cell viability in HEK293T cells, but significantly reduced cell viability in MCF7, DU145 and HeLa S3 cells[39]. For the assay described herein, HEK293T cells are used as an example (**Figure 4**); however, the same assay has also been done in MCF7 cells to evaluate potential toxicity in a cell line that is more sensitive to small RNA-induced toxicity[16].

Since anti-HIV-1 RNAs cannot be processed and presented to the adaptive immune system, they are considered less immunogenic compared to anti-HIV-1 proteins or peptides. However, depending on their sequence or structure, they can elicit innate immune responses, and several immune sensors and signaling pathways have been identified that can respond to small RNAs (reviewed in[40]). In the immune activation assay described here, the levels of phosphorylated PKR and IRF3 were compared in cells transfected with small RNAs as an indication of PKR or TLR3 activation, respectively. Both RNA sensors are present in a wide range of cell lines and their activation can lead to the production of type 1 interferons and, in the case of PKR, a shut-down in translation. To evaluate the potential for anti-HIV-1 RNAs to activate the production of type 1 interferons by alternative pathways, levels of the interferon-stimulated gene ADAR1 (p150) were also compared in cells transfected with anti-HIV-1 RNAs. As demonstrated by the effects of the long dsRNA positive control, Poly I:C, all of these responses were active in HEK293T cells (**Figure 5**) and similar effects were observed in MCF7 cells[16]. Since these responses could inhibit HIV-1 production in the absence of effects on cell viability, the immune activation assay provides additional validation for the efficacy of new anti-HIV-1 RNAs. Further measurements that could be added to this evaluation include measuring the production of inflammatory cytokines and type 1 interferons in cell culture supernatant, and measuring the expression of more interferon-stimulated genes. Since HIV target cells such as $CD4^+$ T cells and macrophages may express different levels of innate immune sensors, candidates identified from the assays described in this protocol should also be evaluated for potential immune stimulation in these cell types.

For all of the assays described, the critical step for obtaining reproducible and accurate results is the preparation of the DNA or RNA transfection tubes (step 1.3.). The plasmid DNA or RNA should be of high purity with concentrations accurately determined. It is also critical to include the appropriate controls. For evaluating new test RNA expression plasmids in the viral production assay, an appropriate negative control is the empty expression plasmid. For antisense-based RNAs, an additional negative control plasmid expressing a non-targeting RNA should also be included. Sequences for a non-targeting ribozyme and shRNA that do not affect HIV production are provided in[13]. For test siRNAs, the only negative control that can be used is a non-targeting RNA and an appropriate non-targeting siRNA sequence for the viral production assay is provided in[16]. For the cell viability and immune activation assays, the negative control should be cells treated with the transfection reagent alone and it is critical that a positive control such as Poly I:C is included to confirm that the cells are responsive to RNA-induced toxicity or immune activation. For RNA expression plasmids, the empty plasmid should also be included to ensure that the vector itself is not having toxic effects on the cells.

Overall, the assays described herein represent a good first step toward the identification of safe and effective RNA therapies for use in HIV-1 gene or drug therapy. For molecules targeting HIV-1 RNA, it is also important to consider the conservation of their target site in HIV-1 circulating strains, and detailed methods to calculate sequence conservation have been previously published[15]. To move a molecule forward into clinical trials, long-term toxicity and efficacy studies should be performed in primary human cells and in animal models to confirm that identified candidates will be safe and efficacious in the clinic.

Disclosures

The authors have nothing to disclose.

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