Effective Inhibition of HIV-1 Production by Short Hairpin RNAs and Small Interfering RNAs Targeting a Highly Conserved Site in HIV-1 Gag RNA Is Optimized by Evaluating Alternative Length Formats

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We have previously identified a target site in HIV-1 RNA that was particularly accessible to a ribozyme and a short hairpin RNA (shRNA). To design small interfering RNAs (siRNAs) targeting this site, we evaluated the effects of siRNAs with different lengths on HIV-1 production. The potency and efficacy of these siRNAs were dependent on the length of their intended sense strand with trends for symmetrical and asymmetrical formats that were similar. Although a typical canonical format with a 21-nucleotide (nt) sense strand was effective at inhibiting HIV-1 production, Dicer substrate siRNAs (dsiRNAs) with the longest lengths (27 to 29 nucleotides) were the most effective. Induction of double-stranded RNA immune responses and effects on cell viability were not detected in cells transfected with different siRNAs, suggesting that the differences observed were not related to indirect effects on HIV-1 production. For the corresponding shRNA designs, a different trend in potency and efficacy against HIV-1 production was observed, with the most effective shRNAs having stem lengths from 20 to 27 bp. Our results highlight the importance of evaluating different designs to identify the best siRNA and shRNA formats for any particular target site and provide a set of highly effective molecules for further development as drug and gene therapies for HIV-1 infection.

RNA interference (RNAi) is a highly conserved process by which small double-stranded RNAs (dsRNAs) use cellular proteins to degrade or repress a complementary target RNA. First described in 1998 (1), RNAi plays major roles in cellular defense against pathogenic RNAs and posttranscriptional gene regulation in diverse eukaryotic cells (2). In human cells, the endonuclease Dicer, in complex with the *trans*-activation response (TAR) RNA binding protein (TRBP), cleaves precursor microRNAs (pre-miRNAs) into mature miRNAs, which associate with the RNA-induced silencing complex (RISC) (3). One of the miRNA strands, called the guide or antisense strand, then directs the RISC to a complementary target in an mRNA where it can repress translation or target the mRNA for degradation. Human Dicer can also cleave complementary dsRNAs into small interfering RNAs (siRNAs) of 21 to 23 nucleotides (nt) (4, 5) that can direct the RISC protein Argonaute 2 (Ago2) to cleave a specific target RNA sequence. These Dicer products are often called canonical siRNAs and can be delivered to mammalian cells to elicit an effective and specific RNAi response (6, 7). Over 22 synthetic siRNAs have entered clinical trials targeting human and viral RNAs (8, 9), and the potential to use siRNAs to treat HIV-1 infection has been validated in preclinical mouse models using different delivery strategies (10–12).

Several noncanonical siRNAs have been designed with various nucleotide modifications and different structural formats (reviewed in reference 13). In 2005, it was shown that longer siRNAs, which can serve as the substrates for the Dicer enzyme, were up to 100 times more potent than their sequence-matched canonical formats (14). These siRNAs are called Dicer substrate siRNAs (dsiRNAs), and a common design includes a 25-nt sense strand and a 27-nt antisense strand with a blunt end and two DNA modifications at the 3' end of the sense strand (25D/27 format) (15). This format helps direct Dicer to process the desired canonical siRNA product (16, 17) and results in better RISC assembly and

more loading of the antisense strand into Ago proteins than canonical designs (18). Although several reports have observed superior efficacy of dsiRNAs over that of canonical siRNAs, one study reported no major difference in efficacy between a large set of molecules and provided evidence that dsiRNAs have greater effects on cell viability (19). In another study, dsiRNAs were shown to have major effects on cell viability in DU145, MCF7, and HeLa S3 cells compared to those of canonical designs (20). In addition to dsiRNAs, several effective siRNAs with lengths shorter than those of canonical designs have been described (21–23), and the optimal length and format of therapeutic siRNAs remains an area of debate and ongoing research.

In addition to the preclinical development of siRNAs targeting HIV-1 replication for use as drug therapies, several genes expressing short hairpin RNA (shRNA) precursors of siRNAs have been developed for use as gene therapies (reviewed in references 24 to 26). shRNA transcripts are composed of two complementary strands that form a stem, separated by a short sequence that forms a loop. They are similar in structure to pre-miRNAs, except that

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the intended antisense strand is perfectly complementary to its target RNA and the corresponding sense strand in the shRNA stem (27). shRNAs are typically expressed from RNA polymerase III type 3 promoters, which allow for a defined transcript with 2 to 4 uridines at the 3' end from the polymerase termination signal (28, 29). These transcripts are exported from the nucleus by the pre-miRNA transporter exportin 5 and processed by Dicer to yield 21- to 23-nt siRNAs. Variations in shRNA design such as different loop sequences and lengths have demonstrated that alternative structural formats can have marked effects on the efficacy of an shRNA (reviewed in reference 27). Similar to conflicting reports regarding the optimal length of therapeutic siRNAs, some studies have reported that longer shRNAs are more effective gene silencers (30–32), while others have reported that shorter shRNAs are superior (33). In a study evaluating shRNAs with different stem lengths targeting several sites in HIV-1 RNA, McIntyre et al. concluded that there is no fixed correlation between stem length and suppressive activity (34), suggesting that empirical data are necessary to identify the optimal format for any particular target site.

We previously screened HIV-1 RNA for conserved ribozyme target sites (35, 36) based on the specific on/off adaptor (SOFA) hepatitis delta virus (HDV) ribozyme motif (37–39). A target site in the Gag coding sequence was identified that was highly conserved and accessible to a ribozyme and an shRNA (36). We named this target site Gag 1498 based on its starting position in HIV-1 NL4-3 DNA (GenBank accession number M19921). Our results suggested that an shRNA targeting this site had therapeutic potential similar to that of an shRNA targeting a site in the Tat-Rev coding sequence starting at position 5983, which has advanced to clinical trials for HIV-1 gene therapy (40; ClinicalTrials.gov registration no. NCT01961063). In this study, we evaluated the antiviral effects of several different siRNAs and shRNAs targeting the Gag 1498 site to identify the most effective formats for further development. Our results show that for the Gag 1498 target site, 27- to 29-nt-long dsRNAs have HIV-1 inhibitory effects superior to those of the typical 25-nt dsRNA format and that they have an increased potency and efficacy over the canonical siRNA format. We also demonstrate that this increase is not due to effects on cell viability or induction of dsRNA immune responses. Finally, we show that siRNAs and shRNAs targeting the same sequence in HIV-1 RNA follow a different length-dependent trend for potency and efficacy against HIV-1 production. Overall, our results provide a set of effective siRNAs and shRNAs targeting the Gag 1498 site for further therapeutic development and highlight the importance of obtaining empirical data to identify the optimal length format for a particular target site.

MATERIALS AND METHODS

siRNA sequences and construction of shRNA vectors. All siRNAs used in this study were purchased from GE Dharmacon (Lafayette, CO). The sequences for sense and antisense strands are provided in Table S1 in the supplemental material. Sequences for si5983-27/29D were obtained from a published sequence called *tat/rev* siRNA (site I [*tat/rev*] 27 mer) (10) and the nonsense siRNA, siNS-25D/27, was adapted from a previous nonsense siRNA called siControl (41). All shRNAs were expressed from the human RNase P H1 promoter in the vector psiRNA-hH1GFPzeo (InvivoGen, San Diego, CA). sh1498-20 and shNS were from our previous study (shRNA1498 and shRNAs, respectively) (36). New shRNA inserts were generated by annealing complementary sense and antisense oligonucleotides at 1.25 μ M each in 40 μ l of 75 mM NaCl for 2 min at 80°C, followed by slowly decreasing the temperature to 37°C. All oligonucleotides were

purchased from Life Technologies (Carlsbad, CA). Sequences for sense and antisense strands are provided in Table S2 in the supplemental material. Inserts were ligated into BbsI-digested psiRNA-hH1GFPzeo and correct insertion into the vector was confirmed by sequencing using a primer located upstream in the vector 5'-TCTACGGGGTCTGACGC-3'.

Cells and transfections. HEK293T and MCF7 cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium with high glucose (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone), 50 U/ml penicillin, and 50 μ g/ml streptomycin (Life Technologies, Carlsbad, CA). Cotransfection of cells with HIV-1 molecular clone pNL4-3 and siRNAs or shRNA-expressing plasmids were performed in 24-well plates seeded 24 h prior to cotransfection at 1×10^5 cells per well. Transfection with siRNAs was performed with 1.5 μ l of DharmaFECT 1 (Dharmacon, Lafayette, CO), and transfection with shRNAs was performed with 1.5 μ l of TransIT (Mirus, Madison, WI) according to the manufacturer's instructions. For cotransfection with shRNAs, an equal amount of DNA was used by topping up lower dose transfections with an irrelevant plasmid [pBluescript SK(+); Stratagene, La Jolla, CA].

For cell viability and protein expression assays, cells were seeded 24 h prior to transfection in 96- and 12-well plates, respectively. HEK293T and MCF7 cells were plated at 2×10^5 cells/ml and transfected with siRNAs or low molecular weight poly(I)-poly(C) (poly[I-C] LMW; InvivoGen, San Diego, CA) using 0.4 μ l (96-well plate) or 3 μ l (12-well plate) of DharmaFECT 1 (Dharmacon, Lafayette, CO). For all transfection experiments, assays were performed 48 h after transfection.

HIV-1 production assay. HIV-1 production was estimated by measuring the activity of the HIV-1 reverse transcriptase (RT) enzyme in culture supernatants of cotransfected cells, as previously described (35). Briefly, 5 μ l of cell supernatant was incubated with a poly(A) template (Roche, Basel, Switzerland), an oligo(dT) primer (Life Technologies, Carlsbad, CA), and [32 P]dTTP (3,000 Ci/mmol; PerkinElmer, Waltham, MA) for 2 h at 37°C in 50 μ l total reaction mixture. Five microliters of the reaction mixture was then spotted onto a DEAE filter mat (PerkinElmer) and washed five times in $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, followed by two washes in 95% ethanol to remove [32 P]dTTP not incorporated into the poly(dT) RT product. Counts per minute (cpm) were calculated for each sample using a microplate scintillation counter (MicroBeta TriLux; PerkinElmer) and are proportional to the amount of HIV-1 RT enzyme present in the reaction mixture.

Cell viability assay. Cell viability was estimated by measuring the metabolism of MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) (Sigma-Aldrich, St. Louis, MO) in cell cultures, as previously described (42). Briefly, cells were incubated for 3 h at 37°C with MTT, followed by the addition of acidified isopropanol to solubilize the blue crystals formed due to the conversion of MTT to formazan by cellular enzymes. Plates were then incubated for 2 h at room temperature and read at 570 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). To evaluate statistical significance, values for RNA-transfected cells were compared to mock-transfected cells using unpaired Student *t* tests (Microsoft Excel).

Protein expression assay. Cell lysates were obtained 48 h after cotransfection in lysis buffer containing antiproteases and antiphosphatases (Roche, Basel, Switzerland). Seventy-five micrograms of total protein was resolved on a 10% denaturing polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, United Kingdom) as previously described (43). Membranes were incubated first with anti-protein kinase R (PKR) (phospho-T446) E120 (Abcam, Cambridge, United Kingdom), anti-IRF3 (phospho S396) 4D4G (Cell Signaling, Danvers, MA), and anti-ADAR1 (from B.L. Bass) at a 1/1,000 dilution. Membranes were stripped by incubation with Re-Blot Plus Strong (Millipore, Billerica, MA) for 10 min and then incubated with anti-PKR 71-10 (from A. Hovanessian) at a 1/500 dilution, anti-IRF3 D614C (Cell Signaling) at a 1/1,000 dilution, and anti-actin C4 (Millipore) at a 1/5,000 dilution. Following primary antibody incubation,

FIG 1 Inhibition of HIV-1 production by different Dicer substrate siRNA (dsiRNA) designs targeting the Gag 1498 and Tat-Rev 5983 sites in HIV-1 strain NL4-3. (a) Sequences of the different dsiRNA designs targeting the Gag 1498 and Tat-Rev 5983 sites in HIV-1 RNA. The intended sense strands are on top in black, and the intended antisense strands are on the bottom in red. DNA nucleotide modifications are indicated by blue lowercase letters. (b) Comparison between dsRNAs 1498 and 5983 effects on HIV-1 production. HEK293T cells were cotransfected with HIV-1 pNL4-3 plasmid DNA (100 ng) and one of the indicated dsRNAs at 25 or 100 nM. Forty-eight hours after cotransfection, supernatants were harvested, and the activity of HIV-1 RT was determined. Data are expressed as percentages of the values obtained for cotransfection with a nonsense siRNA (siNS-25D/27) tested in parallel, shown as relative RT activity. (c) Influence of length and modifications of dsiRNA 1498 effects on HIV-1 production. HEK293T cells were cotransfected with HIV-1 pNL4-3 plasmid DNA (100 ng) and one of the indicated dsRNAs at 500 pM to 100 nM. Relative RT activity was determined as described for panel b. For each concentration of dsiRNA tested, at least two independent experiments with two or three replicate transfections were performed. Data are expressed as the means $\pm$ standard errors of the means (SEM) ($n = 4$ to 6).

membranes were incubated with anti-mouse or anti-rabbit IgG-horse-radish peroxidase (GE Healthcare) at a 1/5,000 dilution for 2 h. Bands were visualized with ECL (GE Healthcare).

RESULTS

dsiRNAs targeting the Gag 1498 site are effective inhibitors of HIV-1 production. To evaluate whether siRNAs targeting the Gag 1498 site described in our previous study (36) can provide effective inhibition of HIV-1 production, we designed two dsiRNAs (si1498-25D/27 and si1498-27/29D). dsiRNAs were named according to the lengths of their intended sense and antisense strands with a D to indicate the strand with two DNA modifications at its 3' end (Fig. 1a, left). si1498-25D/27 followed previously described design principles for dsiRNAs (15), while si1498-27/29D followed a design used for a dsiRNA targeting a sequence in HIV-1 starting at position 5983 in the overlapping Tat-Rev coding sequence of HIV-1 RNA (10). Design-matched siRNAs targeting the Tat-Rev coding sequence (si5983-25D/27 and si5983-27/29D) were used as positive controls (Fig. 1a, left). The effects of siRNAs on HIV-1 production were evaluated by cotransfection of HEK293T cells with HIV-1 molecular clone pNL4-3 and different concentrations of siRNAs. HIV-1 production was estimated by measuring the activity of the HIV-1 RT enzyme in culture supernatants, and data were expressed as percentages of RT activity in cells cotransfected with a nonsense siRNA (siNS-25D/27). All siRNAs dose dependently inhibited HIV-1 production (Fig. 1b).

While the two si5983 designs had similar effects on viral production, the longer si1498-27/29D format was more effective than the si1498-25D/27 format, representing the typical dsiRNA design.

To evaluate the effects of dsiRNA designs that more closely resemble the expected transcription products for shRNAs, si1498-25/27, and si1498-27/29 were designed with two uridines at the 3' end of their intended antisense strands and no DNA modifications (Fig. 1a, right). The potencies and efficacies of si1498-25/27 and si1498-27/29 were indistinguishable from those of the modified designs (si1498-25D/27 and si1498-27/29D) (Fig. 1c), suggesting that the identity of nucleotides in the 3' antisense strand overhang and DNA base modifications are not important determinants for inhibition of HIV-1 production. Rather, the effects of the modified and unmodified designs were dependent on the siRNA length, with si1498-27/29 and si1498-27/29D providing more effective inhibition of HIV-1 production than that provided by si1498-25/27 and si1498-25D/27 (Fig. 1c). As dsiRNA designs with uridines in the 3' antisense strand overhang and no DNA modifications were effective inhibitors of HIV-1 production, this format was used to compare the effects of length on HIV-1 production for different siRNAs targeting the Gag 1498 site.

dsiRNAs targeting the Gag 1498 site with sense strand lengths of at least 27 nucleotides provide improved inhibition of HIV-1 production compared to a canonical design. The typical canonical siRNA design is symmetrical with two 3' nucleotide

FIG 2 Symmetrical and asymmetrical siRNAs with sense strand lengths of 27 and 29 nt targeting the Gag 1498 site are more effective inhibitors of HIV-1 production than the canonical design. (a) Symmetrical and asymmetrical designs for siRNAs with different lengths targeting the Gag 1498 site. The intended sense strands are on top in black, and the intended antisense strands are on the bottom in red. The sequences for si1498-29 and -29/31 are shown, and the positions of si1498-17 and -17/19 are illustrated within the longer siRNAs. Expected sites of Dicer processing for longer siRNAs are illustrated with blue arrows. (b, c) Effects of si1498 length and symmetry on HIV-1 production. HEK293T cells were cotransfected with HIV-1 pNL4-3 plasmid DNA (100 ng) and one of the indicated symmetrical (b) or asymmetrical (c) siRNAs at 500 pM to 100 nM. Relative RT activity was determined as described for Fig. 1b. For each concentration of siRNA tested, at least two independent experiments with two or three replicate transfections were performed. Data are expressed as means $\pm$ SEM ($n = 4$ to 6).

overhangs on each strand, while the recommended dsRNA design is asymmetrical with a blunt end at the 3' nucleotide of its intended sense strand (15) (Fig. 1a). To compare the effects of length on siRNAs targeting the Gag 1498 site, a set of symmetrical and asymmetrical siRNAs were designed with intended sense strand lengths of 17 to 29 nt (Fig. 2a). Antisense strands were designed to be complementary to the target sequence along their entire lengths except for the terminal uridine in their 3' overhang. The trends in potency and efficacy for siRNAs targeting the Gag 1498 site were similar for symmetrical and asymmetrical designs and depended on the length of their intended sense strand (Fig. 2b and c). Most siRNAs provided a dose-dependent inhibition of HIV-1 production; however, siRNAs with sense strand lengths of 17 and 20 nt had low or no efficacy at the concentrations tested. The most effective siRNAs had sense strand lengths of 27 and 29 nt, and these molecules provided the greatest potency and efficacy against HIV-1 production.

si1498 length variants do not affect cell viability or induce dsRNA immune responses in HEK293T cells. A major concern

for the development of siRNA therapeutics is their potential to stimulate dsRNA immune responses, as this can confound the interpretation of experimental results and limit their potential for development as therapeutics (reviewed in reference 44). To determine whether or not the increases in efficacy and potency observed for longer siRNAs targeting the Gag 1498 site were related to toxicity in HEK293T cells, we evaluated the potential for different effective siRNAs to affect cell viability. siRNAs were transfected into HEK293T cells at 100 nM, and their effects were compared to mock-transfected cells and cells transfected with a low-molecular-weight (200 to 1,000 bp long) dsRNA control (poly[I-C]). Cell viability was estimated using a standard MTT colorimetric assay, and data were expressed as relative MTT metabolism compared to mock-transfected cells (Fig. 3a). A minor decrease in cell viability compared to that of mock-transfected cells was apparent for the long dsRNA control, poly(I-C). However, it was only statistically significant at the higher concentration tested (200 μ g/ml). No significant differences in cell viability between mock-transfected and siRNA-transfected cells were observed.

FIG 3 si1498 variants do not affect cell viability, dsRNA immune sensor activation, or interferon gene stimulation in HEK293T cells. (a) Effects of si1498 variants on cell viability. HEK293T cells were mock transfected (M), transfected with 50 or 200 μ g/ml poly(I-C) (PIC 50 and PIC 200), or transfected with 100 nM concentrations of the indicated si1498 variants. Cell viability was estimated by measuring the metabolism of MTT 48 h following transfection. Data are expressed as relative MTT metabolism compared to mock-transfected cells tested in parallel. Three independent experiments with two or three replicate transfections were performed. Data are expressed as means $\pm$ SEM ($n = 7$). Results from unpaired Student *t* tests are shown above each condition that demonstrated a significant decrease over that of the mock-transfected condition (*, $P < 0.05$). (b) Effects of si1498 variants on dsRNA responses. HEK293T cells were transfected as described for panel a, and 48 h after transfection cell lysates were blotted for expression of phosphorylated PKR and IRF3 (P-PKR and P-IRF3), total PKR and IRF3, ADAR p110 and p150, and actin.

In the absence of effects on cell viability, the activation of dsRNA immune responses has the potential to inhibit HIV-1 production and confound the interpretation of experimental results (44). To evaluate the potential for si1498 length variants to activate the dsRNA immune sensors PKR and Toll-like receptor 3 (TLR-3), we next measured levels of phosphorylated PKR and IRF3 (a downstream response to TLR-3 activation) in transfected HEK293T cells. The expression of the interferon-stimulated gene (ISG) ADAR1 p150 was also evaluated to compare potential induction of interferon responses (Fig. 3b). Under conditions where transfection with the long dsRNA control (poly[I-C]) induced phosphorylation of PKR and IRF3 as well as expression of ADAR1 p150, no significant effects were observed in cells transfected with si1498 variants. These results suggest that the differences in anti-HIV-1 effects observed for the si1498 variants in Fig. 2 were related to differences in RNA silencing as opposed to off-target effects on cell viability, dsRNA immune sensor activation, or interferon-stimulated gene expression.

si1498 length variants have minor effects on cell viability and dsRNA immune responses in MCF7 cells. It has been suggested that the length threshold for dsRNA immune responses is cell-type dependent, and reductions in cell viability for dsRNAs

(25/27 format) compared to those of canonical siRNAs have been observed in MCF7, DU145, HeLa S3 (20), and HeLa SS6 (19) cells. For si1498 length variants, cell viability was assessed in MCF7 cells (Fig. 4a). Compared to HEK293T cells (Fig. 3a), MCF7 cells were slightly more sensitive to the toxic effects of poly(I-C), and minor decreases in cell viability were observed in siRNA-transfected cells with significant decreases observed for si1498-21/23 and si1498-27/29 variants. While activation of PKR and TLR-3 was not detected in MCF7 cells transfected with si1498 variants, minor effects on the expression of the ISG ADAR p150 were observed in some conditions (Fig. 4b). As longer siRNAs did not elicit more pronounced effects on cell viability or ADAR p150 expression than those of shorter siRNAs, these results provide evidence that the more effective longer siRNAs do not have greater potential to elicit innate immune responses in different cell types. Experiments in additional cell types are planned to confirm that the potential of longer siRNAs to be used safely in a therapeutic setting is equal to that of shorter siRNAs.

shRNAs targeting the Gag 1498 site with hairpin stems of 20 to 27 bp provide the most effective inhibition of HIV-1 production. The shRNA targeting the Gag 1498 site in HIV-1 RNA identified in our previous study (shRNA1498) (36) had a 20-bp stem

FIG 4 si1498 variants have moderate to no effect on cell viability, dsRNA immune sensor activation, and interferon gene stimulation in MCF7 cells. (a) Effects of si1498 variants on cell viability. MCF7 cells were mock transfected (M), transfected with 50 or 200 μ g/ml poly(I-C) (PIC 50 and PIC 200), or transfected with 100 nM concentrations of the indicated si1498 variants. Cell viability was estimated by measuring the metabolism of MTT 48 h following transfection. Data are expressed as relative MTT metabolism compared to mock-transfected cells tested in parallel. Three independent experiments with two or three replicate transfections were performed. Data are expressed as means $\pm$ SEM ($n = 7$). Results from unpaired Student *t* tests are shown above each condition that demonstrated a significant decrease from that of the mock-transfected condition (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (b) Effects of si1498 variants on dsRNA responses. MCF7 cells were transfected as described for panel a, and 48 h after transfection cell lysates were blotted for expression of phosphorylated PKR and IRF3 (P-PKR and P-IRF3), total PKR and IRF3, ADAR p110 and p150, and actin.

with two to four uridines at its 3' end from the RNA Pol III H1 promoter termination signal (named sh1498-20 in this study). To determine whether the effects of sh1498-20 (Fig. 5a, top) can be improved by introducing target-matched nucleotides at its 3' antisense overhang, we designed sh1498-20-3'c (Fig. 5a, bottom). Consistent with results comparing siRNAs with perfectly (si1498-25D/27 and si1498-27/29D) and imperfectly (si1498-25/27 and si1498-27/29) matched antisense strand 3' nucleotide overhangs (Fig. 1c), the efficacies and potencies of the two shRNAs against HIV-1 production were nearly identical (Fig. 5b). As the addition of perfectly matched nucleotides did not improve the effects of sh1498-20, the original format with imperfectly matched nucleotides in the 3' overhang was used to compare the effects of sh1498 variants with different stem lengths on HIV-1 production.

To determine the optimal stem length for different shRNAs targeting the Gag 1498 site, a set of shRNAs was designed based on sh1498-20 with stem lengths of 17 to 29 bp (Fig. 5c). The loop sequence followed a previously described format of 8 nt, where the nucleotide N is complementary to the target site (45). Contrasting results for siRNAs (Fig. 2), shRNAs with stem lengths below 20 bp were not effective inhibitors of HIV-1 production (Fig. 5d). Beyond 20 bp, a trend toward decreasing potency and efficacy was observed as the stem length was increased to 29 bp.

DISCUSSION

HIV-1 RNA represents an attractive target for the therapeutic development of siRNAs or shRNAs, and more than 750 molecules have been designed with diverse target sites (46). A primary consideration for the design of these molecules is that their target sequence is highly represented in HIV-1-infected populations and that it is accessible enough to allow for the development of effective siRNA or shRNA molecules. Results from our previous study suggested that a novel target site in the Gag coding sequence of HIV-1 RNA was highly conserved across circulating HIV-1 strains and accessible in diverse viral strains and that an shRNA (shRNA1498) targeting it was an effective inhibitor of viral production with low potential to affect human mRNA expression (36). In this study, our aim was to determine the optimal format for siRNAs and shRNAs targeting this site for further development as drug and gene therapies, respectively.

The first study to demonstrate gene silencing by siRNAs in mammalian cells used a canonical format (-21) with two thymidine residues on the 3' overhang of each strand (-21tt) (6). The optimal format of the overhangs was shown to be dependent on their length (2 nt) rather than their identity (target matched, RNA or DNA nucleotides) (47), and the -21tt format became the indus-

FIG 5 shRNAs with 20- to 27-bp stems targeting the Gag 1498 site are the most effective inhibitors of HIV-1 production. (a) Schematic representation of sh1498-20 with a 3' complementary antisense overhang (sh1498-20-3'c). The sh1498 design used in our previous study (36) and the modified sh1498-20-3'c with nucleotides in its 3' antisense overhang complementary to the target RNA are illustrated. (b) Effects of sh1498-20 and sh1498-20-3'c on HIV-1 production. HEK293T cells were cotransfected with 100 ng of pNL4-3 DNA and 10 to 400 ng of shRNA-expressing plasmids. Relative RT activity is expressed as a percentage of the value obtained for cotransfection with an empty shRNA expression vector tested in parallel. Each concentration of the different shRNAs was evaluated in at least two independent experiments with two or three replicate transfections. Data are expressed as means $\pm$ SEM ($n = 4$ to 6). (c) Schematic representation of sh1498 length variants. The intended sense strand is on top in black, and the intended antisense strand is on the bottom in red. The sequence for sh1498-29 is shown, and the position of sh1498-17 is illustrated within the longer shRNA. Expected sites of Dicer processing for longer shRNAs are illustrated with blue arrows. For each length variant, the nucleotide N was complementary to the next position in the target site. (d) Effects of sh1498 length variants on HIV-1 production. shRNAs were evaluated for effects on HIV-1 production as described for panel b. For each concentration of shRNA, at least two independent experiments were performed with two or three replicates, and data are expressed as means $\pm$ SEM ($n = 4$ to 6).

try standard for gene silencing studies. For therapeutic development, several nucleotide modifications at specific internal positions have been shown to enhance the stability of siRNAs without affecting their RNAi activity (48, 49). A comprehensive analysis of modifications to the 3' overhang in the antisense strand of canonical siRNAs showed that, although similar in short-term gene silencing, target-matched RNA nucleotides were superior to thymidine residues in these positions (50), and similar results have been observed for dsRNAs (17). Our results comparing si1498 variants (Fig. 1a and c) and sh1498 variants (Fig. 5a and b) with different nucleotides in the 3' overhang of the antisense strands suggested that the identity of nucleotides in these positions is not an important determinant for effective gene silencing. However, small differences may have been missed because of the short time course of the experiments (48 h), and future studies to determine the optimal identity of nucleotides in these positions are planned.

Although symmetrical siRNAs are commonly used, Sano et al. showed that asymmetrical siRNAs with blunt end modifications at the 3' end of the sense strand can improve selection of the intended antisense strand by the RISC (51). It was also demonstrated that for dsRNAs, the blunt end format at the 3' end of the

sense strand enhances Dicer processing to yield a more homogeneous population of intended siRNA products (16). Our results for shorter and longer siRNAs targeting the Gag 1498 site showed that blunt end modifications do not affect their potency or efficacy against HIV-1 production, and instead, their inhibitory effects were dependent only on the length of their sense strands (Fig. 2). As with effects of the nucleotide identity in the 3' antisense overhang, benefits of the blunt end modification may have been missed due to the short time course of the experiments.

Our results are consistent with previous observations (14, 16, 18) suggesting that longer siRNAs that can act as the substrates for the Dicer enzyme are superior to their sequence-matched canonical formats (si1498-21) (Fig. 2). It has been postulated that, by engaging the Dicer complex, dsRNAs can more efficiently enter into effective RISC complexes (14), and optimal design principles for dsRNAs have been described (15). Our results suggest that for the Gag 1498 target site, the standard dsRNA format (si1498-25/27) may not be long enough to engage Dicer and observe an increase in gene silencing over the canonical format (si1498-21). As several studies have reported highly effective -25/27 dsRNA formats and only one target site was evaluated in this study, we can-

not draw conclusions on the frequency of sequences that would benefit from longer -27/29 and -29/31 dsRNA formats. However, our results suggest that conflicting conclusions reported by Foster et al., showing no benefit of dsRNAs in gene silencing (19), may have been a result of the -25/27 format used rather than the dsRNA concept itself. For the Gag 1498 target site, our results demonstrate that dsRNAs with a sense strand length of at least 27 nt provide the greatest potency and efficacy against HIV-1 production compared to shorter siRNAs. We also provide evidence that the potency and efficacy of different siRNA length variants were not related to differential effects on cell viability, activation of immune sensors, or expression of an interferon-stimulated gene (Fig. 3 and 4).

Studies have shown that lower thermodynamic stability (less GC content) at the 5' end of the intended antisense strand of a canonical siRNA can improve gene silencing by enhancing its selection over the sense strand into effective RISC complexes (52, 53). Therefore, we cannot exclude that the increased effectiveness of longer siRNAs in our study was due to the generation of additional siRNA Dicer products with better thermodynamic properties than those of the intended canonical format. While further studies will be required to elucidate the mechanism behind the observed differences in HIV-1 inhibition, it has been shown that the predominant Dicer product of dsRNAs with an asymmetrical format is the intended canonical siRNA (16). Based on this observation, we can suggest that at least for the asymmetrical siRNAs evaluated (Fig. 2c), the increased effects observed for the longer siRNAs were related to recruitment of the Dicer complex as opposed to the production of improved Dicer products.

As shRNAs already engage Dicer to cleave off their loop sequences, an increase in stem length would not be expected to provide the benefit of recruiting the Dicer complex proposed for longer siRNAs. In agreement, McIntyre et al. showed that for different target sites, no correlation existed between stem length and gene silencing effects (34). For shRNAs targeting the Gag 1498 site, a stem length of at least 20 bp was required to achieve an efficacious effect against HIV-1 production, and as the stem length was increased, the potency and efficacy of shRNAs showed a decreasing trend (Fig. 5c and d). Our results also show that for the Gag 1498 target site, there were major differences in anti-HIV-1 effects between siRNAs with various sense strand lengths (Fig. 2) and shRNAs with various stem lengths (Fig. 5c and d).

The use of shRNAs targeting HIV-1 RNA is currently being evaluated in clinical trials for cell transplant therapy (40; ClinicalTrials.gov registration no. NCT-01961063), and the potential to use siRNAs in drug therapy has been evaluated in preclinical mouse models (10–12). In each of these approaches, combinations of effective molecules will be required to control HIV-1 replication and prevent the development of resistant virus. In this study, we have identified several optimized candidates targeting a highly conserved and accessible site in HIV-1 RNA for use in combination RNA gene or drug therapy. Our results also support the concept that longer dsRNAs are more effective than canonical siRNAs and highlight the importance of evaluating different siRNA and shRNA lengths to identify the optimal therapeutic format for any particular target site.

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