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Efficient inhibition of human immunodeficiency virus replication using novel modified microRNA-30a targeting 3'-untranslated region transcripts

AHMAD NEJATI[1], SHOHREH SHAHMAHMOODI[1], EHSAN AREFIAN[2,3], ZABIHOLLAH SHOJA[4], SAYED-MAHDI MARASHI[1], HAMIDEH TABATABAIE[1], YAGHOUB MOLLAEI-KANDELOUS[5], MASOUD SOLEIMANI[3,6] and RAKHSHANDEH NATEGH[1]

[1]Virology Department, School of Public Health, Tehran University of Medical Sciences, Tehran 14716-13151; [2]Biotechnology Center, College of Science, University of Tehran, Tehran 14176-14411; [3]Department of Molecular Biology and Genetic Engineering, Stem Cell Technology Research Center, Tehran 19977-75555; [4]Virology Department, Pasteur Institute of Iran, Tehran 13169-43551; [5]Immunology Department, Iran University of Medical Science, Tehran 14716-13151; [6]Department of Medical Physics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran 14117-13116, Iran

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Abstract. RNA interference (RNAi)-based gene therapy is currently considered to be a combinatorial anti-human immunodeficiency virus-1 (HIV-1) therapy. Although artificial polycistronic microRNAs (miRs) can reduce HIV-1 escape mutant variants, this approach may increase the risk of side effects. The present study aimed to optimize the efficiency of anti-HIV RNAi gene therapy in order to reduce the cell toxicity induced by multi-short hairpin RNA expression. An artificial miR-30a-3'-untranslated region (miR-3'-UTR) obtained from a single RNA polymerase II was used to simultaneously target all viral transcripts. The results of the present study demonstrated that HIV-1 replication was significantly inhibited in the cells with the miR-3'-UTR construct, suggesting that miR-3'-UTR may serve as a promising tool for RNAi-based gene therapy in the treatment of HIV-1.

Introduction

Currently, ~33.2 million people are living with a human immunodeficiency virus (HIV) infection worldwide, and 2.5 million new cases occur annually, as reported by the Joint United Nations Programme on HIV and acquired immunodeficiency syndrome (AIDS) in 2011 (1). The HIV/AIDS pandemic has become a global health threat and a great deal of effort has been focused on improving the HIV treatment.

Highly-active antiretroviral therapy (HAART) is one of the greatest successes of modern medicine and brings hope to HIV-positive patients by improving their life quality and postponing AIDS; however, emergence of drug resistant variants, side effects and poor adherence demonstrates the necessity for novel therapeutic approaches (2). RNA interference (RNAi) is a highly conserved and sequence-specific post-transcriptional gene silencing mechanism in eukaryotes, which has shown the most promising results in repressing viral replication (3-5). RNAi-based gene therapy has been widely used to inhibit HIV-1 replication in cell culture systems and animal models (6,7). However, emerging HIV-1 escape mutants due to the error-prone nature of viral reverse transcriptase is a major obstacle to therapy (8,9).

Numerous efforts have been made to minimize the emergence of escape mutants. Song *et al* (10) first demonstrated the inhibition of HIV replication using synergistic small interfering RNA (siRNA) targeting cellular C-C chemokine receptor type 5 and viral p24 transcripts (10). In addition, the emergence of viral escape mutants was shown to be delayed by using a multiple short-hairpin RNA (shRNA) approach from a single vector (11). An alternative approach has been applied using the simultaneous expression of multiple siRNAs from polycistronic microRNAs (miRNAs or miRs) (12,13). Although predominantly focused on combinatorial RNAi to reduce HIV-1 viral escape mutations, the multiple RNAi approach may induce side effects, such as cell toxicity and off-target effects. It has been demonstrated that high expression levels of shRNA can induce cell toxicity due to over-saturation of RNAi (14). Furthermore, off-target effects have been shown to increase the occurrence of unwanted cellular mRNA cleavage or suppression (15).

Correspondence to: Dr Shohreh Shahmahmoodi, Virology Department, School of Public Health, Tehran University of Medical Sciences, Keshavarz Blvd, Poursina Avenue, Tehran 14716-13151, Iran
E-mail: shahmahmoodi@tums.ac.ir

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In line with previous studies (16,17), the present study investigated a specific miRNA expression vector targeting 3'-untranslated regions (3'-UTRs), which is highly conserved among the HIV subtypes A, B and C, and present in all HIV-1 transcripts. The main aim of the present study was to design an artificial miRNA with maximum efficacy and minimal side effects (18). Furthermore, the present study may strengthen the evidence in support of controlled clinical trials targeting viral replication in patients infected with HIV.

Materials and methods

Target sequence design. HIV-1 3'-UTR mRNA reference sequences (accession no. AF033819.3) were used to identify candidate target sequences using BLOCK-iT™ RNAi Designer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA USA). Candidate target sequences were subsequently analyzed with all HIV-1 subtypes in the Los Alamos National Laboratory Database (http://www.hiv.lanl.gov) in order to identify the target sequence with the optimal complete identity among the major HIV-1 subtypes, as shown in Table I. The final target sequence (8418-8438 nt) was conserved (100% identity) among HIV-1 clades A-C. Target sequence was then incorporated into the 5' and 3' arm of hsa-miR30a containing a natural loop, and the 5' and 3' flank sequences (Fig. 1A) together with HIV-1 3'UTR target sequence (Fig. 1B), which was referred to as miR-3'UTR in the present study.

UNAFold online software (http://unafold.rna.albany.edu/) was used to predict target accessibility and artificial miR-3'UTR thermodynamic stability. In order to demonstrated that miR-3'UTR was capable of acting directly on HIV sequence, scrambled sequence was designed by Genescrip Bioanformatic online tools (https://www.genscript.com). The scramble sequence was designed according to the target sequence and incorporated into the 5' and 3' arm of hsa-miR30a according to the GC percentage and the melting temperature of the target sequence (Fig. 1C). hsa-miR-29a containing HIV-1 3'UTR seed site was also used as a positive control.

Construction of plasmids. To generate an artificial miR-3'-UTR expression plasmids were generated by Metabion International AG (Steinkirchen, Germany) by annealing two forward and reverse synthetic oligonucleotide strands and introducing them into the *Hin*dIII/*Bam*HI sites of pCDNA 3 and pAc enhanced green fluorescent protein (EGFP) plasmids (Promega Corporation, Madison, WI, USA). The strands were as follows : Forward, AGCTTGGTTGCTGTTGACAGTGAGCGAATG CTAGGCGGCTGTCAAACCTGTGAAGCCACAGATGGG GTTTGACAGCCGCCTAGCATTTGCCTACTGCCTCGGA CTTG and reverse, GATCCAAGTCCGAGGCAGTAGGCA AATGCTAGGCGGCTGTCAAACCCCATCTGTGGCTTC ACAGGTTTGACAGCCGCCTAGCATTCGCTCACTGTCA ACAGCAACCAA. A pre-miR-scrambled expression plasmid was constructed by inserting the annealed forward and reverse strand scrambled oligonucleotides. Target sequence was then synthesized chemically by adding 50 extra nucleotides to the 5' and 3' sites, followed by insertion into the cloning *Xho*I/*Not*I site of the psi-CHECK-2 vector (Promega Corporation). This construct was referred to as psi-CHECK-HIV 3'-UTR in the present study.

Cell culture and transfection. Human embryonic kidney (HEK) 293T cells were purchased from the National Cell Bank of Iran (Tehran, Iran) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml; all Thermo Fisher Scientific, Inc.) at 37˚C. The cells were trypsinized (Thermo Fisher Scientific, Inc.) 1 day prior to transfection, resuspended in DMEM without antibiotics and seeded in 24-well plates at a density of $1.2x10^5$ cells/well.

miR30a, miR-scr and pNL4-3 plasmids were transfected with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) into cells according to the manufacturer's protocol. The transfection efficiency of EGFP expression was measured by fluorescence microscopy (BX51; Olympus Corporation, Tokyo, Japan). Co-transfection with various plasmids was performed as follows. Two groups were used to test target gene expression; one was treated with pNL4-3+ miR-30a, whereas the other was treated with pNL4-3+ miR-scr. Untreated cells served as the control group. Three groups were used to assess viral production: Group 1, pNL4-3+miR-3a experimental group; group 2, pNL4-3+miR29a positive control; and group 3, pNL4-3+miR-scr negative control.

Stem-loop reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and RT-qPCR. Cells were lysed via the freeze/thaw method and total RNA was extracted from the HEK-293T cells expressing artificial miRNAs using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA). Nanodrop 2000 spectrophotometer was used for RNA quantification (Nanodrop Technologies; Thermo Fisher Scientific, Inc.). To digest unwanted DNA, 1 μg RNA was treated with DNase (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Specific artificial miR-3'-UTR stem-loop (5'-GTCGTATCCAGTGCAGGG TCCGAGGTATTCGCACTGGATACGACGTTTGA-3') and small nucleolar RNA stem loop primers (5'GTCGTATCC AGTGCAGGGTCCGAGGTATTCGCACTGGATACGACC CTTCCT-3') were designed using Primer 3 (version 4.0.0; http://bioinfo.ut.ee/primer3-0.4.0/). Using these specific primers, 100 ng of total RNA obtained from the DNase treatment was reverse transcribed into cDNA using avian myeloblastosis virus reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany). To detect artificial miR-3'-UTR, stem-loop qPCR was applied using 2 μl cDNA, specific primers (0.5 μm miR-3'-UTR forward and stem-loop reverse primers) and iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, USA) at a final volume of 20 μl. The PCR conditions were as follows: 94˚C for 15 sec, 58˚C for 30 sec and 72˚C for 30 sec for 45 cycles, followed by melting curve analysis (Rotor-Gene Q; Qiagen GmbH, Hilden, Germany) to assess the specificity of SYBR amplification. Small nucleolar RNA was used as the endogenous control.

RT-qPCR was conducted in order to measure mRNA expression. Total RNA was extracted from the transfected cells and DNase treatment was performed as described above. cDNA was synthesized using 200 ng DNase-treated total RNA and 5 μm random primer with avian myeloblastosis virus-reverse transcriptase (Roche Diagnostics GmbH) in 20 μl reaction mixture according to the manufacturer's protocol. iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) was used to measure the mRNA expression levels with an iQ master containing

Table I. Basic local alignment search tool of target sequence between clades A, B and C.

GenBank accession no.	Subtype	Query: GTTTGACAGCCGCCTAGCATT	Identities
AF538657	A1	549-5'-GTTTGACAGCCGCCTAGCATT-3'-569	100%
JN944919	B	8418--5'-GTTTGACAGCCGCCTAGCATT-3'-8438	100%
JX985439	C	555--5'-GTTTGACAGCCGCCTAGCATT-3'-575	100%

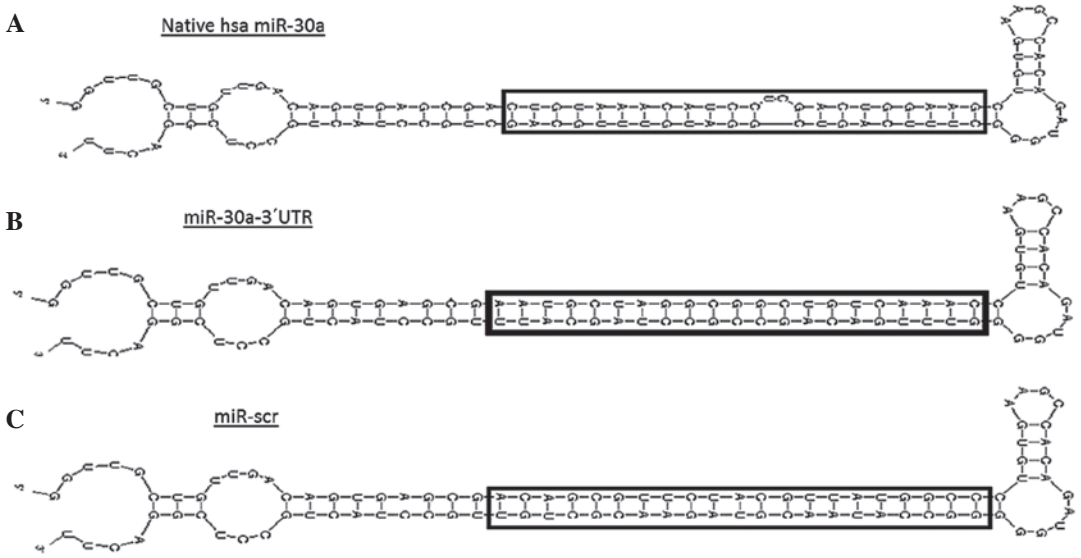

Figure 1. Schematic representation of predicted secondary structure of microRNAs. (A) Natural miR-30a sequences. (B) Artificial miR-3'-UTR. Box indicates sense and antisense 3'-UTR sequences embedded in a miR-30a backbone. (C) miR-scr. Scramble sequences incorporated in a natural miR-30a backbone sequence. miR, microRNA; UTR, untranslated region.

dNTPs, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, 0.5μl cDNA and 0.5 μM of each primer (forward, 3'UTR-GCCGCCTAGCATTTCATCAC-5' and reverse 3'UTR-GAAAGTCCCCAGCGGAAAGT-5'). PCR conditions were 94˚C for 5 min enzyme activation, followed by 94˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec with a melting curve analysis. The $2^{-\Delta\Delta Cq}$ method (19) was used to compare the HIV gene expression levels in cells exposed and unexposed to artificial miR-3'UTR. GAPDH was used to normalize the expression levels of target genes.

Dual luciferase reporter assay. Following the addition of 500 ng psi-CHECK-HIV 3'-UTR and 500 ng artificial miR-3'-UTR to Lipofectamine® 2000, plasmids were co-transfected into HEK-293T cells. To measure luciferase activity, the transfected cells were lysed 24 h post-transfection using passive lysis buffer (Promega Corporation). Firefly and Renilla luciferase activities were determined using a FB 12 tube luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) according to the manufacturer's protocol. Briefly, firefly Luciferase expression was used to normalize target specific *Renilla* luciferase expression. Relative luciferase activity levels were calculated from the ratio of Firefly to *Renilla* luciferase activity.

HIV challenge and p24 antigen assay. To assess the inhibition of artificial miR-3'-UTR on virus production, HEK-293T cells were co-transfected with 500 ng HIV-1 infectious pNL4-3 plasmid clone (Pasteur Institute of Iran, Tehran, Iran), 500 ng artificial miR-3'-UTR and 500 ng miR-scr constructs, using Lipofectamine® 2000. Virus production was measured by Elecsys 2010 (Roche Diagnostics GmbH) 2 days post-transfection. Briefly, 200 μl cell culture supernatant was collected and centrifuged at 300 x g for 10 min to monitor CA-P24 levels using an HIV Combi assay kit (Roche Diagnostics GmbH) according to the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Luciferase activity was measured using ratio comparison and the results of the Combi assay were determined using repeated analysis of variance.

Results

Assessment of expression and processing of artificial miR-3'-UTR. To examine the expression of miR-3'-UTR, EGFP levels were determined by fluorescence microscopy. After 24 h post-transfection of EGFP-miR-3'-UTR into HEK-293T cells, the cells were analyzed for EGFP expression (Fig. 2A). Stem-loop RT-qPCR was also performed on transfected HEK-293T cells to detect miR-3'-UTR expression levels (Fig. 2B). Melting curve analysis was performed to check the accuracy of the experiment (Fig. 2C). In addition,

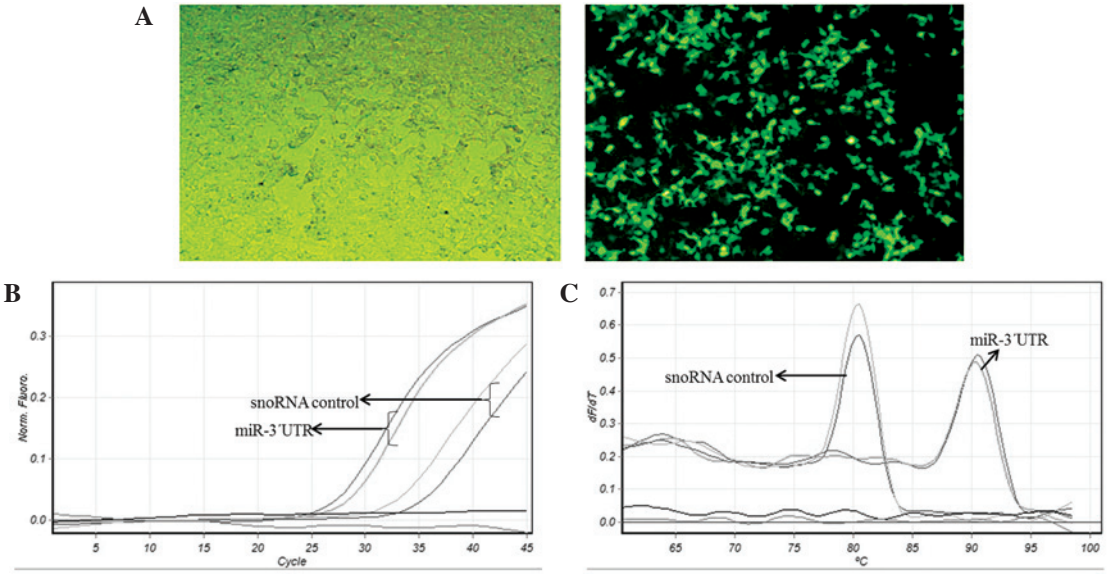

Figure 2. Expression of artificial miR-3'-UTR and snoRNA control. (A) Fluorescence imaging of EGFP expression. HEK-293T cells were transfected with pAcEGFP-artificial miR-3'-UTR. Due to the fact that artificial miR was inserted in the C terminal of the EGFP transcript, EGFP fluorescence indicated artificial miR-3'-UTR expression. (B) Stem-loop real time PCR with primers specific to the 3'-UTR was conducted to analyze the expression of artificial miR-3'-UTR, and small nucleolar RNA was used as an internal control (experiments conducted in duplicate). (C) Melting curve analysis was performed to determine the accuracy of stem-loop real time PCR products (experiments conducted in duplicate). PCR, polymerase chain reaction; miR, microRNA; EGFP, enhanced green fluorescent protein; UTR, untranslated region.

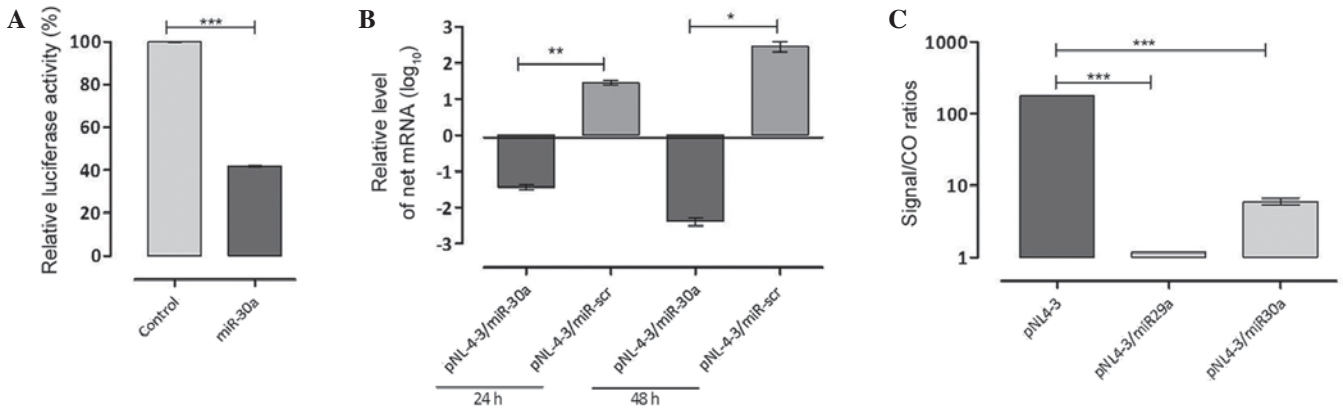

Figure 3. Inhibitory activity of artificial miR-3'-UTR. (A) A dual luciferase assay was used to determine the inhibitory efficiency of artificial miR-3'-UTR, as compared with miR-scr as the control. (B) Reverse transcription-quantitative polymerase chain reaction was used to assess 3'-UTR expression at designated time points (24 and 48 h post transfection). Total RNA was extracted from transfected cells and normalized to the internal control (GAPDH). pNL4-3/miR-30a was compared with pNL4-3/miR-Scr. (C) HEK-293T cells co-transfected with pNL4-3 and artificial miR-3'-UTR. Supernatants were collected 24 h post-transfection for the determine P24 expression levels in order to monitor viral replication. pNL4-3/miR-30a was compared with pNL4-3/miR-Scr. miR-29a was used as a positive control. *P<0.03; **P<0.02 and ***P<0.0001. miR, microRNA; UTR, untranslated region.

the expected size of the stem-loop RT-qPCR product was determined by agarose gel electrophoresis, indicating that processing by Drosha-DGCR-8 and Dicer was performed correctly, to ensure that the anticipated sequences were successfully incorporated in the RNA-induced silencing complex (RISC). No miR-3'-UTR was observed in cells transfected with empty EGFP expression plasmids.

Suppression of specific target sequences by miR-3'-UTR. The knockdown activity levels of miR-3'-UTR were measured using a luciferase activity reporter assay. A 50 nt HIV-1 3'-UTR target sequence was inserted into the 3'-UTR of the hRlu gene in psi-CHECK-2 and co-transfected with artificial miR-3'-UTR into HEK-293T cells.

Dual luciferase assay was used to monitor changes in the expression levels of *Renilla* luciferase contained within the target sequence. The specific suppression activity of miR-3'-UTR was measured by determining the *Renilla* luciferase expression, which was normalized to that of Firefly luciferase. As indicated in Fig. 3A, the artificial miR-3'-UTR was capable of significantly reducing luciferase activity levels (P<0.0001).

Inhibition of HIV-1 replication by artificial miR-3'-UTR in cell culture. To ascertain whether HIV replication was inhibited by the miR-3'-UTR construct, the functional inhibition activity of artificial miR-3'-UTR was analyzed by RT-qPCR. For this purpose, HEK-293T cells were transfected with artificial miR-3'-UTR expression plasmid and, simultaneously, challenged

with a pNL4-3 strain of HIV-1. The miR-scr expression plasmid was used as a negative control. 3'-UTR expression levels were normalized to GAPDH mRNA expression levels (Fig. 3B). The results obtained from the RT-qPCR analysis indicated that cells transfected with the pNL4-3 and miR-3'-UTR showed a significant reduction in 3'-UTR expression levels after 24 and 48 h ($P<0.001$ and $P<0.01$, respectively) compared with cells transfected with the pNL4-3 and miR-scr as a negative control.

The levels of viral capsid p24 in supernatants were subsequently determined in the transfected cells two days post-transfection to monitor virus production. To investigate the inhibitory efficiency, artificial miR-3'-UTR was co-transfected with pNL4-3. Furthermore, co-transfected hsa-miR-29a and pNL4-3 were used as a positive control, whereas transfected pNL4-3 was used as a negative control. As indicated in Fig. 3C, inhibitory activity was observed with artificial miR-3'-UTR as compared with the negative control. However, the inhibitory activity of artificial miR-3'-UTR was relatively low when compared with the miR-29a positive control.

Discussion

The advent of HAART to treat HIV-1-positive patients has improved the lives of patients infected with HIV. Although postponing the development of AIDS in patients with HIV-1 is the main advantage of these drugs (20), there are side effects associated with prolonged use of antiviral drug therapy, including drug toxicity and emerging drug resistant variants (21). Several strategies have been applied to reduce these side effects as well as to inhibit HIV-1 replication in both *in vivo* and *in vitro* systems. Among these, RNAi has recently been considered as a promising gene therapy tool (22,23). Synthetic mature siRNAs were used as first generation anti-HIV-1 RNAi-based gene therapy (24). The short duration of efficacy in mammalian cells has questioned the use of siRNAs to treat chronic diseases such as HIV-1 infection (25). Alternative strategies have been developed using shRNAs and long hairpin RNAs that are predominantly expressed by Pol III promoters, which can be transcribed from expression plasmids (26,27). High expression levels of transcripts induced by the Pol III promoter may result in competition with the cellular RNAi and saturation of the miRNA signaling pathway due to shRNA overexpression. Thus, induced cell toxicity is an obstacle to this approach in clinic trials (28). However, to overcome this problem, decreased cell death has been observed following the expression with Pol II promoter, as compared with Pol III promoter (29). To reduce cell toxicity, an anti-HIV vector under Pol II promoter was constructed in the present study, and the lowest cell toxicity was observed using an miR-3'-UTR construct.

Artificial miRNA has also been introduced as a tool for RNAi-based gene therapy, by incorporating siRNA encoding sequences into a human pre-miRNA backbone (30,31). In the present study, an hsa-pre-miR-30a backbone was used to generate siRNA against HIV-3'-UTR. As artificial miR-3'-UTR has a natural miRNA backbone, this construct was processed efficiently by nuclear microprocessor protein complexes and cytosolic Dicer proteins. The results obtained from the stem-loop RT-qPCR demonstrated that cellular miRNA backbones were able to cause efficient processing of guide strand into RISC. The error-prone nature of viral reverse transcriptase is an obstacle not only to the development of HIV drug treatments, but also to the effective design of anti-HIV RNAi. The use of mono anti-HIV siRNA is limited due to the rapid emergence of viral escape mutants (32,33). To overcome this limitation, the use of simultaneous multiple shRNAs against various conserved regions of HIV-1 has been developed. However, off-target effects are the main limitation of this approach. Increased off-target risk has been described using multiple shRNA anti-HIV strategies (34-36). To reduce the chance of mutant escape, conserved targeting sequences among different virus strains are important.

In conclusion, the use of a single anti-HIV miRNA targeting all HIV-1 transcripts can reduce off-target effects, highlighting the importance of an artificial miR-3'-UTR construct as an efficient and alternative RNA-based gene therapy. These results strengthen the evidence in support of controlled clinical trials targeting viral replication in patients infected with HIV.

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