

Original Research Article

Inhibition of Dengue Virus 3 in Mammalian Cell Culture by Synthetic Small Interfering RNAs Targeting Highly Conserved Sequences

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Abstract

Purpose: To evaluate the inhibition of Dengue virus 3 by synthetic siRNAs targeting the untranslated regions UTR and structural regions of DENV3 genome in Vero-81 cell line.

Methods: Vero-81 cells transfected with synthetic siRNAs were challenged by DENV3. The effectiveness of siRNAs was confirmed by four established virus quantification procedures. Starting with focus assay, DENV3 was quantified using anti-E antibody (Envelope), in which DENV3 was quantified by counting the number of foci per well. Initial results were then confirmed by immuno-fluorescence assay (IFA) as the number of Vero-81 cells displaying DENV3 (Envelope) E antigen had a higher fluorescent intensity in comparison to cells lacking DENV3 replication. DENV3 RNA copy numbers were quantified by real-time quantitative polymerase chain reaction RT-qPCR and in the final step supernatant of Vero-81 cells challenged with DENV3 was collected and protein analysis was performed to determine the presence of DENV3 E protein via western blot analysis

Results: A marked decrease in virus titer of DENV3 in Vero cells was observed with DV3UTR3'siRNA2 targeting the 3'UTR. Focus assay data revealed more than 70 % reduction in DENV3 in Vero-81 cells treated with DV3UTR3'siRNA2. Images showing IFA of infected Vero-81 cells exhibited a major drop in DENV3 titer in the presence of DV3UTR3'siRNA2 and DV3UTR5'siRNA1. DENV3 RNA, quantified by qPCR, DV3UTR3'siRNA2 showed 80 % reduction in DENV3 RNA level in comparison with positive control cells having higher titers of DENV3. Finally, a negligible level of DENV3 E protein was detected in the supernatant of Vero-81 cells containing DV3UTR3'siRNA2. These findings suggest that DV3UTR3'siRNA2 and DV3UTR5'siRNA1 can significantly inhibit DENV3 in mammalian cell line.

Conclusion: Overall, the results demonstrate that DV3UTR3'siRNA2 and DV3UTR5'siRNA1 can become a potential vital component of a therapeutic formulation for major anti-dengue therapy against DENV3.

Keywords: Dengue virus, siRNA, anti-E antibody, Conserve regions, Vero-81 cells

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INTRODUCTION

Dengue virus (DENV) belongs to the genus Flavivirus and are named *arboviruses*, denoting their transmission via mosquitos. DENV transmission involves two vectors *Aedes aegypti*

and *Aedes albopictus*. As per the recent valuation, approximately 50 to 100 million people are infected with DENV annually and 0.5 million progress to DHF/DSS [1]. DENV has a strand of positive sense RNA with the genome size of 11kb. DENV genome encodes a poly-protein,

which is later cleaved into ten proteins [2]. DENV has four distinct serotypes (DENV1-4), which are genetically similar yet diverse on the basis of antigenic variation. DENV serotypes are further divided into genotypes on the basis of 3 % variations within individual serotype [3].

Amongst many antiviral approaches, recent studies show that several RNA viruses are inhibited via RNA interference (RNAi) by targeting naked viral RNA [4]. RNA interference (RNAi) is a mechanism by which a certain RNA is degraded through host cellular machinery. Experimental data from various studies comprising of plasmids, synthetic inhibitors, and RNAi have shown promising results against Japanese Encephalitis virus (JEV), Dengue virus (DENV) and West Nile virus (WNV) [5,6]. In one study, genetically engineered mosquitoes were able to control DENV via RNAi where RNA interference targeted DENV RNA inside the host cell, reducing the copies of viral RNA and decreasing the overall viral load [7]. While targeting the conserved region of Envelope (E) gene in the genome of DENV 2, it was observed that this siRNA can limit DENV 2 replication in human monocytes and macrophages [8]. Synthetic siRNA is usually 19 to 21 bps in length with 3' overhangs of two nucleotides. Highly conserved sequences were later used for siRNA design via different algorithms and then in experiments to observe anti-dengue potential in cell culture [9].

The objective of this study was to target conserve sequences in DENV3 genome by introducing siRNAs (small RNA molecules) which, in turn, will chop target regions via cellular machinery. By interfering with DENV3, genome viral replication can be blocked in mammalian cell line.

EXPERIMENTAL

Viruses and cell lines

On day 1, Vero cells were grown in a growth medium comprising Dulbecco Eagle's medium (DMEM; Cellgro), 10 % heat-inactivated fetal bovine serum (FBS), and panstrep (100 units/ml penicillin and 100 g/ml streptomycin). Vero-81 cells were incubated overnight at 37 °C in a humid atmosphere while 5 % CO₂ was maintained. DENV-3 (strain H87; GenBank accession no. M93130) were obtained from Dr Desilva's laboratory in Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, USA.

Bioinformatics for siRNA design

DENV3 targeting siRNAs were designed by using Dharmacon algorithm (Dharmacon siRNA Design Center), which created optimized siRNAs to be used in anti-dengue assays [9,10]. Dharmacon algorithm generated several sequences on the bases of their likelihood to produce effective siRNAs (data not shown). Six non overlapping sequences were chosen from the top of the scoring list. For designing an siRNA that targets a highly conserved sequence in the DENV virome (conserved siRNA), alignment of complete genomes of reference strains were performed via Tcoffee online software (<http://www.tcoffee.org>). Candidate sequences were aligned with the genomic library by using blastn program. Positive-control siRNA sequence from plant source was also prepared, which was not related to DENV, as shown later in Table 1. Conclusively, to minimize unplanned hybridization, all seven siRNAs were further checked by blastn program against human genomic sequences.

Transfection (siRNA) of cell culture

Transfection for performing cell culture experiments was done by applying reverse transfection in 48-well plates. In each of the experiment, suitable amount of 10 uM siRNA from the available stock (Dharmacon Inc, Lafayette, CO) was mixed with 1 ul of RNAiMAX (Invitrogen) which was later added to 100 ul of Opti-DMEM for 30 min at room temperature [11]. Later, 100 ul of DMEM (with 2 % FBS and no antibiotics) containing 2 x 10⁵ Vero81 cells per well were added and gently mixed. The following day, the medium containing transfection mixture was removed, and virus diluted in 200 ul DMEM (containing 2 % FBS and antibiotics) was used for infecting Vero-81 cells to produce 50 to 70 foci per well in 48 well plate that was kept in CO₂ incubator for 2 h with constant shaking. After two hours of incubation, the medium (containing DENV) was removed and cells were washed twice with Opti-DMEM to completely remove unbound DENV particles concluding with adding 200 ul DMEM (containing 2 % FBS and antibiotics) in each well. Supernatants were collected 48 hours post-infection (p.i.) and FRNT analysis was performed with remaining Vero 81 cells in each well.

Focus assay

Transfected cells (siRNAs) in 48 well plates were infected with 100 ul of diluted DENV3 to obtain 40 to 50 foci per well. A diluent, DMEM (100 ul), was added to each well to completely cover the

surface area. After 1 h of incubation on a rocker, at 37 °C, cells were overlaid with 1 ml 1.0 % methylcellulose in OptiMEM (Gibco), supplemented with 2 % FBS (Cellgro) and antibiotic mix (Gibco Antibiotic-Antimycotic). On the 3rd day of incubation (37 °C, 5 % CO₂), methylcellulose containing overlay medium was removed. Subsequently, washing was performed using PBS, and cells were fixed in 80 % methanol / 1x PBS and left to air-dry. For virus foci visualization, cells were blocked with blocking buffer (5 % instant milk in PBS), followed by incubation with anti-flavivirus MAb 4G2 that was diluted 1 : 60 in blocking buffer for 1 h at 37 °C, on a rocker. PBS was used for washing the wells after which cells were incubated with horseradish peroxidase (HRP), conjugated goat anti-mouse Ab (Sigma), diluted 1 : 500 in blocking buffer for 1 h at 37 °C, on a rocker. Before the addition of 150 ul/well of TrueBlue HRP substrate (KPL), plates were given a final washing. Lastly, viral foci were counted on a light box, enabling the calculation of viral titer by established methods.

Real time PCR

RNA copies were checked by real-time quantitative polymerase chain reaction (RT-qPCR) and quantification was carried out in CFX96™ PCR detection system using the iScript™ RT-PCR kit with SYBRH Green, as shown previously by Alhoot *et al* [12]. A total of three replicates were carried out for each sample along with a template control (negative control).

Western blot analysis

Samples were subjected to electrophoresis in Bio-Rad Mini protean 3, on a 12 % separation gel. 20 ul of samples were loaded to wells of the gel and ran at 100 Volts as per the protocol. After that, the gel was transferred to nitrocellulose membrane using semi-dry method (45 V with 0.35 A). After transfer of the gel, the membrane was washed with washing buffer Tris buffer saline Tween 20 (TBST with 0.2 % Tween20) and later blocked in the blocking buffer (2 % skim milk in TBST 0.05 % Tween). Following that, the membrane was stained with primary antibody 8A1 (1:1000 dilution of 10 mg / ml 8A1 stock). Secondary staining was performed with goat anti-mouse HRP conjugated secondary antibody. Membranes were washed again 3 times and finally substrate was added to develop results on x-ray films.

Immuno fluorescence assay (IFA)

Micro-centrifuge tubes were labeled for each well, and cells were scrapped within an area of 1.5 x 1.5 cm. 1 mL of supernatant was used to briefly wash the scrapped area, after which cells were pelleted in the micro-centrifuge tubes for 3 minutes, at 2500 x g. A 10 uL spot of resuspended pellet was made on each well, with even spreading, without scraping the poly-L-lysine treated surface (coated with 0.0005 % poly-L-lysine diluted in 1X PBS). The slides were air dried in the hood and incubated on ice for 10 minutes. For fixing cells, each slide was immersed in a 50 mL conical tube with 100 % Acetone. Slides were dried and 25 uL Block buffer (5 % Normal Mouse Serum diluted in 1X PBS) was added to each well and incubated for 30 minutes at room temperature. 2H2-488 antibody was used for pre staining diluted 1:200 in Block solution. Staining was performed by adding 25 ul stain solution to each well and incubated for 30 minutes in dark. After decanting, 35 uL of 1X PBS was added and incubated in the dark for 5 minutes. Lastly, one drop of mounting fluid (Polysciences Inc., Aqua Poly/Mount 18606) was added to each well with application of cover slip. Each well of slide (Corning 22 x 50 mm covers all ten wells) was observed under a fluorescent microscope.

Statistical analysis

Statistical analysis regarding siRNA inhibition effectiveness was performed by applying Kruskal-Wallis test using SPSS (SPSS version 17.0 software). Kruskal-W test is a non-parametric test, which involves group analysis of variances that can be implemented to compare scores on continuous variable for a number of groups usually above three. Based on this test, significance is based on *p*-values. If *p* is less than 0.05 (*p* < 0.05), it indicates a significant difference in the groups in the continuous variable, as shown later in Table 2.

RESULTS

Effective siRNA designing and synthesis

More than 20 siRNA sequences were designed from three different regions of DENV3 strain (gene bank accession number JX669500), two each from 5'UTR, 3'UTR and structural regions, respectively. After further evaluation using different algorithms, six siRNA sequences were selected. After checking all the parameters, the following six siRNA sequences were synthesized for inhibition studies in Vero-81 cells, as shown in Table 1.

Focus data

Focus reduction neutralization assay was performed for observing reduction in viral titer of DENV3. Vero-81 cells that were transfected with siRNA were challenged with DENV3. Dilutions of DENV3 were prepared to produce 40-60 foci per well in a 48 well plate. Positive control wells had Vero-81 cells transfected with control siRNA that was not related with DENV, while the negative control well was without any virus infection.

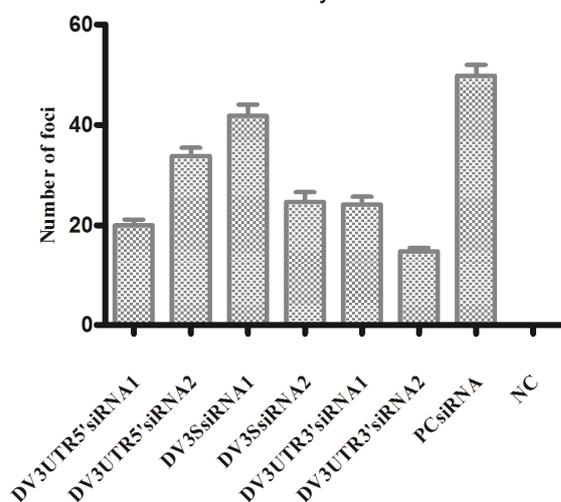


Fig 1: Foci count after 48 h of infection. Vero81 cells transfected with siRNAs designed against DENV3 were later challenged by DENV3 dilution (enough to produce 40 to 60 foci). Bar 1-6 siRNAs targeting DENV3 conserve sequences, bar 7 and 8 are positive control siRNA and negative control respectively. Experiments were repeated twice and similar results were obtained, reducing the likelihood of errors

On the third day post infection, FRNT assay was performed, in which DV3UTR3'siRNA2 was able to reduce virus titer up to 80 %, and siRNA designed from 5'UTR (DV3UTR5'siRNA1) showed marked decrease in DENV3 titer. Structural genes of DENV3 were also targeted by two siRNA sequences. DV3S'siRNA1 from structural region was unable to inhibit DENV3 as efficiently as DV3S'siRNA2 (Fig 1). These results demonstrate that siRNA based inhibition can effectively inhibit DENV3 in Vero-81.

Immunofluorescence

Immuno fluorescence assay was performed to check the level of inhibition achieved by siRNAs against DENV3. Vero-81 cells transfected with synthetic siRNAs were infected with DENV3, followed by performing IFA after 48 hours. Cells for individual wells were scrapped and visualized under a florescent microscope (Fig 2). IFA revealed that DV3S'siRNA2 had the maximum inhibition efficiency against DENV3 in Vero-81 cells. siRNAs designed from the structural regions were less effective with images showing less florescence as compared with siRNAs designed from UTRs. These observations suggest that DV3S'siRNA2 and DV3UTR5'siRNA1 were able to achieve approximately 70 to 85 % reduction in viral titers.

Real time PCR for quantification of DENV3 RNA

Results obtained from Focus assay and IFA were later confirmed with real time qPCR analysis, and DENV3 RNA level was checked by using qPCR. A three-fold difference in DENV3 RNA was observed with DV3UTR3'siRNA2. DV3UTR3'siRNA1 also reduced DENV3 RNA levels, but this decrease in siRNA level was less than the one exhibited by DV3UTR3'siRNA2. While siRNAs designed against the structural region were not able to significantly reduce DENV3 RNA levels in infected Vero-81 cells, DV3S'siRNA2 was on the other hand, able to reduce DENV3 RNA up to 40-50 % (Fig 3). Synthetic siRNA designed from 5'UTR were also highly effective in limiting DENV3 in Vero-81 cells, where a 50 % reduction in DENV3 RNA with DV3UTR5'siRNA1 was observed.

Western blotting

Western blot analysis revealed that siRNAs targeting the 3' and 5' UTR of DENV3 were highly efficient in reducing viral titers in Vero-81 cells. Western blot was done using 8A1 antibody targeting the E protein of DENV3. siRNA

Table1: Location of siRNA sequences in DENV 3 genome

siRNA	Sequence (5'-3')	Positions of siRNA sequence in viral genome
<i>DV3UTR5'siRNA1</i>	UGCUUAACGUAGUGCUGAC	49
<i>DV3UTR5'siRNA2</i>	CUUGCUUAACGUAGUGCUG	27
<i>DV3S'siRNA1</i>	CGACAUGGGCUAUUGGAUA	3001
<i>DV3S'siRNA2</i>	ACACCUGGACAGAGCAAUA	2475
<i>DV3UTR3'siRNA1</i>	GACUAGAGGUUAGAGGAGA	10566
<i>DV3UTR3'siRNA2</i>	GUGUAGCAGACUAGCGGUU	10473
<i>PC'siRNA</i>	ACGUGACGUUCGGAGAAUU	Plant source (control siRNA)

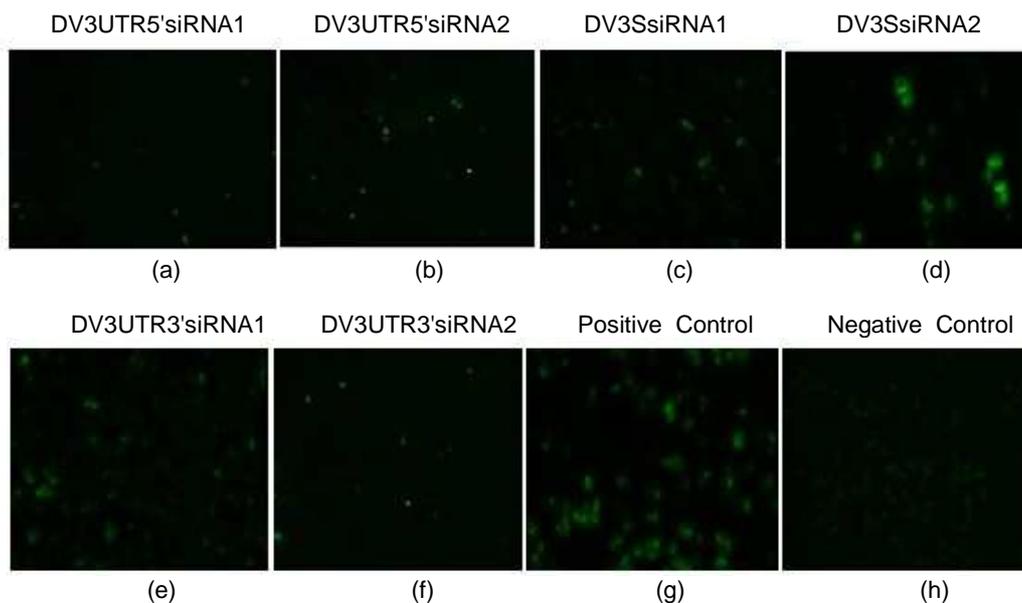


Fig 2: Vero-81 cells transfected with siRNAs stained by anti-E antibody (2H2-488), 48 h post-DENV3 infection. Images (a) and (b) are cells transfected with siRNAs, designed from the 5'UTR, showing marked reduction in viral load. Images (c) and (d) are Vero-81 cells transfected with siRNAs from structural region of the genome. A 30 to 40% reduction in viral load can be seen in the images. Images (e) and (f) display siRNAs that were designed from the 3'UTR of DENV3. Lastly, images (g) and (h) are positive control having PCsiRNA and no PCsiRNA, respectively

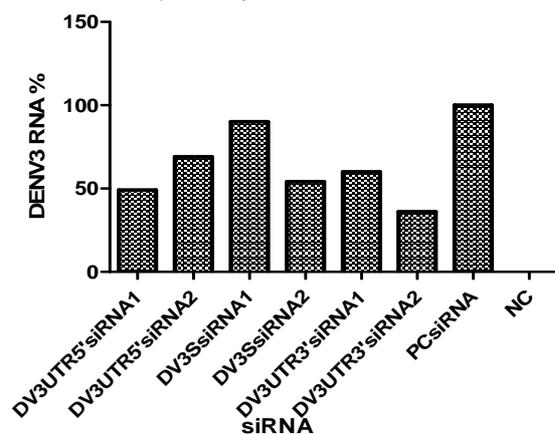


Fig 3: Real time qPCR performed 48 hours post infection. Bar 1 and 2 depict a decrease in DENV3 RNA by siRNAs targeting 5'UTR of DENV3. Bar 3 and 4 exhibit an effect of siRNAs designed from structural region of DENV3 genome. Bars 5 and 6 represent the levels of DENV3 RNA when treated with siRNAs against 3'UTR. Lastly, bars 7 and 8 are positive control containing PCsiRNA not related with DENV3 genome

and negative control without any virus designed from structural regions had no significant effect on virus titer. Samples transfected with siRNAs designed from the structural regions had a higher band visibility.

Statistical data

The focus assay counts were later analyzed statistically using SPSS version 17. Standard deviation and p-value were obtained. The p-value of each siRNA in comparison with the control was ≤ 0.05 , indicating that the variable is statistically significant and the siRNAs were highly effective.

DISCUSSION

With the recent advancements in the field of molecular biology, siRNA technology has become a leading tool in gene therapy with

Table 2: Statistical data for focus assay counts

siRNA	Control	Counts (mean \pm SD)	P-value
DV3UTR5'siRNA1	PCsiRNA	20 \pm 2.82	>0.05
DV3UTR5'siRNA2	PCsiRNA	33.8 \pm 3.9	>0.05
DV3SsiRNA1	PCsiRNA	41.8 \pm 5.4	>0.05
DV3SsiRNA2	PCsiRNA	24.6 \pm 4.7	>0.05
DV3UTR3'siRNA1	PCsiRNA	24.1 \pm 3.8	>0.05
DV3UTR3'siRNA2	PCsiRNA	14.8 \pm 1.7	>0.05

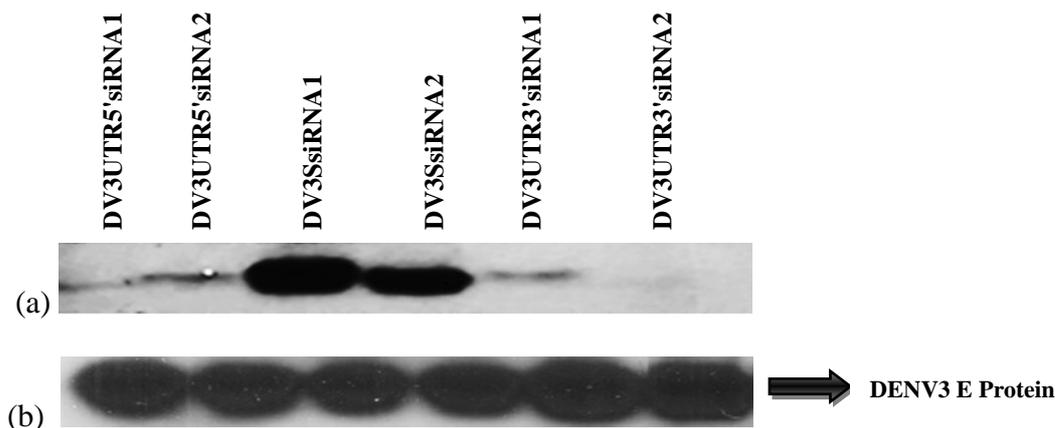


Fig 4: Western blot analysis of samples treated with six synthetic siRNAs. (a) Lane 1 and 2 represent siRNAs designed from the 5'UTR of DENV3. Lanes 3 and 4 show samples treated with siRNAs, targeting the structural region. Lane 5 and 6 are representative of samples treated with siRNAs from 3'UTR, respectively; (b) 1 to 6 represent control, DENV3 E protein

applications such as up-regulation and down-regulation of certain target genes. Based on this enormous potential, siRNA technology is being extensively used for anti-virus drug development. With advantages like specificity, minimum or non-toxicity and efficient mode of action, siRNAs are one of the important components of biopharmaceuticals. Recent studies suggest that siRNAs can be used for blocking virus transmission [12,13]. DENV carrying a single stranded RNA as the genome in the cytoplasm is a probable target for different RNAi based inhibition mechanisms.

In the current study, we observed that synthetic siRNAs that targets the highly conserved regions of DENV3 genome can effectively inhibit DENV3 in Vero-81 cell lines. Reduction in viral loads were observed by immunofluorescence assay (IFA) and focus assay (FRNT), which were later confirmed by quantitative PCR (qPCR) and lastly by western blot analysis. These conserved regions of DENV genome are potential targets for effective antiviral drug designing and targeting. Additionally, this information may possibly be beneficial for future studies involving genome based inhibition of DENV. Several recent studies suggest that 3'UTR of Dengue virus is crucial for proper RNA replication [14,15]. Diego *et al* exhibited that there is a 100-fold decrease in Dengue virus RNA synthesis when deletions were introduced in 3'UTR of Dengue virus [16]. siRNA (DV3UTR3'siRNA2) designed from the conserved 3'UTR region was able reduce DENV3 RNA significantly, demonstrating 80 % reduction in DENV3 titers. The highly conserved 10 nucleotide stretch at the 5'UTR of all Dengue serotypes depicts a pivotal role in Dengue virus replication cycle. RNAi interference

at this location can significantly reduce Dengue virus replication resulting in reduction of virus titers [17-19]. As expected, siRNAs designed from 5'UTR region were also successful in limiting the viral load, where a 2 to 3 folds reduction with siRNA (DV3UTR5'siRNA1) was observed. Ultimately, one of the siRNA from the structural region of DENV genome managed to reduce viral titers in the range of 40 to 45 %.

These results demonstrate that siRNAs can serve as an effective molecular biological tool for inhibiting DENV in mammalian cells. With proper delivery mechanism in place such as, nanoparticles in combination with lipids, these siRNAs can be used for *in vivo* studies [20]. Our data suggests that synthetic siRNAs, when introduced, can reduce Dengue virus titers in the cell cultures. Further studies can enhance the efficiency of these powerful anti-virals by reducing siRNA degradation and sustaining their required levels in the cells.

CONCLUSION

Despite siRNAs being effective for inhibiting virus in cell line, there are many drawbacks associated with siRNAs, including a complex mechanism for delivery, rapid degradation in the cells, inefficient accumulation in the target cells and tissues, and failure to cross various membranes. According to recent studies, lipid nanoparticle (LNP) is the most promising technology for sustained siRNA delivery and currently, it is a part of more than five clinical trials in various phases. A similar mechanism can be applied for deploying these siRNAs for future development of biopharmaceuticals against DENV and other viruses.

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