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Vector-delivered artificial miRNA effectively inhibited replication of Chikungunya virus

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ABSTRACT

Chikungunya virus (CHIKV) has emerged as one of the most significant arboviral threats in many parts of the world. In spite of large scale morbidity, and long lasting polyarthralgia, no licensed vaccine or antivirals are available for the clinical management of CHIKV infection. In this study, a novel RNA interference based strategy has been adopted for effective inhibition of CHIKV. Four artificial microRNAs (amiRNAs) were designed to target different regions of CHIKV genome. These amiRNAs significantly inhibited CHIKV replication in Vero cells at both RNA and protein levels as assessed by qRT-PCR, immunoblotting and immunofluorescence techniques. Further inhibition of the infectious CHIKV up to 99.8% was demonstrated by plaque reduction assay. Concatemerization of amiRNA resulted in higher inhibition of CHIKV than individual amiRNAs. In addition, we studied the effect of combination of RNAi based therapy with other classical antivirals like chloroquine, ribavirin and mycophenolic acid, that helped in understanding the rational selection of RNAi based combination therapy. These findings provide a promising avenue for the development of novel amiRNA or combination based therapeutics against emerging CHIKV.

1. Introduction

Chikungunya virus (CHIKV) has emerged as a major threat to public health in recent years. CHIKV, a member of genus *Alphavirus*, family *Togaviridae*, was first isolated from Tanzania in 1952 (Thiberville et al., 2013). CHIKV is now recognized as a global arboviral threat as numerous major outbreaks were reported from both old and new world since 2005. (Powers, 2015). CHIKV infection in humans is characterized by both acute and chronic form of illness. Acute CHIKV infection is characterised by abrupt onset of fever, headache, fatigue, nausea, vomiting, rash, myalgia and severe arthralgia. Acute-phase symptoms usually disappear after 2 weeks. However, arthralgia and/or myalgia may persist for weeks, months, or even years in some patients. The persistence of CHIKV in joints may be a characteristic of chronic illness (Simon et al., 2011; Labadie et al., 2010; Dupuis-Maguiraga et al., 2012). Severe complications including neurological involvement, mother-to-child transmission and even deaths were reported from recent outbreaks (Chandak et al., 2009; Gerardin et al., 2008).

Despite the occurrence of numerous outbreaks across the world, no approved vaccine or therapeutics are currently available. Many antiviral molecules have been reported to inhibit CHIKV, including arbidol, chloroquine, 6-azauridine, favipiravir, furin inhibitors, monoclonal antibody, interferon-α, mycophenolic acid, 2′ and 5′-oligoadenylate synthase, ribavirin etc. These successes, however, have not fully translated into clinical applications. Multiple strategies involving tissue culture and animal model have been applied for generation of novel therapies against CHIKV (Abdelnabi et al., 2015). Contributing to the accelerating surge of drug discovery against CHIKV, we explored a novel RNA interference (RNAi) strategy to inhibit replication of CHIKV.

RNA interference (RNAi) therapeutics has recently emerged as a new field in drug discovery. Numerous studies involving RNAi have now been published and several RNAi drug candidates have entered clinical trials (Burnett et al., 2011; Davidson and McCray, 2011; Pecot et al., 2011). The last decade has witnessed a major advancement in RNAi based antiviral therapeutic approaches, demonstrating their efficacy against both acute and chronic viral infections, including Ebola virus, Influenza A virus, Severe acute respiratory syndrome (SARS), West Nile virus, Japanese

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encephalitis virus, Hepatitis C virus and Hepatitis B virus etc. (Thi et al., 2015; Tompkins et al., 2004; Ge et al., 2004; Li et al., 2005; Haasnoot et al., 2007). RNAi therapeutics are primarily achieved by utilizing exogenous siRNA, vector-based shRNA or miRNA approaches. RNAi targeting CHIKV using siRNA and shRNA has already been reported (Dash et al., 2008; Parashar et al., 2013; Lam et al., 2012). Artificial miRNA (amiRNA) based approach is now gaining importance owing to its advantages over siRNA and shRNA in terms of increased stability, effectiveness, and lower toxicity (Boudreau et al., 2009; Maczuga et al., 2012; McBride et al., 2008; Xie et al., 2013).

Like natural endogenous miRNAs, amiRNAs are expressed from exogenously transfected plasmid miRNA construct by RNA polymerase II. Primary microRNAs (pri-miRNAs) are transcribed and are trimmed by the microprocessor complex made up of Drosha (RNase III) and DCGR8 in the nucleus into ~70 nucleotide stem-loop precursors, called pre-miRNAs. Following this, pre-amiRNAs are transported to the cytoplasm via exportin-5, where they are recognized by RNA-induced silencing complex (RISC). Pre-amiRNAs are then cleaved into mature amiRNAs by Dicer. The two strands of amiRNA are separated, and the guide strand, complementary to viral gene remains associated with Ago2 which orient it for interaction with the target viral RNA (Carthew and Sontheimer, 2009; Davidson and McCray, 2011; Fischer and James, 2004; Kim and Rossi, 2007; van Rij and Andino, 2006). Target viral RNA is subsequently degraded or translationally inhibited, resulting in post-transcriptional silencing of viral replication. Recently, effectiveness of amiRNA was reported for inhibition of Venezuelan equine encephalitis virus (VEEV), which is a member of genus alphavirus (Bhomia et al., 2013).

In this study, we designed and cloned four amiRNA in commercially available miRNA expression vector targeting CHIKV. The amiRNA plasmid constructs were transfected into Vero cells, and infected with CHIKV. Results revealed that amiRNA either as single or in combination against different targets, can efficiently inhibit CHIKV replication. Growing studies suggest that combinatorial therapy holds significant promise over mono-therapies as it helps to maximize efficacy and minimize the risk of escape mutants. We, therefore, blend our amiRNA based RNAi approach with other classical inhibitors of CHIKV. This work provided information about rational selection of efficient regimes for effective suppression of CHIKV.

2. Material and methods

2.1. Cells and viruses

Vero cells (African green monkey kidney cell line), obtained from National Center for Cell Science (NCCS), Pune, India were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 80 U gentamicin, 2 mM L-glutamine and 1.1 g/L sodium bicarbonate. An Indian isolate of CHIKV (strain DRDE-07; GenBank Acc No. EU372006) belonging to East Central and South African (ECSA) genotype maintained at Virology Division, DRDE, Gwalior was used in the present study. The virus was propagated using standard virus adsorption technique, titrated by plaque assay in Vero cells, aliquoted and stored in −80 °C till further use.

2.2. Target selection and amiRNA design

Four amiRNAs targeting conserved regions of nonstructural and structural ORF of CHIKV were designed using web based BLOCK-iT RNAi designer tool (Invitrogen, USA). The sequences of all oligos along with their genome position, inhibiting gene and ORFs are shown in Table 1. The amiRNA targeting nonspecific/scrambled sequence was also used as negative control in this study.

2.3. Plasmid construction

Top and bottom strand oligos were custom synthesized in HPLC grade, annealed and cloned into pcDNA6.2-GW/EmGFP-miR vector (Invitrogen, USA), which is a cytomegalovirus promoter driven expression construct, co-cistronically expressing emerald green fluorescent protein (EmGFP) with amiRNA constitutively. Positive constructs were confirmed by double pass nucleotide sequencing using vector specific primers (5′-GGCATGGACGAGCTGTACAA-3′ and 5′-CTCTAGATCAACCACTTTGT-3′). These constructs were designated as amiRNA_A, amiRNA_B, amiRNA_C and amiRNA_D.

2.4. Transfection and cytotoxicity assay

To determine cellular toxicity of amiRNAs, Vero cells were seeded in 96-well plate. On 70–80% confluency, different concentrations of amiRNA ranging from 100 to 800ng/well were transfected using Lipofectamine 2000 (Invitrogen, USA), in 8 replicates per concentration. Cell control and mock transfection control were kept along side. Following transfection, the medium was replenished with MEM supplemented with 2% FBS and cells were incubated for 72 h. The cytotoxicity was analyzed by neutral red dye uptake (NRDU) assay (Repetto et al., 2008).

2.5. Virus infection

Vero cells were seeded in a 24-well plate. On 70–80% confluency, cells were washed and transiently transfected with 1 μg/well of each amiRNA. Plate was incubated for 6 h and transfection reagent was replenished with MEM supplemented with 2% FBS. Following 12 h post transfection, the cells were infected with CHIKV at a multiplicity of infection (MOI) of 0.01. After 2 h of adsorption, plate was washed and replenished with MEM with 2% FBS. Mock transfection control, virus control and scrambled amiRNA were kept alongside. Infected culture supernatants were harvested at 24 and 48 h post infection (hpi).

2.6. Quantification of viral genome by real-time RT-PCR

Viral RNA was isolated from infected culture supernatants at 24 and 48 hpi using QIAamp viral RNA kit (Qiagen, Germany). A quantitative RT-PCR (qRT-PCR) assay was used to quantify the CHIKV RNA copies as described previously (Agarwal et al., 2013). Briefly, qRT-PCR was carried out using SS III Platinum one step qRT-PCR kit (Invitrogen, USA) in Mx3005P system (Stratagene, USA). The qRT-PCR reaction was performed in a 25 μl reaction volume, with 2X master mix, CHIKV-E1 gene specific forward and reverse primers, enzyme mix comprising of Taq DNA polymerase and reverse transcriptase, nuclease free water and RNA template. Cycling parameters were reverse transcription at 50 °C for 30 min, initial denaturation at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s. The amplified product was verified by melting curve analysis using Mx3005P software.

2.7. Measurement of viral load by plaque assay

Titer of infectious CHIKV on amiRNA treatment was determined using plaque reduction assay. Vero cells were seeded in a 24-well plate one day prior to the experiment. The 10-fold serial dilutions of the harvested supernatant from each experimental group (virus control, amiRNA_NC and different amiRNAs treatment group) were

Table 1
Target sequences of the amiRNAs. Four sequences were designed against different regions of CHIKV genome as target using Invitrogen Block-iT RNAi Designer. The amiRNA top and bottom oligos were cloned into pcDNA™6.2-GW/EmGFP-miR vector.

Artificial miRNA	Oligo ID & genome position	Sequences (5′–3′)	Target ORF	
amiRNA_A	CKmiRT_855	TGCTGTTAAATGGAACACCGATGGCAGTTTTGGCCACTGACTGACTGCCATCGGTTCCATTTAA	nsP1	ORF1 (Non-structural)
	CKmiRB_855	CCTGTTAAATGGAACCGATGGCAGTCAGTCAGTGGCCAAAACTGCCATCGGTGTTCCATTTAAC		
amiRNA_B	CKmiRT_2562	TGCTGTATTGAAGAAGCCGCACTGCTGTTTTGGCCACTGACTGACAGCAGTGCCTTCTTCAATA	nsP2	
	CKmiRB_2562	CCTGTATTGAAGAAGGCACTGCTGTCAGTCAGTGGCCAAAACAGCAGTGCGGCTTCTTCAATAC		
amiRNA_C	CKmiRT_2733	TGCTGTTAACACGAGGTCTCCAGGGTGTTTTGGCCACTGACTGACACCCTGGACCTCGTGTTAA	nsP2	
	CKmiRB_2733	CCTGTTAACACGAGGTCCAGGGTGTCAGTCAGTGGCCAAAACACCCTGGAGACCTCGTGTTAAC		
amiRNA_D	CKmiRT_8249	TGCTGTTCATTAGCTCCTCCTAAGACGTTTTGGCCACTGACTGACGTCTTAGGGAGCTAATGAA	Capsid	ORF2 (Structural)
	CKmiRB_8249	CCTGTTCATTAGCTCCCTAAGACGTCAGTCAGTGGCCAAAACGTCTTAGGAGGAGCTAATGAAC		

prepared and 200 μl of each dilution was added to respective wells. Following virus adsorption, overlay medium containing 1.25% carboxymethyl cellulose was added and plate was incubated for 72 h. The plate was then washed, fixed with chilled methanol and finally stained with 0.25% crystal violet solution. Numbers of plaques were counted and the titer was calculated considering the volume and dilution factor of the inoculum.

2.8. Immunofluorescence assay

Cell culture conditions, amiRNA treatment and virus infection were same as described above. At 24 hpi, cells were fixed with chilled methanol and permeabilized with 0.1% TritonX-100. Cells were incubated with anti-CHIKV E2 monoclonal antibody (mAb) followed by immunostaining with FITC conjugated anti-mouse antibody (Sigma, USA). Finally, cells were mounted with fluoroshield 4′, 6-diamidino-2-phenylindole (DAPI; Sigma, USA), and images were captured using Fluorescence microscope (Leica, Germany).

2.9. Western blot analysis

Vero cells were transfected and infected with CHIKV as described above. Cells were harvested on appearance of cytopathic effect (CPE) in the virus control and lysed with radioimmunoprecipitation assay (RIPA) buffer (Sigma, USA) containing protease inhibitor cocktail (Sigma, USA). Protein was quantified, resolved on 10% SDS-PAGE followed by electroblotting onto PVDF membrane. Membrane was blocked and probed with anti- CHIKV E2 mAb or β-actin antibody followed by addition of anti-mouse horseradish peroxidase conjugated secondary antibody. The anti-CHIKV E2 mAb was used for detection of viral protein, whereas, β-actin antibody served as an internal protein reference and loading control.

2.10. Construction of concatenated amiRNAs

Combination of two amiRNAs in a single vector construct was carried out as per manufacturer's protocol (BLOCK-iT kit, Invitrogen, USA). Briefly, to generate a construct which express two amiRNAs, first amiRNA segment from a donor plasmid was excised with restriction enzymes, *Bam*HI and *Xho*I, and was ligated to the another amiRNA backbone vector pre-cut with *Bgl*II and *Xho*I. Two vector constructs (amiRNA_BD and amiRNA_CD) were generated. The combined effect of concatenated amiRNAs, in inhibition of CHIKV infection in Vero cells were assessed by qRT-PCR, plaque reduction assay and immunoblotting techniques as described above.

2.11. Combination therapy

The maximum non-toxic dose (MNTD) of antiviral compounds i.e., ribavirin (RIBA), mycophenolic acid (MPA) and chloroquine (CHLO) was determined on Vero cells using NRDU assay. Further, experiments with compounds were carried out using lower doses compared to their respective MNTD. A monolayer of Vero cells were seeded in 24-well plate. Cells were transfected with 1 μg of amiRNA_D in its respective well 12 h prior to infection. Following an overnight transfection, plate was infected with CHIKV at an MOI of 0.01. After 2 h of adsorption, antiviral compounds (20 μM each of CHLO and RIBA, and 10 μM of MPA) in MEM supplemented with 2% FBS, were added to the respective wells in triplicate and plate was incubated at 37 °C with 5% CO_2. The results were analyzed based on the appearance of cytopathic effect (CPE) and infected culture supernatants were harvested at 24 and 48 hpi for analysis by qRT-PCR.

2.12. Statistical analyses

All the assays were performed three times with each sample in triplicate and results were graphed, with error bars indicating the mean ± standard deviation (SD). Data were analyzed using two tailed student t-test and One-way ANOVA with post-hoc Tukey HSD test. The asterisk indicates statistical significance (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001).

3. Results

3.1. Construction of amiRNA plasmids and cytotoxicity testing

The amiRNA oligonucleotides were cloned into vector pcDNA6.2-GW/EmGFP-miR as recommended by the manufacturer's protocol, resulting in four CHIKV specific amiRNA expression plasmids (amiRNA_A, amiRNA_B, amiRNA_C and amiRNA_D) and a negative control miRNA expression plasmid (amiRNA_NC). The positive recombinant plasmids were confirmed by nucleotide sequencing (data not shown). In order to test the toxicity of the amiRNAs, Vero cells in 96 well plate were transfected with different concentrations of amiRNAs (100–800 ng per well), and then assessed by NRDU cell viability assay at 72 h post transfection. No effect on viability of Vero cells was observed up to 400 ng concentration with >90% viability, where as in 800 ng amiRNA concentration, viability was observed up to 75% (Fig. 1A and B). After transfection (~12 h), typical fluorescence-positive cells were observed (Fig. 1C), showing that the transient transfection with EmGFP-amiRNA constructs were suitable as an indicator to test the transfection efficiency.

3.2. Inhibitory effect of amiRNAs on CHIKV replication

The efficacy of amiRNAs was evaluated by transfecting 1 μg of each plasmid into Vero cells 12 h prior to infection with CHIKV. RNA was collected from the infected cell supernatant 24 and 48 hpi for qRT-PCR and plaque reduction analysis. Compared to infected Vero

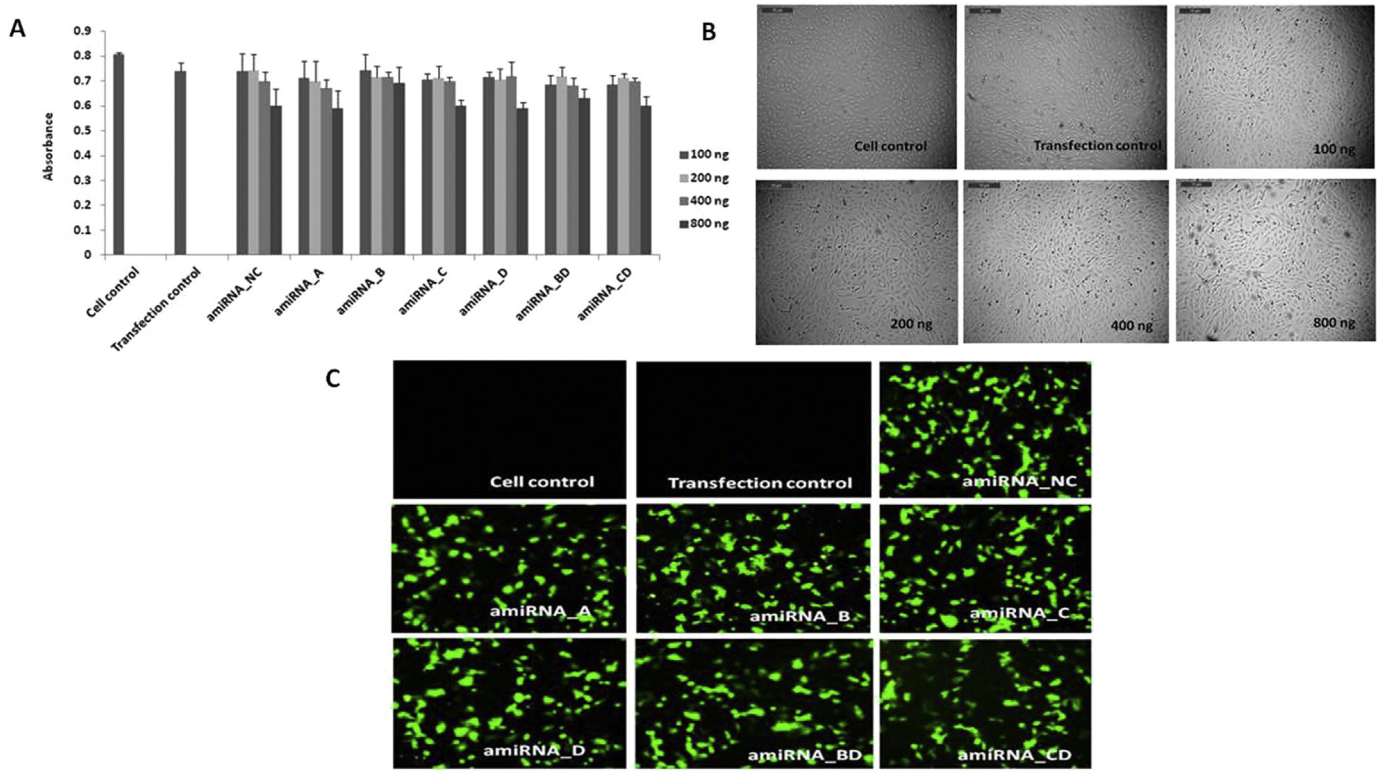

Fig. 1. Cytotoxicity of artificial miRNAs in Vero cells. (A) Cell viability assay (NRDU) was performed to evaluate cytotoxicity of artificial miRNAs on Vero cells. Vero cells were grown in 96 well plates and transfected with 100–800 ng/well of plasmids expressing amiRNA in 8-replicates for each concentration. After 72 h post transfection, neutral red dye was added and absorbance was recorded at 540 nm. (B) Cytopathic effect at 48 h post transfection. No significant cytotoxicity was observed (representative data of amiRNA_D CPE is shown). (C) Assessment of transfection efficiency. Vero cells were transfected with 1 μg amiRNA using 1 μl Lipofectamine 2000/well in a 24 well plate. Cells with amiRNA_NC served as negative control. Fluorescence microscopy images of Vero cells were observed 12 h post-transfection and green fluorescence indicates the expression of EmGFP along with artificial miRNAs indicating the transfection efficiency. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells, cells expressing amiRNA_A, amiRNA_B, amiRNA_C or amiRNA_D exhibited a marked reduction in CHIKV RNA as measured by qRT-PCR up to 48 hpi. The qRT-PCR analysis showed that the RNA load in virus control was $\log_{10}6.5$ and $\log_{10}8.2$ at 24 and 48 hpi respectively. The CHIKV RNA was log105.8, log105.4, log105.5 and log103.5 in amiRNA_A, amiRNA_B, amiRNA_C and amiRNA_D treated cells respectively at 24 hpi. Similarly, at 48 hpi, the viral RNA transcripts were $\log_{10}7.9$, $\log_{10}7.6$, $\log_{10}7.7$ and $\log_{10}6.8$ in amiRNA_A, amiRNA_B, amiRNA_C and amiRNA_D respectively. The relative expression of CHIKV RNA is shown in Fig. 2A. The highest reduction of CHIKV RNA was observed in amiRNA_D treated cells, followed by amiRNA_B, amiRNA_C and amiRNA_A. The relative titer of infectious CHIKV was depicted in Fig. 2B. CHIKV titer in amiRNA_A, amiRNA_B, amiRNA_C and amiRNA_D treated cells was 7.2×10^5, 1.6×10^5, 3.1×10^5 and 4.5×10^3 PFU/ml, respectively at 24 hpi, compared to 2.7×10^6 and 2.6×10^6 PFU/ml in virus control and amiRNA_NC respectively. The analysis through indirect immunofluorescence assay revealed reduction in CHIKV load in amiRNA transfected group compared to control. Notably, fluorescence was barely detectable in the cells treated with amiRNA_D followed by amiRNA_B compared to virus control and amiRNA_NC at 24 hpi (Fig. 3A). Down-regulation CHIKV-E2 protein was also assessed by western blotting. A faint band of the E2 protein (~48 kDa) was observed in amiRNA_D transfected cells followed by amiRNA_B compared to virus control and amiRNA_NC. E2 protein band was not observed in mock cell control. All the cells expressed the β-actin protein at an equivalent level. Nevertheless, adding scrambled amiRNA (amiRNA_NC) construct did not affect viral replication (Fig. 3 B).

3.3. Multiple amiRNA treatment

The combination of two best amiRNAs (amiRNA_BD) resulted in significant inhibition of replication of CHIKV, as assessed by qRT-PCR, immunofluorescence, plaque assay and immunoblotting techniques (Figs. 2 and 3). Overall, the inhibition of amiRNA_BD was higher than amiRNA_B and amiRNA_D alone. However, effect amiRNA_CD was found marginal, compared to both amiRNA_C and amiRNA_D.

3.4. Combination therapy

Next, we investigated the inhibitory effect of amiRNA_D in combination with classical antiviral drugs like RIBA, MPA and CHLO. CHLO treatment of amiRNA_D transfected Vero cells revealed marked reduction in viral load as shown by reduced CPE in comparison to both amiRNA_D and chloroquine alone. However, the cells transfected with negative control plasmid (amiRNA_NC) showed an extensive CPE within 48 h after infection. Further, no combined effect was seen in RIBA and MPA treated amiRNA_D transfected cells. In contrary, effect of combination treatment was less than the inhibitory effect of amiRNA_D and RIBA/MPA alone (Fig. 4A, MPA data not shown; but was similar to RIBA). In addition to the examination of the CPE, the replication of CHIKV through viral RNA load was studied. Viral genome copy number in amiRNA_D, RIBA, MPA, CHLO, RIBA+amiRNA_D, MPA+amiRNA_D and CHLO+amiRNA_D was $\log_{10}3.5$ $\log_{10}3.3$, $\log_{10}3.2$, $\log_{10}$ 3.1, $\log_{10}4.3$, $\log_{10}4.0$ and $\log_{10}2.8$ at 24 hpi and $\log_{10}$ 6.8, $\log_{10}5.9$, $\log_{10}6.3$, $\log_{10}$ 5.8, $\log_{10}$ 7.1, $\log_{10}7.2$ and $\log_{10}$ 4.6 copies at 48 hpi respectively. In contrast, the virus control

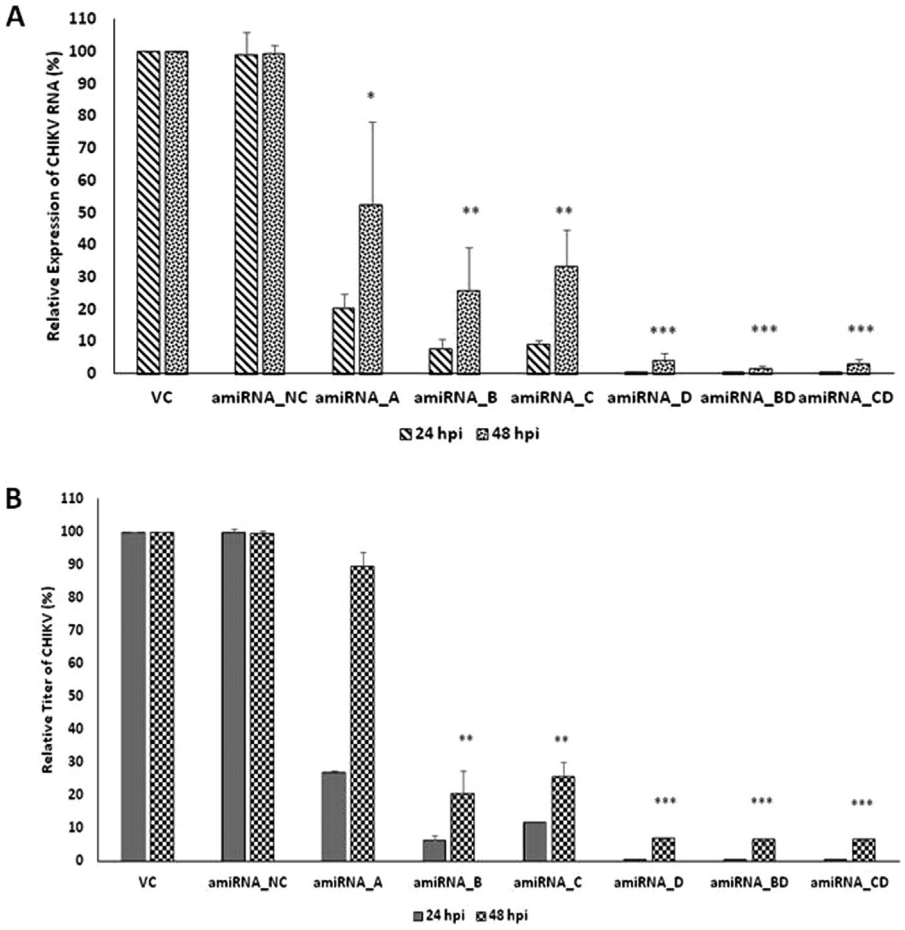

Fig. 2. Antiviral efficacy of both single and concatenated artificial miRNAs in Vero cells. (A) Determination of relative viral RNA copy number by qRT-PCR. Vero cells were treated with artificial miRNAs and infected with 0.01 MOI of CHIKV. Cell supernatants were harvested at 24 and 48 hpi and RNA was isolated. The qRT-PCR was performed with specific primers for CHIKV E1 region. Titer of CHIKV RNA was determined from a standard curve drawn using 10-fold serially diluted *in vitro* transcribed CHIKV RNA. (B) Relative CHIKV titer as assessed by plaque assay at 24 and 48 hpi. Data represents the mean ± standard deviation of three independent experiments. The asterisk indicates statistical significance at 48 hpi ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

revealed a CHIKV RNA titer of $\log_{10}$ 6.5 and $\log_{10}$8.2 copies at 24 hpi and 48 hpi respectively. The relative expression of CHIKV RNA with respect to virus control is shown in Fig. 4B.

4. Discussion

The RNAi therapeutics has been attempted against various genera of viruses in recent past. The expansion of CHIKV across different continents attracts the attention of researchers towards development of an effective therapeutic. The inhibitory effect of siRNA and shRNA has already been demonstrated against CHIKV. However, no report is available regarding inhibition of CHIKV through vector-delivered amiRNA, though miRNA is believed to have more potential than both siRNA and shRNA. Controlled expression of amiRNA by RNA polymerase II results in lower cytotoxicity due to their relatively low concentration inside the cells, leading to a better RNAi response than both shRNA and siRNA (Boudreau et al., 2009; Maczuga et al., 2012; Xie et al., 2013).

In the present study, we have evaluated the effect of vector-delivered miRNA on CHIKV replication in Vero cells. Our results showed that miRNA based RNAi could efficiently inhibit CHIKV replication irrespective of the target ORF/gene, as it ultimately resulted in either translational repression or cleavage of the entire RNA transcript.

One of the most important advantages of miRNA-based expression vector system is its ability to express multiple amiRNAs from a single construct, which is not possible through regular shRNA vectors. We constructed concatenated amiRNA expressing vectors, which allowed simultaneous expression of different amiRNAs. A combination of two amiRNAs against different genomic targets of CHIKV resulted in statistically significant improvement of inhibitory effect compared to individual amiRNAs. It has been previously reported that expressing more than one amiRNA in a single construct resulted in better inhibition of rabies virus replication (Israsena et al., 2009). This combination system has also been effectively utilized in anti-HIV studies to prevent generation of escape mutants, during persistent HIV infection (Haasnoot et al., 2007; Zhang et al., 2012).

Success of RNAi based therapeutics suggests that it can suppress viral replication very efficiently (Ibrisimovic et al., 2013; Jacque et al., 2002; van Rij and Andino, 2006). However, there is an apprehension that amiRNA based RNAi may face the same obstacles as seen by other mono-therapies in the past, leading to the development of resistance. To address this concern, the study was further extended to evaluate the inhibitory effect of amiRNA in combination with conventional antiviral drugs like chloroquine and ribavirin against CHIKV infection. The inhibitory effect of chloroquine-amiRNA combination was found much greater than

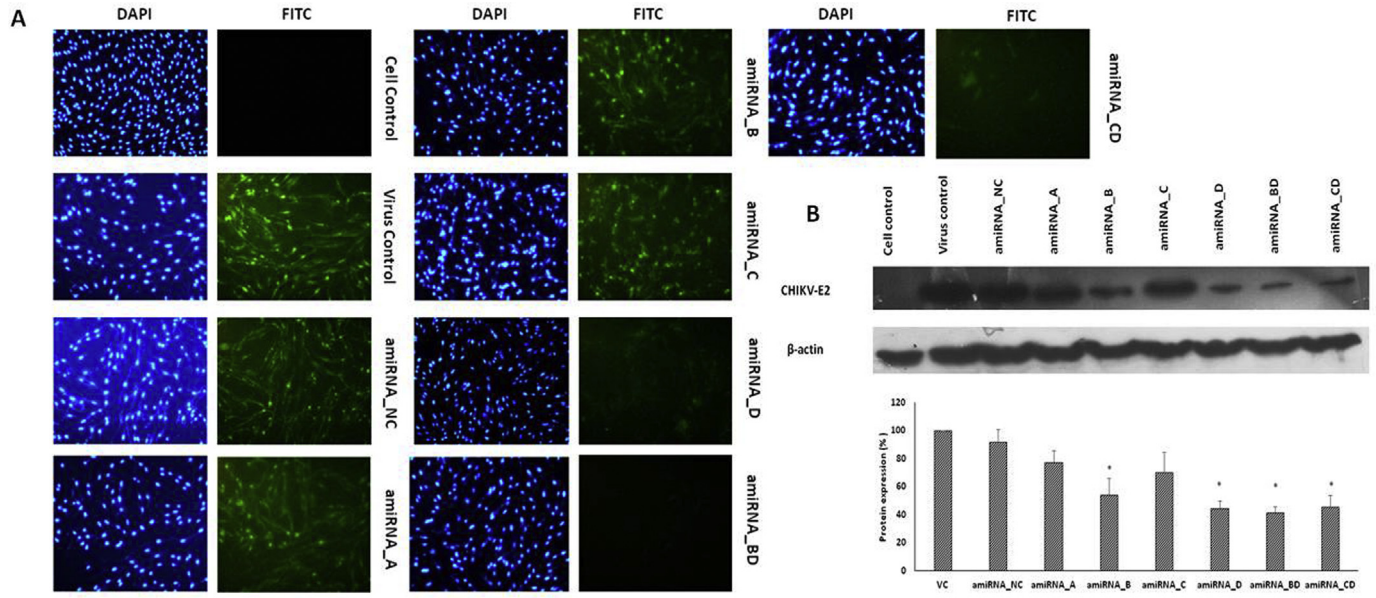

Fig. 3. (A) Immunofluorescence assay. Cells were observed at 24 hpi, green fluorescence indicates the virus load as assessed with anti-CHIKV E2 mAb and secondary antibody conjugated with FITC and blue fluorescence indicates the nuclear staining with DAPI at 20X. (B) Western blot showing amount of viral E2 glycoprotein in Vero cell lysate after treatment with different amiRNAs. Total cell lysate containing 50 μg protein per sample was loaded. β-actin served as an internal quantity and loading control. The results of western blots were quantified as an average optical density (OD) from three independent experiments using image analyser programme of Bio-Rad Quantity One software. CHIKV E2 protein signals were normalized against the loading control (β-actin) and percent reduction in E2 expression was calculated with respect to virus control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

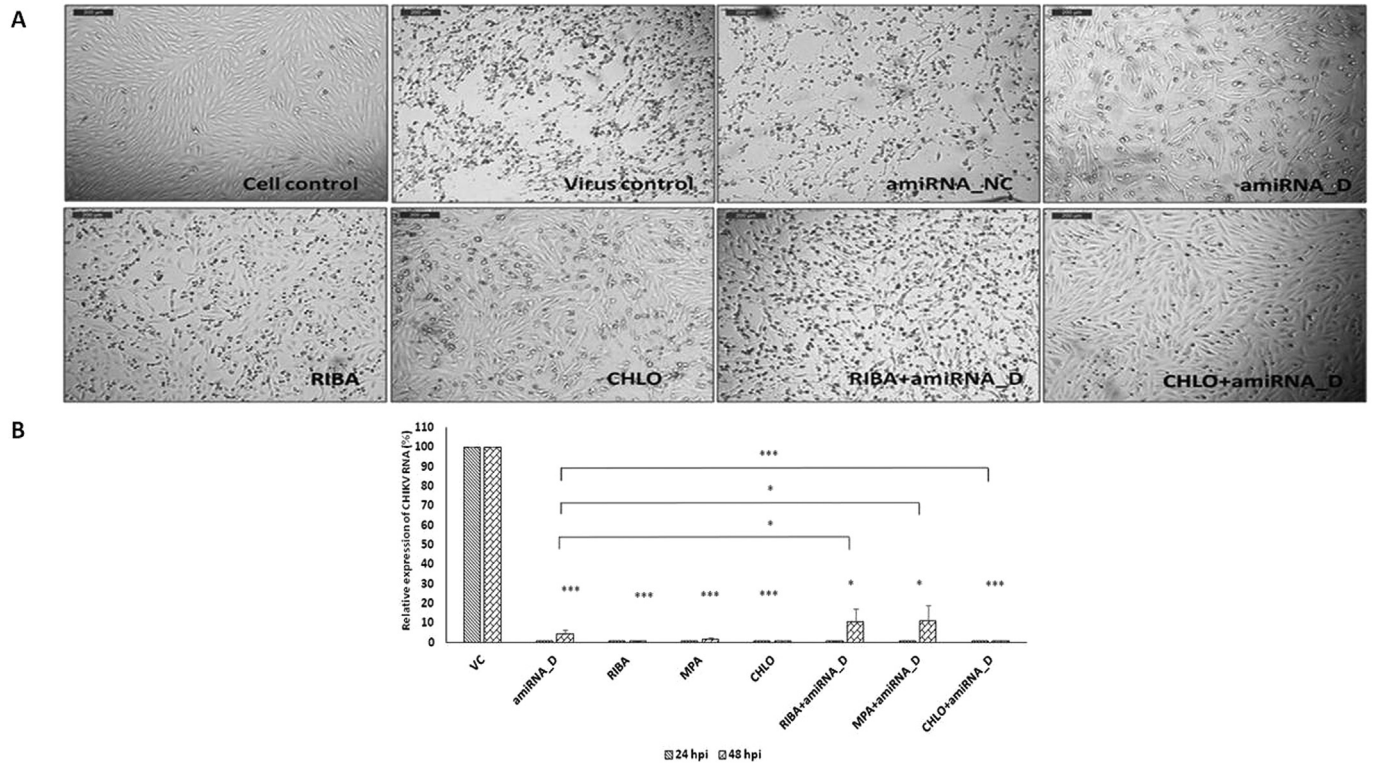

Fig. 4. Combination therapy. CHIKV infected Vero cells were treated with amiRNA, RIBA, MPA and CHLO alone or in combination RIBA+amiRNA, MPA+ amiRNA and CHLO+amiRNA. The combination treatment of CHLO+amiRNA significantly inhibited CHIKV replication whereas amiRNA+RIBA showed less inhibition compared with individual treatments. (A) Microscopic images showing morphology of Vero cells at 48 hpi after respective treatment. Data shown here is representative of one of the three experimental repeats. (B) Relative expression of viral RNA copy number by qRT-PCR. Data represents the mean ± standard deviation of three independent experiments. The asterisk indicates statistical significance at 48 hpi ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

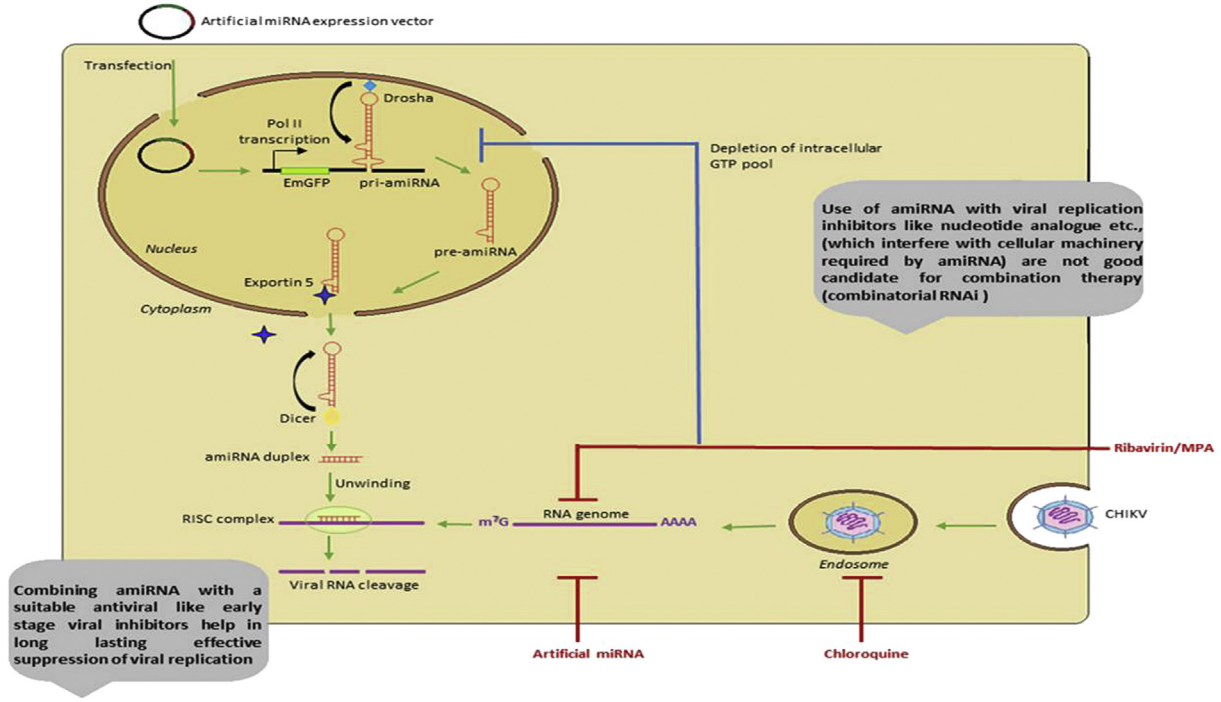

Fig. 5. A schematic representing the targets of RNAi based therapy and classical antiviral drugs, and the hypothetical idea behind the rational selection of RNAi based combination treatment. Use of amiRNA with viral replication inhibitors like nucleotide analogue etc. (which interfere with cellular machinery required by amiRNA) are not good candidate for combination therapy (combinatorial RNAi). Whereas, combining amiRNA with a suitable antiviral like early stage viral inhibitors help in long lasting effective suppression of viral replication.

individual compounds. This could be due to the fact that the combination of these two compounds led to targeting of the virus life cycle at both early and late stage by chloroquine and amiRNA respectively. Chloroquine interferes with fusion of viral E1 protein with endosomal membrane by raising the endosomal pH whereas, amiRNA acts through post-transcriptional silencing of viral replication. Earlier, ribavirin in combination with INF-α and doxycycline, revealed synergistic inhibitory effect against CHIKV (Briolant et al., 2004; Rothan et al., 2015). However, in this study, ribavirin-amiRNA combination did not exhibit desired effect compared to both ribavirin and amiRNA. The major mechanism of ribavirin is to inhibit inosine monophosphate dehydrogenase enzyme (IMPDH), resulting in the depletion of intracellular GTP pool (Khan et al., 2011; Abdelnabi et al., 2015). Thus, ribavirin might interfere with the miRNA pathway as well, which also require cellular nucleotide pool for its expression. Nevertheless, we tested this possibility by using another nucleotide analogue mycophenolic acid and found similar result. Collectively, these results indicate, use of inhibitors of viral genome replication in combination with amiRNAs, may interfere with the expression of amiRNAs resulting in ineffective suppression of viral replication. However, combining amiRNA based RNAi approach with a suitable early viral replication inhibitor will significantly help in suppression of viral replication (Fig. 5).

So far amiRNA has not been tested *in vivo* for acute viral infection. However, RNAi was recently used to inhibit lethal infection by Ebola virus in various animal models. Intravenous delivery protected animals from death, including those that received treatment even after the onset of ebola infection (Thi et al., 2015). In this study, we have used lipofectamine based protocol for *in vitro* transfection. Nevertheless, lipid-based delivery for RNAi have yielded successful advances *in vivo* and to an extent in clinical trials (Bobbin and Rossi, 2016; Dong et al., 2014; Torrecilla et al., 2014; Wittrup and Lieberman, 2015). Moreover, RNAi therapeutic (TKM-Ebola) has also been successfully used during 2014 Ebola epidemic in Western Africa (Bobbin and Rossi, 2016). All these reports confirm the potential utility of RNAi in acute viral infections. Further, plasmid DNA as a vector for gene therapy has been successfully employed in arthritis model using various physical and chemical methods. Delivery of plasmid DNA in arthritis gene therapy showed its promise as a therapeutic strategy, which may be further explored for the systemic delivery of amiRNA, particularly during chronic phase of CHIKV infection in humans (Evans et al., 2006; Subang and Gould, 2010).

This is the first report to successfully apply vector-delivered miRNA in inhibition of replication of CHIKV. The efficient *in vitro* inhibition of CHIKV replication by amiRNA makes it a promising candidate for development of anti-CHIKV therapeutics. Targeting of the conserved sequences across all the genotypes of CHIKV makes it an excellent candidate. Combinatorial RNAi will further give the insights about the rational selection of combination regimes in the future. However, the efficacy of these amiRNAs remains to be tested *in vivo*. Although our knowledge of RNAi therapeutics and combination therapy has increased in the recent years, but several important issues must be studied carefully like safe delivery methods, immune response, dose of combinations, etc., if this knowledge is to be extended further for the development of anti-CHIKV therapeutics.

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