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Suppression of the PB1 and PA Genes of the Influenza A Virus Using siRNAs Targeting Conserved Accessible Regions

[1]Sara H. Mahmoud, [2]Rabeh El Shesheny, [1]Mahmoud El Hefnawi, [3]Naglaa Abdallah, [3]Abdulhadi Abdallah and [2]Mohamed Ali

[1]Biomedical Informatics and Chemoinformatics Group, Informatics and Systems Department, Division of Engineering Research and Centre of Excellence for Advanced Sciences, National Research Centre, Dokki, Giza, Egypt
[2]Influenza Research Laboratory, Environmental Research Division, National Research Centre (NRC), Dokki, Giza, Egypt
[3]Genetics Department, Faculty of Agriculture, Cairo University, Giza, Egypt

Abstract: Influenza A viruses infects birds, animals and humans and it has the ability to mutate and develop mutant variants which may cause unexpected events. There is an urgent need for new medications to combat influenza pandemics. Using the genome sequences of the influenza A virus performed previously, we designed and performed a combinatorial exhaustive systematic methodology for optimal design of universal therapeutic short-interfering RNA (siRNA) molecules targeting all diverse of the virus strains. By using the bioinformatics tools, we designed siRNA targeting influenza A virus polymerase genes. The selection and validation of optimal siRNAs was also studied. The influenza A virus subtype H1N1 and H5N1 were propagated in MDCK cells. $TCID_{50}$ and HA assays were performed and real time PCR for assessment of viral load was used. Time-dependent and dosage-dependent usage were performed and showed high decrease in viral load.

Key words: Bioinformatics tool · Influenza A virus · Influenza polymerases · MDCK cell line · siRNA efficacy · siRNA designing.

INTRODUCTION

Influenza A virus (IAV) is a negative sense RNA virus comprised of eight viral segments encoding ten major proteins [1]. In April 2009 a novel flu strain evolved that combined genes from human, pig and bird flu. Initially dubbed "Swine flu" and also known as influenza A/H1N1, it emerged in Mexico, the United States and several other nations. The World Health Organization officially declared the outbreak to be a pandemic on 11 June 2009. The WHO's declaration of a pandemic level 6 was an indication of spread, not severity, the strain actually having a lower mortality rate than common flu outbreaks [2]. Cross-species infection is thought to be the fundamental mechanism of initiation of human influenza pandemics [3]. In 1997, a highly pathogenic avian influenza (HPAI) H5N1 virus crossed the species barrier to directly transmit from birds to humans in Hong Kong and caused 18 cases of illness including six deaths [4]. Influenza A virus belongs to Orthomyxoviridae family (a group of RNA viruses). Influenza is a major cause of morbidity and mortality worldwide [5]. RNAi is natural pathway which was firstly discovered in *C. elegans* that works on post-transcriptional gene regulation. RNAi has been used as a powerful research tool in functional genomics, seems to be a promising way to discover new antiviral drugs. It is a universal phenomenon of post-transcriptional gene silencing in which siRNA (small interfering RNA) molecule is utilized to promote homology dependent mRNA degradation. This process leads double stranded RNA to a specific degradation of homologus RNAs resulting in generation of 21 nucleotide

Corresponding Author: Mohamed Ali, Influenza Research Laboratory, Environmental Research Division, National Research Centre (NRC), Dokki, Giza, Egypt.

small interfering RNA (siRNA). siRNA triggers the formation of RNA induced silencing complex (RISC) in which double stranded RNA is in corporated and unwounded then binds to mRNA target sequence resulting in its cleavage from the genome when introduced into mammalian cells [6]. The design and selection of optimal siRNAs for all diverse strains of the Influenza A virus is performed by integration of factors that affect the functionality, specificity and potency of the siRNA. Efficient siRNA design is very important to increase potency and hence decrease dosage and possible side effects. Advantage of siRNA molecules, due to the ability of effective and specific inhibition of disease causing genes, elicited great expectations in therapeutic applications and drug discovery [6]. Anti-influenza drugs have great role in influenza control with their independence with the antigenic changes in the virus. The influenza RNA polymerase complex is made up of three subunits, PB1, PB2 and PA. The phenotype of classical temperature-sensitive mutants suggested that PB1 is responsible for all RNA synthesis, while PA and PB2 would be involved in RNA replication and transcription, respectively [7]. The PB2 has a role in replication [8] and PA is involved in cap-snatching [9]. Also, PB1 function considered as the classic polymerase action in which it is responsible for polymerization and endonuclease. The variability in IAV genomic due to phenomena of antigenic shift and drift, poses major challenge in front of this novel field of therapeutic agents. RNA interference is considered as one of gene therapy methods to inhibit the replication of the influenza virus, in which the mechanism of siRNA was used in the degradation of mRNA [6]. We previously performed a systematic methodology for design and selection of optimal siRNAs targeting diverse strains of influenza a virus [10]. Here, we select the optimal siRNAs for the PB1 and PA segments based on their scores and thermodynamic parameters and validate them in vitro cell lines replicating the influenza virus subtypes H1N1 and H5N1.

MATERIALS AND METHODS

An effective siRNA must have certain features related to the sequence, structure, target accessibility, specificity and the targeted mRNA region (conserved regions). Here, we show a novel protocol for design and selection of the optimal siRNAs using an integrative bioinformatics approaches and state of art tool techniques.

In Silico Methods

Sequences Analysis: The sequences of influenza virus PB1 and PA were collected and downloaded from NCBI's Influenza Virus Resource (IVR) database [11]. Then, sequences were grouped to swine, avian, human and other strains infecting species influenza A viruses.

Multiple Sequence Alignment and Conserved Regions Identification: The sequences alignment process was carried out using MUSCLE version 3.6 [12] and the conserved regions were extracted using Bioedit [13] and analyzed by EMBOSS [14].

siRNA Design: The siRNAs were designed using different tools using the conserved regions consensus sequences as an input in order to design all possible siRNA that targets the viral mRNA. The ISCORE software designs and scores the efficacy of siRNAs according to many different first and second generation algorithms and has its own score. The siRNA Scales and DSIR are also based on linear regression models of positional preferences and have comparable ROC curves and correlation coefficients to BIOPREDSI. Primary filtering using threshold scores and exact off-target matches was performed using three selection criteria; mapping threshold scores for 70% and 90% experimental inhibition and filtering the siRNAs below the average threshold scores of all eleven utilized algorithms, off targets and deleting siRNAs with undesirable nucleotide properties. siRNA thermodynamic properties, target site terminal ends single strandedness and target accessibility were calculated using the ISCORE Designer which allows to calculate the whole and partial dG of each siRNA. Also, the SFOLD program was used to calculate the target accessibility of the influenza A virus using the consensus of each segment.

Cells and Viruses: Madin-Derby canine (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen/strep) mixture (Invitrogen). Cells were grown in T-75 tissue culture flasks at 37°C in 5% CO_2. A/chicken/Egypt/Q1995D/2010 (H5N1) and A/H1N1/PR8 viruses were inoculated in 10-day-old SPF embryonated chicken eggs (SPF Eggs Production Farm, Agricultural Research Center, Ministry of Agriculture, Egypt) and incubated for 48 h at 37°C then chilled at 4°C for 4 h before harvesting. The allantoic fluid was harvested, clarified, tested for hemagglutination and then stored at-80°C until use.

Cells Transfection and Influenza Infection: All siRNAs are from Dharmacon (ThermoScientific) and were custom-made. Lyophilized siRNA duplexes (Sigma) were dissolved in RNase free water (Sigma) to generate a storage stock at 20ìM according to product manual instructions. For siRNA introduction, logarithmic-phase MDCK cells were trypsinized, washed and resuspended in growth medium without antibiotic and cultured in 24 well tissue culture multi-well plates and incubated till 30-50 % confluency at the time of transfection. siRNAs were introduced into cells at defined concentrations (20, 40, 50, 100 and 150 pmol) usingLipofectamine®2000 (Invitrogen), according to the manufacturer's instructions. After 6-8h of incubation at 37°C, influenza infection experiments were carried out. Briefly, MDCK cells were infected with influenza virus at MOI=0.001 and 0.0001 for Influenza H5N1 (A/chicken/Egypt/Q 1995D/2010) and MOI=0.1 and 0.01 for Influenza A H1N1/PR8. Infection Opti-MEM® medium was supplemented with 4% BSA and 1% pen/strep (Sigma). Influenza viruses were titrated by HA according to World Health Organization manual on animal influenza diagnosis and surveillance [15].

Quantitative Assay of Virus Genome by Real Time Rt-PCR: RNA was extracted from infected MDCK cells by RNeasy Mini Kit (Qiagen) according to product manual instructions. Q-RT-PCR enables monitoring the amplification of a particular transcript quantitatively at each cycle and hence allow determination of the exact cycle threshold (Ct) at which the amplification enters its linear phase.

RESULTS AND DISCUSSION

***In Silico* Results:** The design and targeting of individual siRNA to conserved regions across different influenza species based on the in-depth analysis of the influenza A viral segments, an exhaustive approach was performed for the design of all possible siRNAs targeting conserved regions. The siRNAs were designed using siVIRUS and using other tools (siRNA software design SDS version 3, ISCORE, SCALES, DSIR, Thermo19 and Thermo 21. The strategy was to design siRNA that will target all species utilized. All siRNAs were 19 nucleotides long with 2 nucleotides overhangs. The siRNAs designed with siVIRUS were done using their intrinsic influenza A virus sequences and the input being the start and end point of the conserved regions as input, while the consensus of the conserved regions was used as input to the other siRNA design tools.

For the siRNA extrapolated from the siVIRUS dataset, 807 siRNA targeted the PB1 and 447 targeted the PA. The siRNAs were scored using the ISCORE designer tool, which uses nine different algorithms, the Thermal 19 & 21 and the scales tool. These include the 3 best performance tools (DSIR, ISCORE and Biopredsi), as well as other algorithms. Furthermore, the siRNAs were also scored using the Thermal 19 & 21 algorithms, which are thermodynamically based and the SCALES program, which is based on nucleotide preferences. Selection of best siRNA in the influenza genome which target functional motifs and are 100% conserved in all diverse influenza sequences. Two siRNAs were selected from the final stages of siRNA design tools which have GC content is less than 50% and whole dG is about -27 to -38.7

Experimental Results: Two siRNAs were selected from the final stages of siRNA design tools, (si 17) and (si 19). siRNA 17 is capable of abolishing the expression of PB1 protein derived from influenza A virus and siRNA 19 inhibits the expression of PA. As a target, it was used the A/chicken/Egypt/Q 1995D/2010 E4 H5N1 and PR8 E1 HA 1:2048 H1N1,since siRNA 17 and siRNA 19 were supposed to inhibit the expression of such PB1 and PA according to *in silico* analysis. For the transfection, it was used MDCK cells that have high susceptibility to infection with various influenza strains. Also, Madin Darby Canine Kidney (MDCK) cells have been widely used as a substrate for influenza virus isolation and vaccine production.

MDCK cells are standard cells for influenza isolation and support the replication of different influenza strains. MDCK cells were infected by A/chicken/Egypt/Q 1995D/2010 E4 H5N1 influenza at MOI=0.001 and 0.0001. Also, MOI=0.1 and 0.01 for PR8 E1 HA 1:2048 H1N1. Culture medium was collected at 6, 12, 24and 48 hours and influenza replication was assessed by haemagglutination assay (HA). Then the cells were transfected by the two siRNAs (si 17 and si 19) that targeting both PB1 and PA sequences to inhibit the replication of the virus.

HA Results: Here we show the suppression of decrease in the viral load of influenza MDCK cells transfected using si 17 and si 19 respectively vial the HA assay. For different minimal concentrations of 20, 40 100 and 150 pmol, respectively were used in these experiments. The MOI of infection was 0.1 for the 20 and 40 pmol experiments and 0.01 for 100 and 150 pmol experiments. Experiments were done by infection of both H1N1 PR8 and H5N1 strains. The highest inhibition was achieved

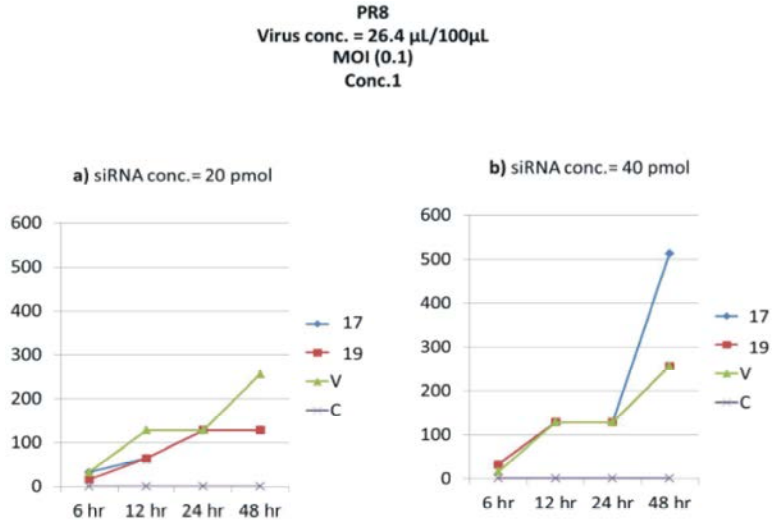

Fig. 1: HA results of PR8 E1 HA 1:2048 H1N1 using two different concentrations from siRNA 20 and 40 pmol.
a) PR8 E1 HA 1:2048 H1N1 virus strain was infected into MDCK cells at MOI=0.1 (26.4 µl/100µl) and siRNA concentration = 20 pmol
b) PR8 E1 HA 1:2048 H1N1 virus strain was infected into MDCK cells at MOI=0.1 (26.4 µl/100µl) and siRNA concentration = 40 pmol

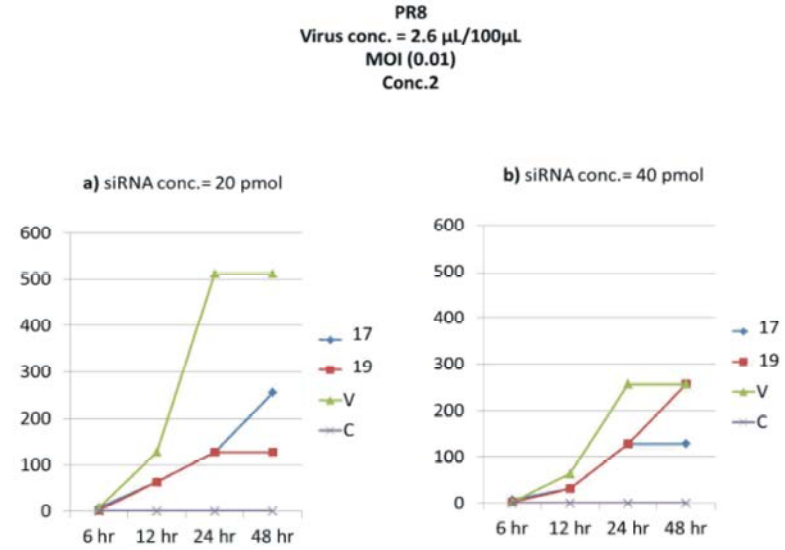

Fig. 2: HA results of PR8 E1 HA 1:2048 H1N1using two different concentrations from siRNA 20 and 40 pmol.
a) PR8 E1 HA 1:2048 H1N1 virus strain was infected into MDCK cells at MOI=0.01 (2.6 µl/100µl) and siRNA concentration = 20 pmol.
b) PR8 E1 HA 1:2048 H1N1 virus strain was infected into MDCK cells at MOI=0.01 (2.6 µl/100µl) and siRNA concentration = 40 pmol.

after 12 hours in which the viral load dropped in comparison to the virus control which was not transfected with siRNAs. The use of very low concentrations and virulent virus strains prevented complete suppression of the virus. Also, suppression of both the H1N1 PR8 and H5N1 relative to the control was achieved.

Fig. 1 shows the inhibition of H1N1 strain by using two different siRNAs, one targets the PB1 gene which is si 17 and the other is si 19 which targets the PA gene in two different concentrations from siRNAs: 20 and 40 pmol. It shows high inhibition after 12 hours from the infection of virus of high concentration 26.4µl/100µl.

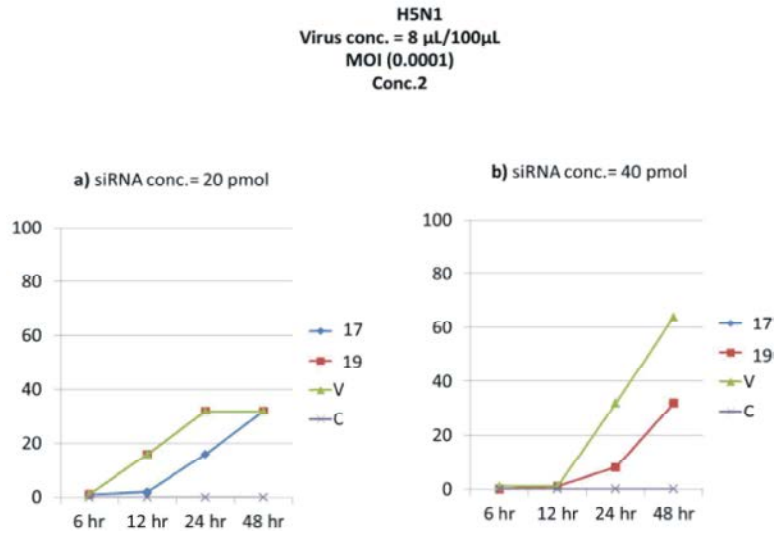

Fig. 3: HA results of A/chicken/Egypt/Q1995D/2010 H5N1using two different concentrations from siRNA 20 and 40 pmol
a) A/chicken/Egypt/Q1995D/2010 E4 HA 1:256 15-3-2012virus strain was infected into MDCK cells at MOI=0.0001 (8 µl/100µl) and siRNA concentration = 20 pmol.
b) A/chicken/Egypt/Q1995D/2010 E4 HA 1:256 15-3-2012virus strain was infected into MDCK cells at MOI=0.0001 (8 µl/100µl) and siRNA concentration = 40 pmol.

Fig. 2 shows the inhibition of H1N1 strain by using two different siRNAs, one targets the PB1 gene which is si 17 and the other is si 19 which targets the PA gene in two different concentrations from siRNAs: 20 and 40 pmol. It shows high inhibition after 24 hours from the infection of virus of high concentration 2.6µl/100µl in compare with virus control that is in green color.

Fig. 3 shows the inhibition of H5N1 strain by using two different siRNAs, one targets the PB1 gene which is si 17 and the other is si 19 which targets the PA gene in two different concentrations from siRNAs: 20 and 40 pmol. si 17 shows highly inhibition after 24 hours from the infection of virus of high concentration 8µl/100µl in compare with virus control that is in green color and with siRNA concentration 20 pmol. si 19 shows highly inhibition after 48 hours from virus infection with siRNA concentration 40 pmol.

Fig. 4 shows the inhibition of H5N1 strain by using two different siRNAs, one targets the PB1 gene which is si 17 and the other is si 19 which targets the PA gene in two different concentrations from siRNAs: 100 and 150 pmol. si 17 shows highly inhibition after 24 hours from the infection of virus of high concentration 1.1µl/100µl in compare with virus control that is in green color and with siRNA concentration 100 pmol. si 19 shows highly inhibition after 24 hours from virus infection with siRNA concentration 40 pmol.

$TCID_{50}$ Results: Fig. 5 shows the inhibition of H1N1 strain by using two different siRNAs, one targets the PB1 gene which is si 17 and the other is si 19 which targets the PA gene in low concentration from siRNAs: 20 pmol. It shows highly inhibition after 12 hours from the infection of virus of high concentration 2.6µl/100µl in compare with virus control that is in green color.

Real-Time PCR Results: The results of $TCID_{50}$ and HA were confirmed by the real-time PCR. Real-time PCR data were analyzed through the calculation of the ratio between the two by the following equation:

$$\text{Ratio}_{(\text{test/calibrator})} = E^{C_T(\text{calibrator}) - C_T(\text{test})}$$

E is the efficiency of the reaction and can be determined. If we assume that the assay has perfect amplification efficiency (that is, the template is doubled in each amplification cycle), then the equation above becomes:

$$\text{Ratio}_{(\text{test/calibrator})} = 2^{C_T(\text{calibrator}) - C_T(\text{test})}$$

or:

$$\text{Ratio}_{(\text{test/calibrator})} = 2^{\Delta C_T}$$

where $\Delta C_T = C_T(\text{calibrator}) - C_T(\text{test})$

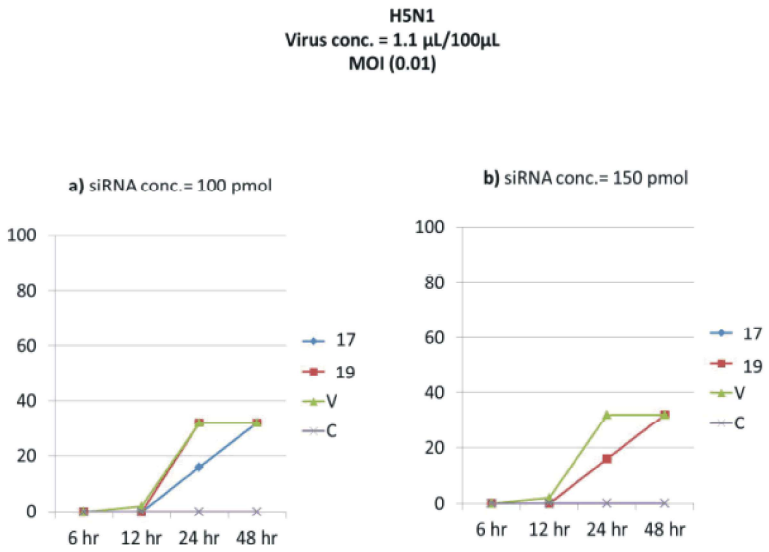

Fig. 4: HA results of A/chicken/Egypt/Q1995D/2010 H5N1using two different concentrations from siRNA 100 and 150 pmol
a) A/chicken/Egypt/Q1995D/2010 E4 HA 1:256 15-3-2012virus strain was infected into MDCK cells at MOI=0.01 (1.1 µl/100µl) and siRNA concentration = 100 pmol
b) A/chicken/Egypt/Q1995D/2010 E4 HA 1:256 15-3-2012virus strain was infected into MDCK cells at MOI=0.01 (1.1 µl/100µl) and siRNA concentration = 150 pmol

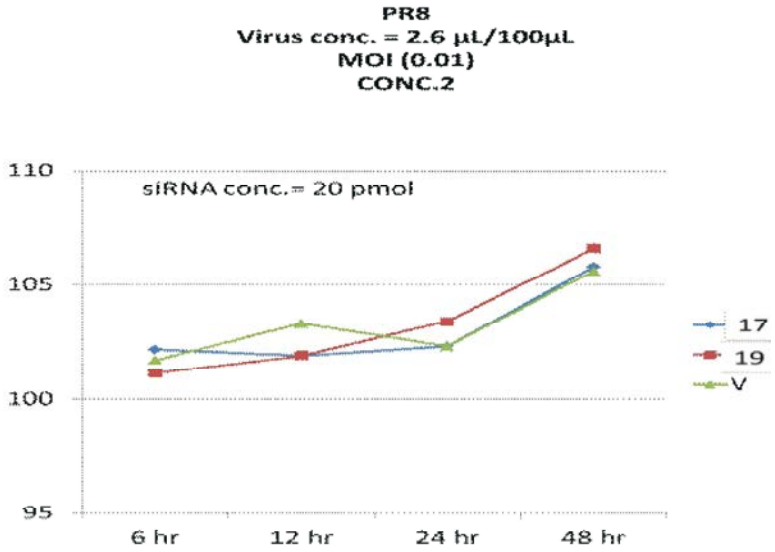

Fig. 5: $TCID_{50}$ results of PR8 E1 HA 1:2048 H1N1 using low concentration from siRNA 20 pmol.
The result of $TCID_{50}$ were calculated for PR8 H1N1 strain with MOI = 0.01 (2.6 µl/100µl) and siRNA concentration = 20 pmol at 6, 12, 24, 48 hours

This method for calculating relative expression is often referred to as the $_{\Delta}C_T$ method.

Suppression of the PB1 and PA gene expression was achieved below in Figure 6 we show perfect silencing of the PB1 and PA genes for the virulent H5N1 strain using only 40 pmol of siRNA. The gene expression decreases with time reaching almost zero after 48 hours.

Fig. 6 shows viral load decrease with time using the H5N1 strain (A/chicken/Egypt/Q1995D/2010 E4 HA 1:256 15-3-2012virus strain) even with the very small concentration of siRNA =40 pmol. Influenza A virus cause widespread infection in animals and human. However, the usage of many antivirals in the therapy against the influenza virus is limited because of concerns of side

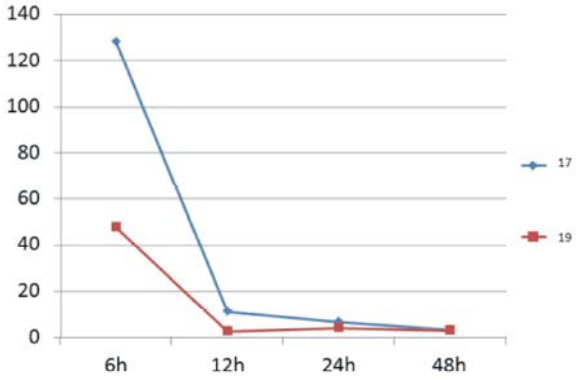

Fig. 6: Real-time PCR result for H5N1 strain using low concentration from siRNA 40 pmol and two different siRNAs si 17 and si 19 at different times 6, 12, 24 and 48 hours

effects, compliance and the emergence of resistant virus strains. Here we show the strategy for designing a novel drug that inhibits the replication for both H1N1 and H5N1 influenza strains. The design of optimal therapeutic small interfering RNA (siRNA) specifically targeting conserved regions of the influenza virus genome that would yield potent inhibitors of influenza virus replication and generated the potential best set of siRNAs for each of the influenza segments (PB1 and PB2). Ge *et al.* [16] formulated a broad inhibitor of influenza A virus H1N1 strain NP1496 and PA 2087. Our strategy was to study all diverse strains of influenza in different hosts, find common conserved regions and use the design power of the best recommended second generation algorithms. This was complemented by target accessibility analysis of the siRNAs using different approaches. Additionally, we selected siRNAs that target functional and completely conserved motifs to ensure a comprehensive exhaustive methodology for efficient potent siRNA selection. Out of the total designed siRNAs using siVirus and second generation tools, more than half remained after filtering the 70% inhibition scores and omitting siRNAs with less than 2 mismatches to human mRNAs. The RISC off target effect was eliminated by eliminating all siRNAs with less than 2 mismatches to human mRNAs. Following the 70% average experimental inhibition percent to scores mapping a wide selection, in hundreds, of siRNA remained that covered the 5 influenza genome with the largest number targeting PB1 (the largest segment). Cropping the list to the scores corresponding to the 90% inhibition reduced the numbers of siRNAs by more than a half, with still more than a hundred siRNA for each segment. Zhang *et al.* [17,18] have designed a siRNA that targets the PA gene and inhibits viral replication of H5N1 strain. Also, PB1 is the critical protein subunit of the influenza virus RNA polymerase and encoding this protein, PB1, is highly conserved among different subtypes of IAV and was therefore chosen as the target in the study of Li *et al.* [19], in which they designed a small interfering RNA that targets PB1 region in influenza A virus. In addition, Cheng *et al.* [20] using the same strategy in designing small interfering RNA (siRNA) targeting overlapping gene of PB1 gene of the influenza A virus and investigated its effect against influenza A virus infection. Zhou *et al.* [21] have tested H5N1 targeting siRNAs.

CONCLUSION

Best siRNAs that target segments PB1 and PA of Influenza A virus are recommended as novel potent specific viral silencers. These siRNAs were designed and analyzed using the consensus of different tools, targeting highly conserved and accessible regions of the genome. Finally, our initial findings and assessment showed high dose-dependent decrease in viral load in comparison to virus infected in control cells at the lowest transfection concentrations of 20, 40, 100 and 150 pmol. This could serve as potential universal therapeutics for the influenza A viral strains. The primary results of inhibition using HA experiments showed inhibition of the virulent H5N1 strain.

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