https://doi.org/10.48047/AFJBS.6.15.2024.11165-11179

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

A design and therapeutic approach of West Nile Virus: in silico method

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Article History

Volume 6, Issue 15, 2024

Received: 25 Aug 2024

Accepted: 24 Sept 2024

Published: 20 October 2024

doi: 10.48047/AFJBS.6.15.2024.11165-11179

Abstract

West Nile Virus (WNV) is a single stranded RNA virus that causes west nile fever. Culex mosquitoes are considered the key vectors of WNV. The virus replicates in skin Langerhans dendritic cells and causes brain damage, permanent muscle weaknesses and death. No vaccinations exit to prevent WNV yet, an alternative therapy is essential.NS2A and NS5 nonstructural proteins of WNV are virulent and NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 are caused for the viral replication. Here-after the aim of this study was the NS5 protein silencing by the RNA interference technique. In the study siRNA was designed against NS5 nonstructural protein for five different strains of WNV using several computational methods. Si Direct 2.0 tool was used to design target specific siRNA. Using BLAST, off target similarity screening was carried out to ascertain the secondary structure and guanine cytosine contents (GC) of the siRNAs that were designed. Using RNA cofold and Intra RNA, the RNA-RNA interaction was computed. The DNA melt web server was then used to determine the duplex concentration and heat capacity. The identified siRNA molecule had no off target in human genome and lower number of GC content that results efficient siRNA function. The selected siRNA molecule exposed exact thermodynamic characteristics for silencing the NS5 nonstructural protein.

Keywords*: West Nile Virus (WNV), Small interfering RNAs (siRNAs), RNA, Guanine cytosine contents (GC), Basic local alignment search tool (BLAST), RNAcofold, DNAmelt*

1. Introduction:

Arthropod-borne viruses possess a large part of global health problem. These are the member of *Flavivirus* genus and cause for serious diseases of humans and animals. They mainly transferred into hosts by mosquito.[1]West Nile Virus (WNV) is such a member. WNV is a single-stranded positive sense enveloped RNA virus. It belongs to *Flaviviridae* family and *Flavivirus*genus. The virus is mosquito-borne and closely relates to other important human pathogens, like dengue (DENV), yellow fever (YFV), Japanese encephalitis virus (JEV) these viruses are also endemic in several region of the world. West Nile fever caused by WNV. About 11kb genome encodes a polyprotein precursor of 3,430 amino acids that cleaved into three structural proteins (capsid, C; precursor membrane, prM/M and envelope, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).[2]

The virus completes a zoonotic cycle between birds and mosquitos. It can infect other animals as well. All over the world WNV has emerged as an important pathogen for human and domestic animals.[3]According to the Centers for Disease Control and Prevention (CDC), about 80% of infected people do not show any symptoms, 20% people show moderate symptoms like fever, vomiting, headache, rash and only 1% people develop severe symptoms, like encephalitis and meningitis that associated with neck stiffness, seizures and confusion.[4]For animal, West Nile virus vaccines (WNVV) are available but there is no recognized vaccine for human. It is needed for a novel effective antiviral therapy against WNV.[5]

The virus contain 10 genes flanked by 5′and 3′noncoding regions (NCR) and no polyadenylation tail at the 3′end.[6] The coding regions encode three structural proteins (C, prM/M, E) and seven non-structural proteins.[7]In the genome, the structural proteins are located at the 5′end and produces mature protein with the help of proteases. Nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) are associated with the viral replication.

According to epidemiological studies, the severity and frequency of west Nile virus illness increases with age.[8] The transit of WNV can get in at any ages and immunocompromised patients including diabetes, cancer, kidney disease, hypertension and transplant recipients are in higher risk. The rate of fatality is greater in aged people and the higher risk arise over the 65 year aged people. After inoculation of WNV, replication occurs in skin Langerhans dendritic cells. Then the cells migrate to the lymph nodes, results in primary viremia and primary infection occurs in peripheral tissues like kidney, spleen etc. After a week, the WNV removed from the serum and peripheral organs and CNS infection occurred in immunocompetent animals.[4]The virus have the capacity to cross the blood-brain barrier (BBB) and can cause infection of CNS. The permeability of BBB increased because of the replication of WNV in astrocytes, microglia and neurons and they trigger the immune mediators. As a result, infection of meninges occurs, CNS cell damaged and correspondingly CNS disease occurred.[9]

Different antiviral therapy used in modern era. In molecular biology, gene knockdown or knockout is also an important technique that connected with different biological pathways. The gene knockout can be attained with the RNA interference (RNAi) technique. Small interfering RNAs (siRNAs) or silencing RNAs are double stranded RNA molecules containing 20-25 base pairs. They follow the RNAi pathways, there they intervene with gene expression of particular genes by hybridizing to complementary mRNA molecules. This restraint transcription and suppress gene expression.[10] It is a valuable tool in basic and clinical research. An important role of siRNA molecules is post-transcriptional gene silencing. So, siRNA can be designed to inhibit the nonstructural protein translation and it can be repressed the West Nile virus replication. Small interfering RNAs could be a targeted therapy for viral diseases.si RNAs contain higher specificity and flexibility as a new therapeutics than other conventional drugs. Now many companies are interested in siRNA technology than others because siRNAs contain more specificity and lower concentrations needed to show the biological effects.[11]

siRNAs are synthesized in mammalian cells by endonucleolytic processing of the ribonuclease Dicer. A cytoplasmicribonucleaseIII–likeenzyme, dicer is called‟molecular ruler″. It recognizes dsDNA and cleaves it into 21-23 nucleotide fragments which include 2-3 nucleotide overhangs.The 3′ end contain a dinucleotide overhang, and the 5′ end terminates in a monophosphate group. This siRNAs then bind to a multiprotein complex called RNA-induced silencing complex (RISC) in which dsRNA become linearized and separated.Single stranded RNA that bind with the RISC are called the guide strand that can hybridizetocomplementarymRNA.

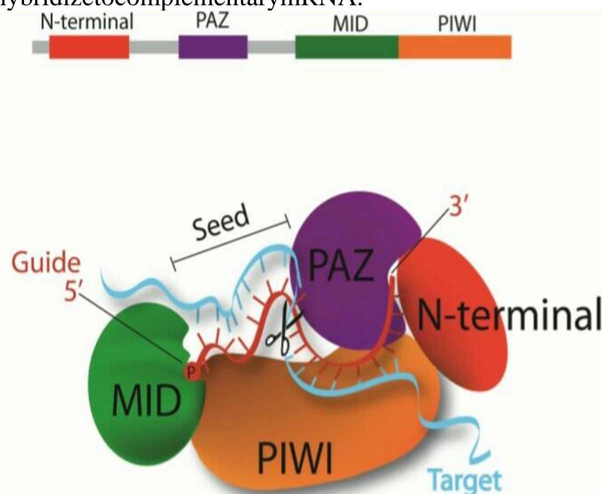

Figure 1.1: AGO-2 mediated siRNA guide-strand tethering and the recognition of target mRNA and slicing. (Source: Gavrilov and Saltzman: Therapeutic siRNA)

Four different argonaute proteins (AGO 1-4) present in human. Argonaute protein is the heart of the RISC complex and it is principal executer of siRNA-mediated silencing. SiRNA silencing occurs by argonaute-2 (AGO-2). AGO2 typically tack the guide siRNA strand and through down the passenger strand then several cycles occur for recognition of the target mRNA. After binding guide strand, cleaves occurs as the translation of mRNA is not possible. AGO2 contain three functional domains these are PAZ, MID, and PIWI. PIWI resorts an RNaseH fold and is the powerhouse behind RISC's “slicer” activity. Particularly, the 3′ dinucleotide is recognized by the PAZ domain of argonaute. The overhang caves into a hydrophobic pocket of the domain, in which terminal nucleotide base can stack with an aromatic ring of one of the

several aromatic residues that line the pocket. At this time, the 5′ phosphate group inserts in the MID and PIWI domains and binds to a magnesium ion that is coordinated to the proteins C-terminus (**Figure 1.1**). Argonaute can select the guide strand and slices the passenger strand for ejection.

In RISC complex, 2-8 guide strand bases (from the 5′ end) remain uncovered and they are free for take part in WatsonCrick base-pairing with target of the mRNA. This "seed region," is important for particular target recognition. In the slicer active site, this region placesthe target's scissile phosphate group.

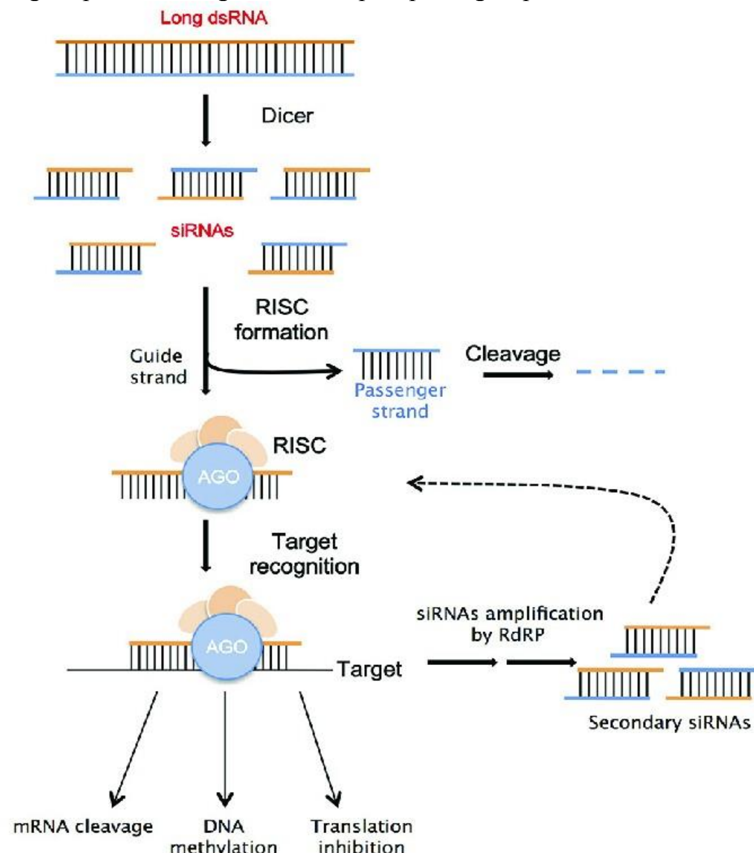

Figure 1.2: Mechanism of siRNA. Firstly, double-stranded RNA (dsRNA) is cleaved into small interfering RNA (siRNA) fragments, which then degrades the complementary messenger RNA (mRNA). (Source: Schematic illustration of RNAi mechanism, researchgate.net)

In the meantime, base-pairing occurred between guide and target strand. Then the silencing occurs with 5′end of the guide strand. The guide strand which is intheRISC complex, can also repeatedlybind to the complementary mRNA and amplify the degradation of the target mRNA.[12,13]

In a research carried out by Beasley et al. in 2002, it was found that West Nile virus (WNV) containvirulence and neuroinvasion properties.[14] WNV is endemic in different parts of Africa, Europe, the Middle East, Asia and America. For animals, vaccines are available but there is no lisenced vaccine for human use.[15]In 2009, Ribavirin and Mycophenolic acid were used as a therapy against WNV by Diamond et al. But these were not selected as therapeutic candidates against WNV infection. Best et al. used interferon-α for treating WNV in 2005 but IFN-α failed to improve outcomes. Antibody based therapy showed promosing result in the first phase but in the next trail it is not effective.[16]Although many vaccines has been discovered for treating human WNV in several times, no one is effective therapeutic agent. Various vaccine candidates for the protection of humans from WNV have been developed in last two decades. Hydrovax-001 is a vaccine candidate that remains in phase I clinical trail and this inactivatesthe virus using hydrogen peroxide.[17] Another candidate is inactivated WNV which is now in phase II clinical trail and it inactivates the virus by fomaldehyde.[18]Recombinant yellow fever vaccine strain (ChimeriVax-WN02) that express the prM/E-fragment of WNV is also now in phase II trail.[19]Another recombinant attenuated DENV expressing the prM/E-fragment of WNV type vaccine (rWN/DEN4Δ30)are designed. This vaccine is remain in phase I clinical trail. Other two vaccines are in phase I clinical trail. These are HBV-002 and VRC WNV.[20]

Nowadays, siRNAs are used as a therapeutic approach for treating different disease. In basic research it becomes an extensive exprimental tool. In mammalian cells, synthesized siRNAs has the ability of gene silencing.[21]It is an effective therapy against cancer,[22] kidney diseases,[23]central nervous system (CNS) diseases,[13]viral diseases, blood genetic

diseasesand so on. In 2018, a lipid based siRNA therapy has been apprved for the treatment of hereditary transthyretin-mediated (hATTR) amyloidosis, approved by United States Food and Drug Adminstration and European Commision.[24]More than 30 siRNA based therapheutics are now in clinical trail that are investigated for curing different diseases including Ebola and Hepatitis B virus infection.[25]The potentiality of the siRNA to silence particular gene is already approved against various viruses including hepatitis C virus,[26]40 HIV-1 infection[27] and herpes simplex virus 2 infections.[28]A lipid nanoparticle based siRNA confirmed 100% protection in Rhesus monkeys against lethal dose of Ebola virus which is approved by FDA for the further evaluation of its efficacy in EBOV infected patients.[29]siRNA also designed targeting NS5 region of Japanese encephalitis virus that particularly inhibit the virus.

Several studies were completed to determine that siRNA could act efficiently as a therapeutic by administering it after viral challenge. Although siRNA could protect against lethal infectionand reduced WNV replication and infection founded by Geiss et al., in 2005. Because of the limitations of siRNA delivery system, the siRNA therapy are not effective.[16]Another vector based siRNA also designated in 2006 but it contain limitations because of vector.[30]Nowadays, delivery systems are modified.Another study revels that West Nile virus replication in vero cultured cells is inhibited by bifunctional siRNA targeting the NS2A and NS5 conserved region of Eg101 strain.[31] So, siRNA can be designed against different strains of WNV.

siRNA with novel delivery systems can be a effective therapy against WNV infection in human. In this study, five strains of WNV genome sequence is used to design siRNA. NS5 nonstructural protein sequence is conserved in these five strains of WNV. The designed siRNA targets the NS5 nonstructural protein.NY99 associated strains first introduced in United States. These are indicated as a lethal strain by exporing in mice.[32] Different bioinformatics tools are used for designing predicted siRNA in this study. The more efficient siRNA can be identified by further evaluation.

RNAs (siRNAs) are double-stranded RNAs that are able to silence their target genes by enzymatic cleavage of target mRNA. siRNA is the method of sequence-specific gene-silencing that can be used as a new therapeutic therapy for treatment of a different types of diseases.For controlling immedicable diseases, now siRNAs are used rather than conventional drugs. Many attempts have been produced for overcoming the problems that are necessary for delivery, stability, off-target gene silencing and immune stimulatory effects of siRNA. Different efforts have carried out to improve in vitro and in vivo delivery of naked or formulated siRNAs. So, therapeutic approaches involving siRNA can be a solution for reemerging West Nile virus outbreak worldwide. The virus contain various endemic strains. In this study we designing siRNA, evaluation of designed siRNA using different tools and delivery of siRNA.

2. Materials and Methods:

2.1: Selection of Viral Strain:

West Nile virus and their related informations (family, genus, host, pattern of transmission, pathogenecity, genome etc.) are selected by ViralZone (https://viralzone.expasy.org/) of the ExPASY bioinformatics tool.

2.2: Retrieval of Sequence and Evolutionary Analysis:

Five complete cds of nonstructural protein (NS) of different West Nile virus (WNV) strains were collected from NCBI nucleotide database (https://www.ncbi.nlm.lnih.gov/nucleotide) that are available at NCBI (https://www.ncbi.nlm.nih.gov/)[33] The accession numbers of selected strains cds are DQ211652.1, AF196835.2, AF260967.1, FJ151394.1, FJ411043.1. Then multiple sequence alignment was done of these retrieved five sequences by Clustal Omega at EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.3: Target Identification and Designing Rational siRNA Molecule:

Efficient and target specific siRNAs were designated by siDirect 2.0 (http://sidirect2.rnai.jp/) web server.[34] The Amarzguioui, reynolds and Ui-Tei rules were maintained for design probable siRNA.[35,36,37] As the melting temperature is the reflection of thermodynamic stability, melting temperatures (Tm) were optimized below 21.5°C. This temperature is the optimal to design functional siRNA duplex.

Table 2.1 represent the important parameters and corresponding algorithms. GC content is the most important parameters for designing siRNAs because lower numbers of GC content caused for weak and unspecific binding and higher numbers opposed unwinding of siRNA duplex with the help of helicase and RISC complex.[38]
Various acceptable range have been established by different studies. Amarzguioui rules suggests for 31.6% - 57.9% GC content.[37] On the other,Reynolds rules demonstrated 36 - 52% GC content.[36] Besides, Ui- Tei rules suggest that in the antisense strand’s GC content between the second to the seventh and the eighth to the eighteenth nucleotides should be respectively 19% and 52%.[35]

2.4: Checking of Off Target Similarity:

It is important to find out that the designated siRNA can bind to other sequence on whole genome on Human. Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast) used to identify off target similarity with any sequence on whole genebank datasets.[39] The accepted thresh-olds value is 10 that means 10 matches are expected to be found only by chance, in according to the stochastic model.[40] BLOSUM 62 matrix was also used to detect weak similarities.

Table 2.1: Parameters for designing functional siRNA from various rules:

Rules	Parameters
Amarzguioui rules[37] **Reynolds rules**[36] **Ui- Tei rules**[41]	Asymmetrical nucleotide content in the duplex (More A/U at 5′-end of antisense sense strand and more G/C at 5′-end of sense strand)
Amarzguioui rules[37] **Ui- Tei rules**[41] **Jagla rules**[42]	Weak base pairing at 5′-end of antisense (existence of A/U)
Reynolds rules[36] **Ui- Tei rules**[41]	Non-existence of internal repeats
Amarzguioui rules[37]	A is sixth position of the antisense strand
Reynolds rules[36]	A is third and nineteenth position of sense strand
Reynolds rules[36] **Jagla rules**[42]	In sense strand non-existence of G at thirteenth position and G/C at nineteenth position
Reynolds rules[36] **Jagla rules**[42]	U is the tenth position of sense strand

2.5: GC Content Calculation and Determination of Folding Pattern:
For calculating GC content of predicted siRNA, Oligo Calc33 Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html)was used. After that, RNAfold Webserver of the Vienna RNA WebSuite (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was used to determine folding patterns of siRNA.[43] The free energy of folding with RNA sequence transcribed from the target sequence using Transcription and Translation Tool (http://biomodel.uah.es/en/lab/cybertory/analysis/trans.htm).

2.6:Calculation of RNA-RNA Interaction:
To study the thermodynamics of interaction between predicted siRNA and target gene, RNAcofold program35 (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi) was used. It helps in calculating the hybridization energy and base-pairing form of two RNA sequences. The server works as extension of McCaskill's partition function algorithm for computing the probabilities of base pairing, realistic interaction energies and equilibrium concentrations of duplex structures. Then the web server IntaRNA 2.0 (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) was used to crosscheck the outcomes.[44]

2.7:Determination of Heat Capacity:
Heat capacity and concentration plot of siRNA was computed by using DINAMelt web server (http://unafold.rna.albany.edu/?q=DINAMelt/Hybrid2).[45]The collective heat capacity (Cp) is plotted as the function of temperature, by the melting temperature Tm (Cp). The inclusive heat capacity plot explains the contributions of each molecules to the collective heat capacity. On the other, the concentration plot (Tm Conc.) is the point at which the concentration of double-stranded species of one-half of its maximum value defines the melting temperature Tm (Conc).

2.8: Prediction of Secondary Structure:
The UNAfold program of the mfold web server (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) was used to expect the secondary structure that is established between RNAi guide strands and mRNA. The UNAfold web server (Unified Nucleic Acid Folding and hybridization package) is now a conjugation of two existing web servers: mfold & DINAMelt. The program aims to integrate the existing servers and to expand by developing algorithms and software which will provide more effective results.

2.9: Others Criteria for Designing siRNAs:
Target gene, length of siRNA and specificity of siRNAs are important factors for designing siRNA molecules. DNA microarrays are used for the identification of the specificity of designed siRNAs.[46]

2.9.1: Target sites for siRNA:
Single nucleotide polymorphism (SNP) locations are avoided in siRNA designing because they may be caused for variation in gene silencing strategies. Intronic parts, 5´ UTR and 3´ UTR are also avoided because UTRs and the sequences that are close to start codon can interfere with RISC complex and gene silencing. For silencing, 50-100 nucleotides downstream of start codon are the perfect target sites.[47] Accessibility of target site for secondary structure of mRNA is the indicator of siRNA functionality. Any variations of target sites can be influenced the effectivity of siRNA.[48,49]Continuous four unpaired bases at the 5´ or 3´ ends in the target site has silencing effect of siRNA.[50]

2.9.2: siRNA length:
Smaller siRNAs are very useful for mammalian cells because lengthy siRNAs induce mammalian immune response. siRNAs should be 21-29 nucleotides for obtaining better result. Short siRNAs involved in unspecific binding but 21-29 base containing siRNAs have the same efficiency in silencing.[51]

2.9.3: Specificity:
To abate the risk of unintended gene, the designated siRNAs should be checked by BLAST. The acceptable range of BLAST result must be; higher E value, query coverage may be less than 78% with other genes and ⩽15 nucleotides out of 19 matching with the respective siRNA.[52]Palindromic sequences and internal repeats are screened for siRNAs.The antisense strands 5´ end must have lower internal stability that is important for unwinds siRNA duplex and enter into the risk complex.[53]

3. Results:
3.1: Conserved Sequence Identification amongFive Different Strains:
NS5 nonstructural protein of five strainsfrom West Nile virus were collected from the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/) and used to design a siRNA molecule against the target protein. The conserved sequences and phylogenetic tree were analyzed by using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) from different strains coding region. For this the designated siRNA targets all these five strains. A portion of the conserve sequence is added in **Figure 3.1**and this portion contain the target for siRNA.

```
TGGGTGAGTCGAGCTTCAGGCAATGTGGTACATTCAGTGAATATGACCAGCCAGGTGCTC
TGGGTGAGTCGAGCTTCAGGCAATGTGGTACATTCAGTGAATATGACCAGCCAGGTGCTC
TGGGTGAGTCGAGCTTCAGGCAATGTGGTACATTCAGTGAATATGACCAGCCAGGTGCTC
TGGGTGAGTCGAGCTTCAGGCAATGTGGTACATTCAGTGAATATGACCAGCCAGGTGCTC
TGGGTGAGTCGAGCTTCAGGCAATGTGGTACATTCAGTGAATATGACCAGCCAGGTGCTC
TGGGTGAGTCGAGCTTCAGGCAATGTGGTACATTCAGTGAATATGACCAGCCAGGTGCTC
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Figure 3.1:A portion of conserve sequence.

The phylogenetic analysis revealed that there had two common ancestor among the four sequences and they reserved some similarity at the time of evolution. The phylogenetic tree represented in **Figure 3.2**.

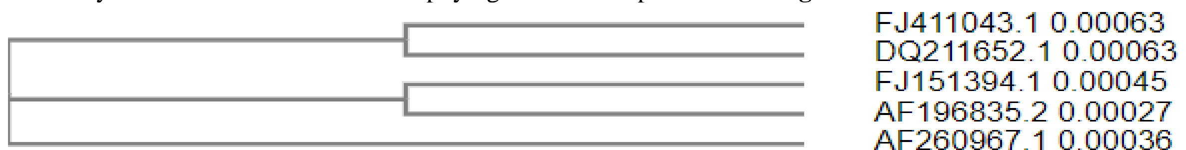


Figure 3.2: Phylogenetic tree of the used strains of WNV.

3.2: Effective siRNA Design by siDirect:
One most effective siRNA is predicted using the web-based online tool siDirect that follow Ui-Tei, Amarzguioui and Reynolds rules. The software computes highly effective siRNA with maximum target specificity from the retrieved sequences. Melting temperature is a reflexion of thermodynamic stability and in gene silencing, experiments suggest the melting temperatures below 21.5°C is optimal.[41] The designated siRNA is presented in **Table 3.1 and Figure 3.3**.Thedesignated siRNAmaintains all the criteria.The formula for calculating the Tm is: $Tm = \{(1000 \times \Delta H) / (A + \Delta S + R\ln(CT/4))\} - 273.15 + 16.6 \log[Na+]$

Here, the sum of the nearest neighbor enthalpy change is ΔH (kcal/ mol), A is the helix initiation constant (-10.8), the sum of the nearest neighbor entropy change isΔS.[54] R is gas constant (1.987 cal/deg/mol), and CT is the total molecular concentration of the strand (100 µM). [Na+] was fixed at 100 mM.

target position	target sequence 21nt target + 2nt overhang	RNA oligo sequences 21nt guide (5'→3') 21nt passenger (5'→3')	functional siRNA selection: Ui-Tei Reynolds Amarzguioui	seed-duplex stabilty (Tm): guide	passenger
19-41	AGGCAATGTGGTACATTCAGTGA	ACUGAAUGUACCACAUUGCCU GCAAUGUGGUACAUUCAGUGA	U R A	☐ 18.1 °C	18.1 °C

Figure 3.3: Designated effective siRNA candidates.

3.3: Elimination of Off-Target Similarity:

The siRNAs are crosschecked by Basic local alignment search tool (BLAST) for any sequence homology with human genome. This is done for discarding any off-target binding of siRNA.

NIH U.S. National Library of Medicine
National Center for Biotechnology Information

BLAST ® » blastn suite » results for RID-0YN2U5DK016

< Edit Search | Save Search | Search Summary

Job Title	Nucleotide Sequence
RID	0YN2U5DK016 *Search expires on 01-04 21:48 pm* Download All
Program	Citation
Database	nt See details
Query ID	lcl|Query_54951
Description	None
Molecule type	rna
Query Length	42
Other reports	

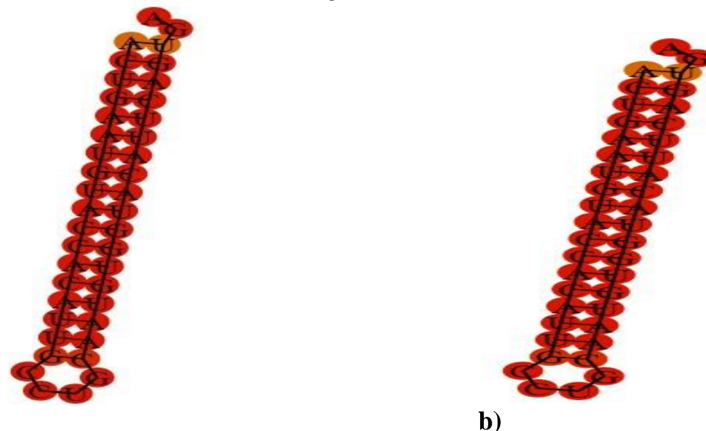
No significant similarity found. For reasons why, click here

Figure 3.4:BLAST result for proposed siRNA.

BLAST result eliminates the off-target binding possibility.BLAST similarity for the retrieved sequences that are used in the study result no possible chance of off-target binding for designated siRNA.

3.4: Evaluation of GC Content and Secondary Structure:

Lower number of GC content results unspecific binding and higher GC content prevent the release of passenger strand. For this reason the GC content range set up for the predicted siRNA is 40-60%, calculated by OligoCalc33 Oligonucleotide Properties Calculator. The effective designed siRNA revels 43% GC content that displays in **Table 3.1**.



a) **b)**

Figure 3.5: Secondary structure of the siRNA. (a) MFE secondary structure and (b) Centroid secondary structure.

Molecular structure is important for the utility of siRNA. RNAfold WebServer is used for detecting secondary structure of RNA. Here, the server was used for detecting folding pattern and minimum free energy. **Table 3.1** represent these values of designated siRNA.

3.5: Determination of Target-guide Strand Interaction by Thermodynamics:

The predicted siRNAs free energy of binding with targets were analyzed by two server (RNAcofold and IntaRNA) and the result showed the highest binding of the predicted siRNA molecules with the target sequences.

The free energy that released for binding of siRNAs with target is represented in **Table 3.1**. The interaction of siRNA molecules with their target is also represented in **Figure 3.6**.

Table 3.1: The Proposed siRNA molecules with GC%, free energy of binding with target.

Target No	Target Position in mRNA	Target sequence	Predicted siRNA duplex	GC Content (%)	Free energy of folding with target (kcal/mol)	Tm Cp (°C)	Tm Conc. (°C)
1.	19-41	AGGCAATGTGGT ACATTCAGTGA	ACUGAAUGUACC ACAUUGCCU GCAAUGUGGUAC AUUCAGUGA	43.0	-34.32	76.0	78.5

3.6: Heat Capacity and Concentration Plot Duplex:

The values for Cp as a function of temperature and concentration (Conc.) as a function of temperature are represented in **Table 3.1.** The effectivity of RNAi molecules depends on the high the value.[55]The higher Tm value conduct to the best siRNA and our predicted siRNA revealed high Tm value.

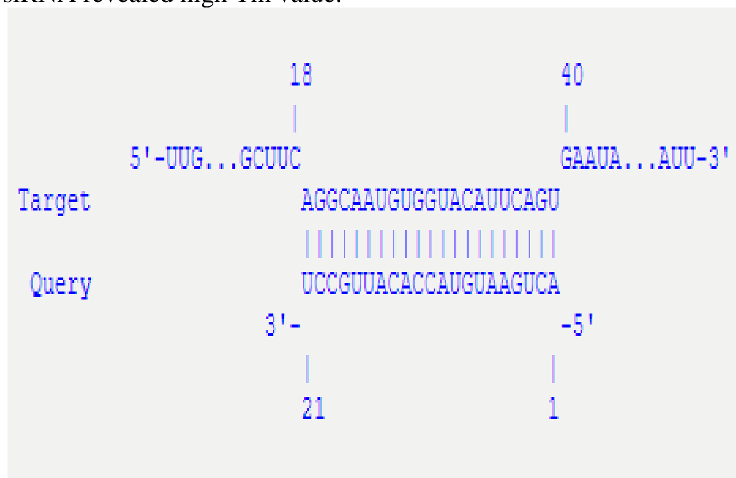


Figure 3.6: Interaction of siRNA with target.

3.7: Prediction of Secondary Structure:

The secondary structure was predicted by the Mfold Web Server of UNAfold program. The structure of the designated siRNA molecules are represented in **Figure 3.7**.

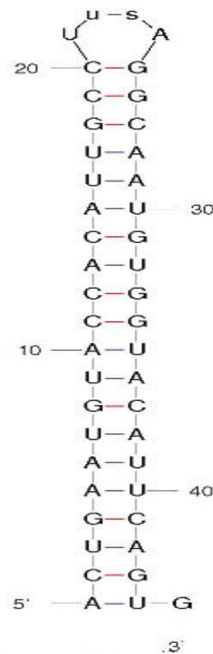


Figure 3.7: Secondary structure of target siRNA duplex.

3.8: Delivery of Designated siRNAs:

The gene silencing agent siRNAs has no utility if it cannot be delivered to its intentional cells.

Proper and effective distribution is necessary for systemic administration of siRNA therapeutics. When naked siRNAs are delivered into cells, it faces two main obstacles.[56]

1. siRNA molecules contain high negative charge, hydrophilicity and molecular weight which make transportation through the biological membranes difficult.
2. They may be degraded after the cellular uptake in the endosome/lysosome compartment and cannot able to bind the RISC within the cytoplasm.

For overcoming these hindrances, liposomes and nanoparticles are used for the delivery of the designated siRNA.

3.8.1: Liposomes:

Liposomes contain a phospholipid bilayer that surrounds an inner aqueous portion which is a prominent strategy for delivery of siRNA to target cells. Liposomes help in proficient internalization of siRNA by membrane fusion with the host cell.[57]Biocompatibility of the constituents and facile assembly of the complexes are important for mixing and incubation of components.So, Lipid encapsulation is an effective delivery method.[58]It is also important for specific delivery to conjugation of targets and lipid molecules prior to the liposome production. Neutral lipids are absolutely non-toxic and are not activate an immune response. Two highly used neutral lipids are 1, 2-Oleoyl-snGlycero-3-phosphocholine (DOPC) and 1, 2Dioleoyl-sn-Glycero-3-phosphoethanolamine (DOPE).Mixing DOPC with siRNA results in more than 65 percent encapsulation. If neutral lipids show lower transfection, cationic lipids, such as 1-oleoyl-2[6-[(7-nitro-2-1, 3-benzoxadiazol-4-yl) amino]hexanoyl]-3-trimethylammonium propane (DOTAP) is used for producing complex electrostatically with siRNAs. It is used to producing a highly effective liposome because of the positively charged lipids help in promoted cell entry and increase the protection against serum enzymes. The incorporation of positive charge to increase transfection efficiency should be balanced carefully against inflammatory effects that create polycations in vivo. Theunwanted interaction with negatively charged serum proteins must be balanced .Because this interaction helps in opsonization and clearance of the lipocomplex.[59]

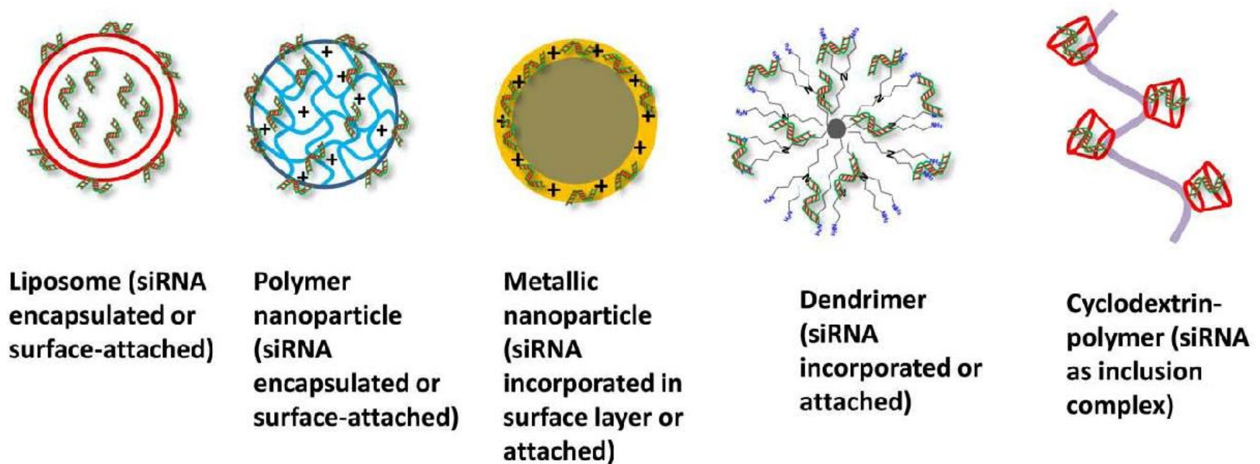


Figure3.8:siRNA Nano carriers. (Source: nanoparticles for siRNA based gene silencing in tumor therapy)

Another delivery system based on lipid, has demonstrated that include synthetic lipid-like materials (lipidoids). These form complexes with siRNA or miRNA which helps in intracellular delivery of the oligonucleotides.[60] Lipidoids are not effective individually but effective when formulated together as binary combinations into single delivery vehicles.[61]

3.8.2: Nanoparticles:

Polymeric nanoparticles are efficient gene delivery approach as they maintain stability and controlled release. Nanoparticles contain other effective properties; these are capacity to encapsulate large amounts of genetic material, surface modification to enhance stability, transport properties, targeting, or uptake and also allow co-delivery. Biodegradable, biocompatible, and non-toxic polymers produce efficient candidates to design in vivo delivery vehicles. Nanoparticles generally used in delivery system are chitosan, cyclodextrin, dendrimers, and metallic core nanoparticles, polyethyleneimine (PEI), poly(lactic-co-glycolic) acid (PLGA). The metallic core nanoparticles is popular for use in delivery approach.[57]

Chitosan is natural nanoparticles that is cationic polysaccharide and can be separated from crustacean exoskeletons. It act as effective biomaterial because of its biocompatibility, mucoadhesive properties, and nuclease resistance.[58] This particle already used in silencing GAPDH and EGFP in the lungs of mice.[62]Cyclodextrin-based polycations (CDPs) are also non-toxic polymer-based complexes that is used to deliver siRNAs. These constructs contain cationic polymer that complexed with siRNA duplexes and interdigitated with funnel-like cyclodextrin molecules. This cyclodextrin then can be linked to functionalized adamantane molecules. Alabi CA, et al suggest that these materials can produce RNAi in humans.[63] Synthetic polymer polyethyleneimine (PEI) have the capacity to condense siRNAs and protect from degradation by RNases, and also facilitate their cellular uptake via endocytosis. [64] As it shows toxicity in vivo, the polymer has been modified with polyethylene glycol (PEG) and the modified PEI-PEG/siRNA complex reduced toxicity.[65]

Another FDA approved, favourable strategy of siRNA delivery is degradable polymer PLGA. It contain the capacityof intracellular delivery of siRNAs, biocompatibility and can also be easily assembled into a carrier system for large amounts of siRNA that offers controlled and sustained release.[66]

Dendrimers polymeric molecules are also used in siRNA delivery because of its nano-sized, spherical structures.In its structure, it packaged siRNAs and can be accomplished by positively charging the core at the time of abolishing surface charge.[67]siRNAs become closed up within dendrimer polyplexes by disulfide linkages that provide controlled release. By combining them with targeting ligands the strategy can be more effective.[68]

Metallic core nanoparticlesinclude another delivery method.[69]Metal cores of iron oxide, iron cobalt, iron gold, or iron nickel are coated with a layer of sugars or other polymers and generates a core-shell structure in which siRNA can be conjugated by the linking molecules like thiols, dextran, cationic polymers or biotin-streptavidin. Iron oxide nanoparticles-dendrimer complexes used effectively deliver siRNA.[70]

3.8.3: Targeting molecules:

Selective targeting diseased cell is important for specificity and avoiding off-target effects. Targeting can be achieved by combining siRNAs to ligands.These ligands includeantibodies, aptamers, small molecules, and peptides that specifically interact with corresponding surface moieties of target cells.[71]

Antibodies are effective molecules for targetingbecause of specificity, diversity, and ability to modulate bio-distribution. Aptamers are also used in targeted siRNA delivery that are able to bind their respective ligand molecules with an affinity and specificity. Aptamer-based targeting may become more widespread in siRNA delivery.[58]

Cholesterol and its derivatives have been effectively used in targeting ligands as they include stability and bioavailability.[72]

4. Discussion:
West Nile virus responsible for neuroinvasive disease, may be deadly in severe cases. There is no licensed vaccine and no siRNA based therapy available to prevent West Nile virus infection in human. For this, siRNA therapy would be helpful against WNV infection. In this study, conserved NS5 nonstructural protein sequences of five different strains of WNV are used that are available in gene bank database.The consensus target sequence was identified by multiple sequence alignment of five different strains of West Nile virus through Clustal Omega.These lethal strains were chosen to measure the ability of the proposed siRNA molecule. To design an effective siRNA molecule targeting a specific gene have various challenges that includes stability of siRNA,efficient delivery method, off-target silencing and activation of immune response.[12]

For overcoming these obstacles, the online web-server siDirect is used for providing target specific, efficient, thermodynamically stable siRNA. Using suitable algorithm, the software decrease off target silencing capacity and considers the thermodynamic stability of the seed-target duplex. The server also follow the Amarzguioui, Reynolds and Ui-Tei (URA) rules. siDirect revealing siRNAs were reviewed through this three rules. URA rules proposed only one siRNA that maintains 21.5°C maximum seed duplex stability of the both guide and passenger strand.

After primary selection, the siRNA was checked in blastn and there have no off-target similarity with the human genome. This result indicates that the targeting site of nonstructural protein will not mediate in silencing any genes of host.
GC content is the most importantparameter for designing functional siRNA.Lower number of GC content results in inhibitory properties. The standard GC content for siRNA action is 40-60%. The GC content of the selected siRNA molecule is 43% that supported the range of 40-60%. The calculated server is OligoCalc33, Oligonucleotide Properties Calculator. Higher GC containing siRNAs were excluded because of their possibility of forming secondary structure.
Another determinant for the utility of siRNA is molecular structure. Using RNAfold web serverthe minimum free energy of secondary structure of the siRNA was predicted.[43]The server is used because of predicting the most appropriate and exceptional system of RNA structure which is free energy minimization.[73]RNAfold web server measured the folding minimum free energy for determination the stability of the guide strand. The summarized lowest free energy of proposed siRNA's are added in **Table 3.1**.

Thermodynamics stability of RNA-RNA interaction is another important parameter for siRNA efficiency. This predicted by the RNAcofold server from Vienna RNA web services. The RNA–RNA interaction thermodynamics is explained by the summation of two energy contributions, one energy is essential to open the binding position and another energy that is gained from hybridization.[74]Considering a combination of thermodynamics and RNA-RNA interaction kinetics, RNAcofold predicts the structure. Here, ΔGA is the free energy of the heterodimer of sequence A (target sequence in mRNA) and ΔGB is the free energy of sequence B (siRNA).ΔG of binding was calculated by using the equation, $\Delta G = \Delta GAB - \Delta GA - \Delta GB$; that has been presented in **Table 3.1**.[75]After that, IntaRNA 2.0 crosschecked the thermodynamics of RNA-RNA interaction.[44] Here, the uniformthermodynamicscheme of proposed siRNA molecule had been audited and the predicted interactions are enlisted in **Figure 3.6**.

The DINAMelt web server calculates the melting temperature of proposed siRNA. The server supplies the whole equilibrium melting profiles as a function of temperature. It computes ultraviolet absorbance at 260 nm, heat capacity change Tm (Cp) and concentrations of different molecular species as the function of temperature. Tm (Conc.), is the temperature where the total concentration of all dimers is half of its maximum value at low temperature can be successfully predicted using this server.[45] The efficiency of siRNA molecule depends on the high Tm value.[55] Higher Tm value reveals the effective siRNA and the proposed siRNA represented high Tm value. The revealed Tm (Cp) and Tm (Conc.) enlisted in **Table 3.1**.

The Mfold server of UNAfold web server is widely used comprehensive tool for predicting of nucleic acid folding and hybridization. Minimum free energy of folding andhybridization are computed by energy minimization approaches. This approaches also figure out suboptimal folding. The secondary structure helps in RISC cleavage and inhibit the siRNA's activity. So, higher stabled secondary structure of the proposed siRNA is found out by minimizing folding free energy.The structure was proposed at the 37°C particular temperature. The proposed siRNA's secondary structure is shown in **Figure 3.7**.

There are some challenges for therapeutic siRNA. These are siRNA stability and targeting, off target silencing and activation of immune response. As the proposed siRNA contain short nucleotide sequence and validated by different server it may overcome these challenges.

After the above discussion it can be called that the proposed siRNA maintains all the intended criteria that considered for effective siRNA approach and could play a significant role in silencing NS5 nonstructural protein of different strains of West Nile virus. The designated siRNA may be used as effective candidate in the dynamic treatment of West Nile fever by silencing nonstructural protein. The highestefficiency of the siRNA may be due to this NS5 regions of WNV genome codes for RNA dependent RNA polymerase enzyme for virus replication. This region is highly conserved in WNV andhopeful target for antiviral therapy and their corresponding siRNA may reveal proficient inhibition of WNV replication. This designated siRNA might play an important role in silencing the NS5 nonstructural protein of used five strains of WNV with the liposome and nanoparticle mediated delivery of the siRNA. Liposome and nanoparticle mediated delivery are most effective and significant approaches for siRNA delivery within host cell. The predominance of siRNA therapy is sequence specific gene suppression.

5. Conclusion:

When conventional therapeutic approaches failed to treating diseases, molecular therapy becomes promising alternative therapeutic system.Some diseases are difficult to cure but siRNAs already proved particular and effective cure of these diseases. In this study, we designated a siRNA molecule to be used as a therapeutic against West Nile virus. We computedvarious parameters to support the functioning and permanency of the proposed siRNA molecule. As molecular therapies are specific to DNA sequences, these therapies sometimes become nonfunctional against different strains and for delivery limitations. The siRNA moleculeis expected to overcome theseissues of being nonfunctional against different strains of West Nile virus by targeting a conserved sequence among five lethal WNV strains and associated with the liposome and nanoparticle mediated delivery of the siRNA.The future prospects of this research are in vitro and in vivo delivery of the siRNA, vaccine and antiviral drug design against WNV. There is needed extensive time and cost-saving prospects for development of therapeutic based on this siRNA molecule. Although, in vitro, in vivo validation and verification of biological safety are needed, this siRNA based drugs may give an identical facility to supremacy unique drug development. Rather than all interruptions, we hope that the designated siRNA molecule will provide an efficient treatment system against the targeted West Nile virus strains.

6. Acknowledgments: None

7. Conflict of Interest:
There is no conflict of interest to declare.

8. Financial Support: None

9. Ethics Statement: Not applicable

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