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Original Article

In-Silico Prediction of Common siRNA Targeting Protein Coding Sequence of *NS5* Gene of West Nile and Japanese Encephalitis Virus

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HIGHLIGHTS

- Promising siRNA target identified for dual protection against WNV and JEV.
- Efficient siRNA molecule S2 shows high binding affinity with human AGO2 protein.
- siRNA targeting NS5 gene shows promise for treating WNV and JEV-mediated infections.

ABSTRACT

West Nile virus (WNV) and Japanese Encephalitis virus (JEV) are two major mosquito-borne flaviviruses that share almost similar symptoms after infection, e.g., flu-like symptoms. Though JEV is the most common cause of encephalitis (brain inflammation), recent studies have discovered approximately 30% involvement of the WNV in this. Furthermore, both viruses share similar genetic constituents with more than 70% homology. Therefore, these two viruses sometimes cause misdiagnosis due to their co-circulation with the same vector, and no solid protective treatment has yet been discovered against them. As a result, in this study, we used small interfering RNAs to provide dual protection against both viruses. The siRNAs have high demand as a potential treatment option for genetic treatments, and antiviral or antibacterial therapeutics for many diseases. In this study, we concluded that a single highly potent siRNA (5'-UCUCUUUCCCAUCAUGUUGUA-3') could be effective in silencing the coding sequences (CDS) of both WNV and JEV by utilizing several computational assays such as GC content, free energy of binding, free energy of folding, melting temperature, siRNA efficacy prediction, and molecular docking. After structural analysis (molecular modeling and docking), we found that this siRNA effectively binds with the human Argonaute-2 (AGO2) protein. Consequently, this siRNA could be a potential therapeutic development target against both viruses by silencing the CDS. Our research aims to develop genome-level therapies. The results can be applied to develop RNA molecules as a drug against WNV and JEV.

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Introduction

Two flaviviruses of West Nile virus (WNV) and Japanese Encephalitis virus (JEV) are mosquito-borne viruses globally, posing substantial medical concerns. The ability of these viruses to infect non-endemic areas by mosquito vectors has already significantly affected too many people's lives. Both of these viruses can cause a wide range of clinical symptoms, including nonspecific fever, muscle weakness, myalgia, vomiting, neurologic dysfunction, lower back pain, loss of motor function, and in the case of JEV, encephalitis (Daep et al., 2014).

West Nile virus is a zoonotic infection that first emerged in the Western Hemisphere in New York City (1999) before swiftly expanding across North America, infecting wild birds, horses, and people (Guy et al., 2010). Since the first recorded case in 1999, WNV has been spread among the US population, with yearly issues ranging from 20 to 5,674 between 1999 and 2012 (Daep et al., 2014). However, the WNV has lately arrived throughout South America, including Brazil and Argentina. Furthermore, neuroinvasive disease affects 30% of cases of WNV, ranging in severity from mild febrile exanthema to fatal encephalitis. In addition, since its introduction, WNV infection has been linked to approximately 30,000 points and over 1,000 fatalities worldwide (Lindsey et al., 2008; Murray et al., 2008).

On the other hand, approximately 3 billion people living in endemic areas are at serious risk from JEV, the most common cause of viral encephalitis in Asia. JEV has also invaded areas of Australia that were previously uninhabited (Van den Hurk et al., 2008; Van den Hurk et al., 2009). Every year, it is thought to be responsible for 50,000 reported cases of illness and 10,000 fatalities, with children being especially at risk (Halstead and Jacobson, 2012). Roughly, 1–20 cases of acute encephalitis occur for every 1,000 infections, and 29% of these cases result in death (Guy et al., 2010). With up to 70,000 cases reported annually (Campbell et al., 2011), JE is also the main viral cause of encephalitis in Asia.

WNV has also been shown to belong to the JE serogroup, thus these two viruses can be targeted for cross-protective medication administration. The genome organizations are also similar in both of these viruses (Gaunt et al., 2001). Three structural proteins (C, M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are encoded by the 11 kb (+)-sense ssRNA genome of the WNV (Heinz and Stiasny, 2012) which are also found in the JEV with similar structure (Tesh et al., 2002).

RNA interference (RNAi), a biological regulatory mechanism, inhibits mRNA through post-transcriptional gene silencing. Then, it is hypothesized that human viral infections can be managed with the help of RNAi (Levanova and Poranen, 2018). There are several non-coding RNA have been found till now that have RNAi activity e.g., small interfering RNAs (siRNAs) or miRNA. These types of non-coding RNAs can hybridize with corresponding mRNA molecules and neutralize mRNA to decrease gene expression (Lam et al., 2015). The siRNA is an RNA duplex that is between 19 and 25 base pairs long with two nucleotide overhangs on the 3′ end. Post-translational gene silencing (PTGS) is caused by the target complementary mRNA's binding to it and the resulting reduction in its enzymatic stability (Méndez et al., 2015).

Inhibiting gene expression through siRNA is a complex process. The siRNA duplex enters the cell and is separated by dicer, an enzyme similar to RNase III. It then becomes a part of the RNA-induced silencing complex (RISC), which is responsible for silencing genes (Xu et al., 2019). The RNA strands within RISC are cleaved by the ATP-dependent RNA helicase domain. RISC eliminates the sense strand of the target mRNA. The catalytic RISC protein, which is an Argonaute (AGO) protein, can position RISC on the target mRNA and then cut both strands of the mRNA (Dana et al., 2017).

In this investigation, we found the siRNAs against the WNV, which is also conserved and homologous to the JEV and could therefore be speculated to generate cross-effecting gene silencing for both viruses *in vivo*. While scientists are trying hard to develop a specific treatment for WNV and JEV, no approved definitive vaccinations or medications are currently available. Currently, there are only a limited number of flavivirus vaccines that have been approved for human use globally, and in the United States, there are only two available. These include the inactivated JE vaccine, which was developed in Japan by BIKEN and is marketed in the United States by Connaught Laboratories, as well as live yellow fever (YF) 17D vaccine, which is produced in the United States by Aventis Pasteur (Heinz and Stiasny, 2012). Additionally, researchers have used a variety of animal models to study the issue of cross-protection amongst

heterologous, closely related members of the Flaviviridae family, and they are currently working to build common cross-protective treatment protocols (Goverdhan et al., 1992; Tesh et al., 2002; Williams et al., 2001). Furthermore, in the mouse model, the inactivated JE vaccination showed a modest protective effect against deadly WNV infection (Takasaki et al., 2003). Therefore, we hypothesized our study would aid in developing a similar therapeutic technique against both of these viruses.

Materials and Methods

Sequence retrieval and multiple sequence alignment

The genomic sequence of both of the virus mRNA was obtained from the NCBI virus database under the accession numbers NC_009942 (WNV) and NC_001437 (JEV). These are the RefSeq data of both viruses. After sequence retrieval, multiple sequence alignments were performed on the different genes of both viruses to find the best homologous gene. We then selected the highest homologous gene's coding sequences for further siRNA prediction analysis.

Designing of siRNA from the coding sequences of NS5 gene

To identify the siRNA molecule from the coding sequences (CDS) of the highest homologous gene of both viruses, siDirect (https://sidirect2.rnai.jp/) was used (Naito et al., 2004; Naito et al., 2009). First, the retrieved FASTA sequence of the highest homologous gene's (here in this study, *NS5* protein) CDS was submitted in the siDirect web server, in order to identify potential siRNAs. The seed duplex's melting temperature (Tm) is default set by the web server to be below 21 °C. Improving the efficacy of siRNAs depends on the seed duplex melting temperature to reduce off-target effects (Naito et al., 2009). Each of the three algorithms selected for siRNAs prediction has distinct characteristics and employs several established rules (Amarzguioui et al., 2004; Reynolds et al., 2004; Ui-Tei et al., 2004). For example, the Ui-Tei algorithm follows specific rules, such as i) 5′ the antisense/guide strand's terminus must include an A/U nucleotide, ii) G/C nucleotides must be present at the sense/passenger strand's 5′ end, iii) there must be at least 4 A/U nucleotides of 7 base pair in the sense/passenger strand's 5′ terminal, and iv) a maximum of 9 nucleobases should separate each GC base pair (Ui-Tei et al., 2004). Meanwhile, Amarzguioui rules include the parameters such as A/U differential of the duplex end must be greater than zero, position 6 should always contain A, position 1 must contain any base apart from U, position 19 must contain any base besides G, and position 5′ sense/passenger strand binding must be robust (Amarzguioui et al., 2004). Reynolds algorithm also follows several criteria, such as i) between positions 15 and 19, the sense/passenger strand needs to maintain three base pairs, ii) maintaining the intended siRNAs GC content between 30% and 52%, iii) the sense/passenger strand must contain A in positions 19 and 3, iv) the target site's internal stability must be low, v) sense/passenger strand should contain U at position 10, vi) there must be a base other than G at position 13 of the sense/passenger strands (Reynolds et al., 2004).

Investigation of parameters for siRNA refinement

Several refinement procedures were performed for highly effective siRNA selection to identify the most effective siRNAs. GC content of the siRNA molecules was calculated through the OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) (Kibbe, 2007). In order to predict the siRNAs secondary structure and free energy of folding, we subsequently used the RNA structure website (Bellaousov et al., 2013). Any siRNA that showed negative free energy of folding in the web server needs to be excluded from further study. Then, we predict the RNA-RNA interaction between the target and guide strand of siRNAs. A stronger contact between the target and guide strand is indeed a better predictor of siRNA efficacy. As a result, using the Bifold tool on the RNA structure website, the thermodynamic interaction between the target and guide strands was anticipated (Bellaousov et al., 2013). The DINAMelt web server (http://www.unafold.org/hybrid2.php) was then utilized to produce the heat capacity and concentration plots (Markham and Zuker, 2005). The detailed heat capacity figure displays the melting temperature [Tm (Cp)] and the ensemble heat capacity (Cp) in a temperature-dependent manner. Meanwhile, the concentration plot can be used to calculate the Tm (Conc). It is determined at the point where the concentration of double-stranded molecules is half of their maximum value. Finally, the SMEpred web server was used to validate the final siRNAs (Dar et al., 2016). SMEpred was used for predicting the efficacy of chemically modified siRNAs. The predicted siRNAs are evaluated on two datasets:

regular siRNAs (2182) and cm-siRNAs (3031 cm-siRNAs), both of which have been experimentally confirmed. SMEpred was also utilized to do a Support Vector Machine (SVM)-based 10-fold cross-validation.

Conservancy checking against other strains and human genomic transcript

Conservancy checking was performed against the 91 strains for WNV and 96 strains for JEV through the multiple sequence alignment (MSA) of CLC Drug Discovery Workbench 3.0 (https://digitalinsights.qiagen.com/products/clc-drug-discovery-workbench/latest-improvements/previous-line/). Furthermore, we did a single blast analysis in NCBI to compare the resulting siRNAs to human genomic transcripts. The *e-value* was reduced to 1e-10 in order to reduce the stringency of the search criterion and therefore improve the chance of arbitrary matches.

Molecular docking of guide siRNA and argonaute-2 protein

The RISC complex protein, mainly the human Argonaute (AGO) protein, requires the correct interaction with the siRNA duplex, specifically the guide strand, to initiate an effective antiviral response via RNAi-mediated viral gene silencing (Jana et al., 2004). Molecular docking of the siRNA guide strand with AGO2 protein was conducted using the HDOCK (http://hdock.phys.hust.edu.cn/) (Yan et al., 2020). However, we also docked a 20nt RNA molecule (retrieved from the PDBID: 4Z4D) with AGO2 protein as a control. Before conducting molecular docking, we used the Robetta web server (Kim et al., 2004) to predict the 3D models of both the siRNAs and the human AGO2 protein. This is a homology modeling web server that employs deep learning algorithms, RoseTTAFold and TrRosetta, as well as an integrated reporting facility for specific sequence alignments for homology modeling. For predicting the 3D structure of the siRNA guide strand, we used Mfold (http://www.unafold.org/mfold/applications/rna-folding-form.php) and RNA Composer (https://rnacomposer. cs.put.poznan.pl/) (Popenda et al., 2012; Zuker, 2003). Mfold web server calculates DNA/RNA folding patterns while RNA Composer models RNA 3D structures autonomously. We used a method that utilizes the automatic translation concept and the RNA FRABASE database as a lexicon to connect RNA secondary and tertiary design components. Our ultimate goal was to dock the siRNA with the RISC complex (AGO2) by modeling the guide siRNA and human AGO2 protein through molecular docking. After docking we visualize the interaction pattern through PDBsum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) (Laskowski et al., 2018). PDBsum provides structural information on Protein Data Bank entries (PDB), protein secondary structure, a protein ligand, and DNA binding proteins.

Results and Discussion

Sequence retrieval and multiple sequence alignment

RefSeq CDS of all proteins of both the WNV (NC_009942) and JEV's (NC_001437) were retrieved from the NCBI web server. After sequence retrieval, we conducted multiple sequence alignments between the genes of both of these viruses through the Clustal W web server. It showed that the coding sequence of *NS5* (one of Non-Structural protein)/RNA-dependent RNA polymerase shared the highest homology which is 72.4% (Table S1).

siRNA prediction from the CDS of NS5 gene

The coding sequence of the *NS5* gene was used for further siRNA prediction analysis as this could be a potential target due to its highest homology between both of the viruses. Therefore, any siRNA predicted from this *NS5* gene could be similar in both viruses. siDirect web server was used to identify the possible siRNAs from the *NS5* CDS of both viruses. It identified a total of 46 siRNAs for WNV and 43 siRNAs for JEV. Among them, only 3 siRNAs (S1, S2, and S3) target the sequence of both of the viruses with the highest similarity in sequence alignment analysis through ClustalW. For the S1 siRNA molecule, 19 out of 23 bases are found as similar, for the S2 molecule 22 out of 23, and S3 molecule 20 out of 23 bases are identical (Table 1 and Fig. S1). It has been shown that the inhibition of gene expression can be achieved by directing a mismatched RNA, of up to 3-4 nucleotides, to a specific location within the coding sequence of an endogenous gene. This process works by suppressing translation (Saxena et al., 2003). Hence, we selected those 3 siRNA that were effective against both of these viruses, while there was a slight dissimilarity (3- 4 nt mismatch). Furthermore, the siDirect web server predicted that the S2 molecule out of these 3 siRNAs,

followed the rule of Ui-Tei, Renol, and Amarguioui. The other 2 siRNA molecules followed only the Ui-Tei and Amarguioui rules. Consequently, S2 molecules could be a better candidate among the others (Table S2). Subsequently, the seed-duplex Tm of these siRNAs under 21.5 °C was predicted by the siDirect web server, which means that these siRNAs have less off-target effect (Naito et al., 2009).

The following equation was applied to get Tm (Ui-Tei et al., 2008):

$$Tm= (1{,}000\ H) / (A + \Delta S + R \ln (CT/4)) - 273.15 + 16.6 \log [Na+]$$

Where R is the gas constant (1.987 cal/deg/mol cal deg^{-1} mol^{-1}), CT is the total molecular concentration of the strand (100 M), A is the helix initiation constant (-10.8), S is the sum of the nearest neighbor entropy change, and H (kcal/mol kcal mol^{-1}) is the sum of the nearest neighbor enthalpy change. Na^+ was maintained at 100 mM.

Then the 3 siRNAs of WNV were subjected to different computational analyses to find out the best possible candidate. We selected only the siRNAs of WNV for further analysis as these siRNAs could also be effective against the JEV (as there is little dissimilarity).

siRNA refinement assessment and GC content calculation of the predicted siRNA

To refine the predicted siRNAs, we first analyze the GC content of all siRNAs. It is often recommended to choose siRNA sequences with low GC content, usually between 30% and 52% (Safari et al., 2017). This is because a lower GC content may limit the effective identification and hybridization of target mRNA. On the contrary, higher GC content may cause to take a longer time to unwind the siRNA duplex (Amarzguioui et al., 2004). GC content of all our 3 siRNAs was predicted in the 39.1- 43.5% (Table 2).

Table 1: siRNAs with the highest base similarity of the target sequences predicted by siDirect web server for both West Nile and Japanese Encephalitis Virus.

Alias	Target sequence in West Nile Virus (21nt target + 2nt overhang)	Target position in mRNA (NC_009942)	Target sequence in Japanese Encephalitis virus (21nt target + 2nt overhang)	Target position in mRNA (NC_001437)	Base similarity
S1	CACTTGCATTTACAACATGATGG	9029-9052	TACATGTATCTACAACATGATGG	9026-9048	19/23
S2	TACAACATGATGGGAAAGAGAGA	9040-9062	TACAACATGATGGGAAAAAGAGA	9036-9058	22/23
S3	CTCCAAAAACTGGGTTACATCCT	9220-9242	GTCCAAAAGCTGGGATACATCCT	9216-9238	20/23

Table 2: Calculation of GC content, free energy of folding, free energy of binding, heat capacity, and the concentration plot of the Effective conserved siRNA molecules for both West Nile and Japanese Encephalitis Virus.

Alias	RNA oligo sequences 21nt guide (5′→3′) 21nt passenger (5′→3′)	Seed duplex (Tm) Guide	Seed duplex (Tm) Passenger	GC%	Free energy of folding	Free energy of binding	Tm (Conc)	Tm (Cp)	Validity (binary)
S1	AUCAUGUUGUAAAUGCAAGUG CUUGCAUUUACAACAUGAUGG	20.5 °C	20.0 °C	39.1	-0.6	-31.4	81.3	82.7	58.2
S2	UCUCUUUCCCAUCAUGUUGUA CAACAUGAUGGGAAAGAGAGA	19.0 °C	20.5 °C	39.1	1.8	-35.4	88.3	89.1	86.5
S3	GAUGUAACCCAGUUUUUGGAG CCAAAAACUGGGUUACAUCCU	14.6 °C	5.6 °C	43.5	-1.7	-35.1	86.9	86.2	61.1

Secondary structure prediction of siRNA

The free energies of folding for all 3 siRNA molecules were calculated. Previous research suggests that RNA molecules typically have the highest free energy of folding (Vickers et al., 2000). Lower folding free energy in siRNA molecules may cause the formation of secondary structures, which can hinder the cleavage of the target by the RISC complex, as per the findings of the study (Vickers et al., 2000). That is why free energy of folding with associated secondary structure prediction is crucial for functional siRNA selection. Previous research has suggested that an RNA molecule must have a low free energy of folding in order to be stable (Singh et al., 2012). Therefore, siRNA molecules with positive energy may be more receptive to the target site and have a higher propensity to attach to the target, resulting in effective gene silencing. Our study found only positive free energy of folding for the S2 molecule (Table 2). The other two molecules (S1 and S3) have negative free energy of folding (Fig. 1). The computational RNA-RNA interaction, also known as the free energy of binding with the target, plays a crucial role in determining the efficacy of RNAi. The binding energies of siRNAs to their respective target mRNAs are directly proportional to their effectiveness in gene silencing. Hence, the free energy of binding is an important parameter to consider in the design and evaluation of siRNAs for RNAi-based therapeutics. Lower binding energy indicates stronger interactions and, hence, a higher probability of blocking the target.

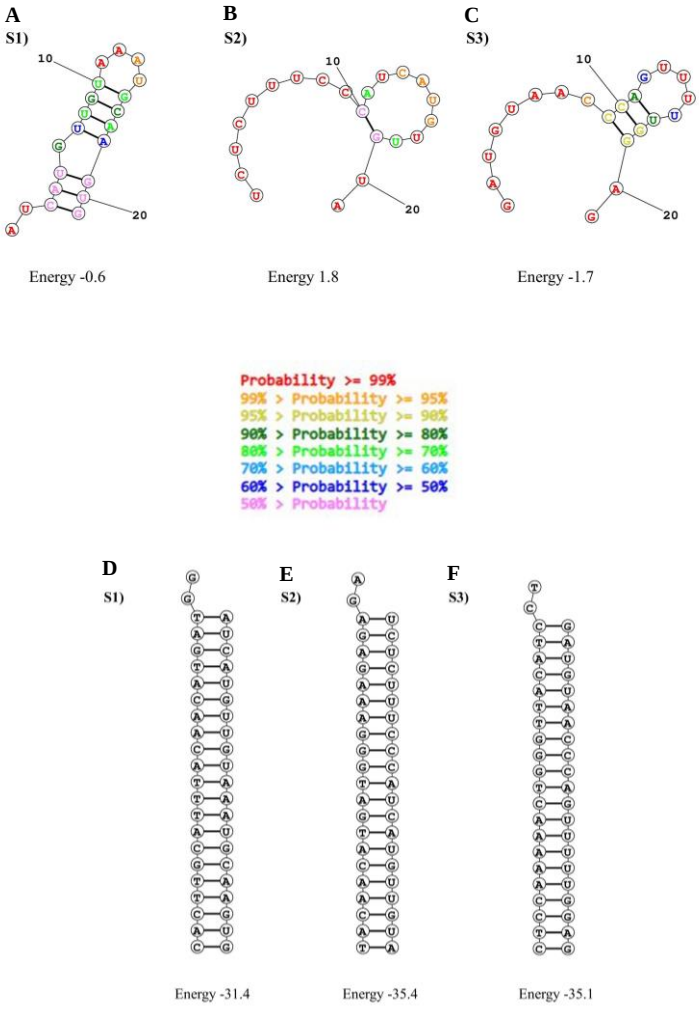

Figure 1. Prediction of free energy of folding and free energy of binding of the putative siRNAs. The free energy of folding of the three siRNAs S1, S2, and S3 is shown in (A), (B), (C), and the free energy of binding of the subsequent siRNAs (S1, S2, and S3) is shown in (D), (E), (F). Among them, siRNA S2 showed the highest free energy of folding (Energy 1.8) and lowest free energy of binding (Energy -35.4) indicating its better efficacy than the other two siRNAs.

RNA-RNA binding computation, heat capacity, concentration plot and validation

All 3 siRNAs analyzed in this study, showed negative free energy of binding; e.g., -31.4 for the S1 molecule, -35.4 for the S2 molecule, and -35.1 for the S3 molecule (Table 2). S2 molecule again outcompeted the other two siRNAs according to RNA-RNA interaction. The effectiveness of siRNAs (Conc) was implied by the higher values of Tm (Cp) and Tm. Tm (Cp) represents the relationship between Cp and Tm, which is shown in the heat capacity plot as Cp varying with temperature. Similarly, to a concentration plot, Tm (Conc) represents mole fractions plotted as a function of temperature. At the point known as Tm (Conc), the concentration of the double-stranded molecule is half its maximum value. Calculations of the equilibrium melting profiles throughout a range of temperatures were performed by utilizing the online server DINAMelt. It was observed that the Tm (Cp) and Tm (Conc) values were positively correlated with the quality of RNAi molecules, indicating that our siRNAs were of high quality as illustrated in (Table 2 and Fig. S2).

Calculation of off-target effect

The inhibitory efficacy of the anticipated siRNAs was determined using the SMEpred website. Here, the siRNAs (S1, S2, and S3) showed inhibition efficacy greater than 80%. S2 siRNA again showed better efficacy in the SMEpred web server than the other siRNAs with a value of 89.1. We confirmed that our siRNAs have a reduced off-target binding, as the seed-duplex Tm of all these siRNAs is under 21.5 °C. To confirm the off-target silencing effect, BLASTn was used for the final two siRNAs against the human genomic transcript. This revealed that our projected siRNAs are unique and have no connection to any human genomic target. According to the above analysis, the S2 siRNA molecule is sure to be better than the other 2 siRNAs. Moreover, the target of S2 siRNA of the WNV has one base mismatch with the JEV. Consequently, it could be assumed that the S2 siRNA molecule could be a better candidate for RNAi activity against both viruses (Table 2).

Evaluation of conservancy against other strains and human genomic transcript

In the last step before molecular docking, we also employed MSA against all other strains of both viruses, e.g., 91 strains for WNV and 96 strains for the JEV. The MSA also revealed that the target sequence of the S2 siRNA molecule is highly conserved (Fig. S3). Therefore, it can be stated that S2 siRNA could be a highly efficient molecule against all the other strains of both of these viruses.

Molecular docking of guide siRNA and AGO2 protein

The molecular modeling of the final siRNA molecule (S2) was performed. Firstly, we fed the five guide siRNA sequences into the Mfold web server to create the RNAdraw format. This RNAdraw format was then used in the RNA Composer web server to generate the final 3D structure of the siRNA molecules. Once we had designed the 3D models of the siRNAs, we created a 3D structure of the human AGO2 protein using the Robetta homology modeling web server. To model the AGO2 protein, we used the RefSeq sequence of the human AGO2 (UniProtKB: Q9UKV8). The 4Z4D crystal structure (human AGO protein bound to t1-G target RNA) was selected as the template for homology modeling, as it showed maximum sequence similarity with our AGO2 sequence. The modeled protein was then refined with the GalaxyRefine web server (https://bio.tools/galaxyrefine). The quality of the model was checked using Ramachandran plot analysis of the ZLab web server (Anderson et al., 2005). Ramachandran's analysis of AGO2's 3D structure showed 99.062% of residues in the highly preferred region, with only 0.938% in the preferred region and none in the questionable part (Fig. 2).

Molecular docking of the S2 siRNA molecule and human AGO2 was performed using the HDOCK web server. *In-silico* molecular docking is a sophisticated technique utilized in many studies to investigate vaccine docking, epitope docking, or small molecule docking with various protein complexes (Hoque et al., 2021; Islam et al., 2020; Islam et al., 2021; Khan et al., 2021A; Khan et al., 2021B; Rahaman et al., 2022). Results suggested that siRNA targeting the CDS could help in regulating transcript levels through AGO2-mediated transcript cleavage. Additionally, when siRNA complementary to the 3′ untranslated region (UTR) of mRNA is targeted, it results in translational repression, which is mediated by AGO1, AGO3, and AGO4 (Rivas et al., 2005; Su et al., 2009; Safari et al., 2017).

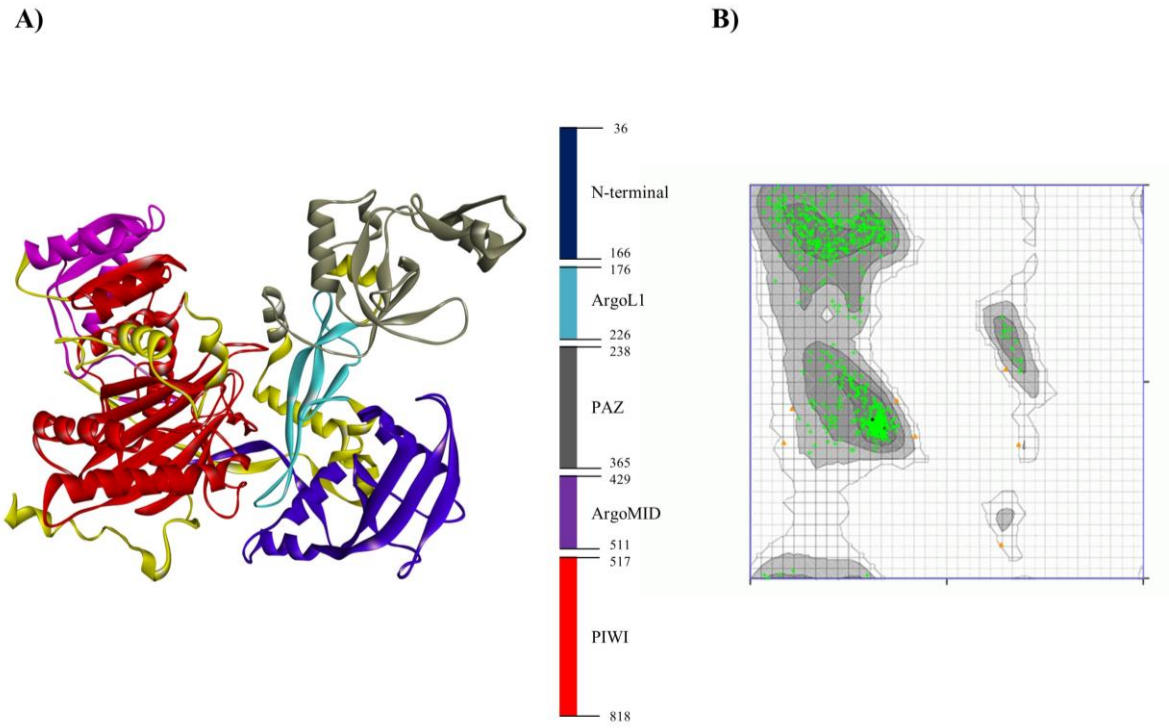

Figure 2. (A) Homology modeling and (B) Ramachandran plot analysis of human argonaute-2 protein. Ramachndran plot analysis revealed 99.062% residues in the highly preferred observation, 0.938% residues in the preferred region, and no residues in the questionable part.

In addition, we also docked a 20nt length RNA (UUCACAUUGCCCAAGUCUUU) with our AGO2 receptor as a control. The RNA used as a control is the ligand of the human AGO2 protein of PDB ID: 4Z4D. We docked this 20nt RNA with our modeled AGO2 protein to find out if the docking was successful or not. Our docking analysis through the HDOCK docking server revealed control RNA binds in the same pocket (Energy: -1081.52, ligand RMSD: 0.21) resembling 4Z4D Human AGO2 protein with t1-G Target RNA. After docking the control RNA, we docked our final siRNA molecule S2 with our modeled human AGO2 protein. The docking energy of this complex (S2-AGO2) was found as -327.03. We also found that the S2 molecule bound in the same cavity of AGO2 protein as resembled the control and it placed mostly between the PAZ and PIWI domain of AGO2 (Table S3). Some interacting residues are also found similar to the control RNA bound with AGO2 e.g., PAZ: ILE365, THR368, ARG375 and PIWI: LEU522, GLY524, LYS525, THR526, TYR529, GLN545, CYS546, VAL547, GLN548, LYS566, THR599, HIS600, LYS709, ARG710, ARG714, GLN757, GLY758, THR759, SER760, ARG761, ARG792, TYR804, PHE811, ARG812, TYR815, ALA859. In addition, some residues of the docked complex are similar to previously reported studies such as ALA221, ILE365, THR526, TYR529, LYS566, ARG635, ARG710, THR759, ARG792, and ARG812. So, it can be stated that these residues are conserved for binding of the siRNAs with human AGO2 protein (Fig. 3).

In the current work, one possible siRNA molecule against both viruses was identified that satisfied all of the characteristics, suggesting that siRNA can be more effectively bound to target mRNA. As a result, by suppressing the *NS5* gene of distinct strains of both viruses, this siRNA molecule might be exploited as a possible option in the advanced treatment of WN fever or disease linked with JE. This high-quality research will aid in meeting the need for the same treatment strategy against diverse infections. RNAi, a unique post-transcriptional gene silencing approach, has been utilized successfully against various viral infections, including hepatitis B and C, and SARS-CoV-2 infection (Chen et al., 2008; Chowdhury et al., 2021; Hasan et al., 2021; Motavaf et al., 2012; Shawan et al., 2021).

Despite their novelty in the RNAi process, siRNA is considered more effective than miRNA in gene silencing due to its ability to specifically target sequences and its simpler insertion method into cells. Targeted gene silencing using siRNAs has promising clinical potential, but it may face obstacles such as siRNA instability, poor cellular uptake, and the absence of a dependable delivery method (Tanaka et al., 2010). A promoter-controlled vector that is suitable for delivering therapeutic genes to the desired cell is essential for effective gene therapy. In plasmid form, vector-based siRNA can be used to target specific genes within a particular cell line, allowing the efficacy of a newly developed siRNA to be determined (ElHefnawi et al., 2016).

In our study, we have just identified the possible siRNA molecules for RNAi activity from the NS5 gene of both the WNV and JEV. However, to test our proposed two siRNAs, additional vector-based *in vitro* research is required. We believe that our research will be a valuable addition to this field. Moreover, the development of this siRNA therapeutic approach could be an alternative to traditional vaccine design for slowing down WN and JE-borne diseases.

Conclusion

RNA interference is a cutting-edge technique used to develop multiple siRNA molecules that silence genes post-transcriptionally in different organisms. Our study has found a promising siRNA molecule that effectively inhibits the expression of *NS5* or RNA-dependent RNA polymerase in various strains of WNV and JEV. This synthetic compound can be a potential revolutionary antiviral therapy that can help create antiviral medicines at the genome level. This finding provides a strong foundation for researchers in the academic and pharmaceutical sectors to combat viral infections.

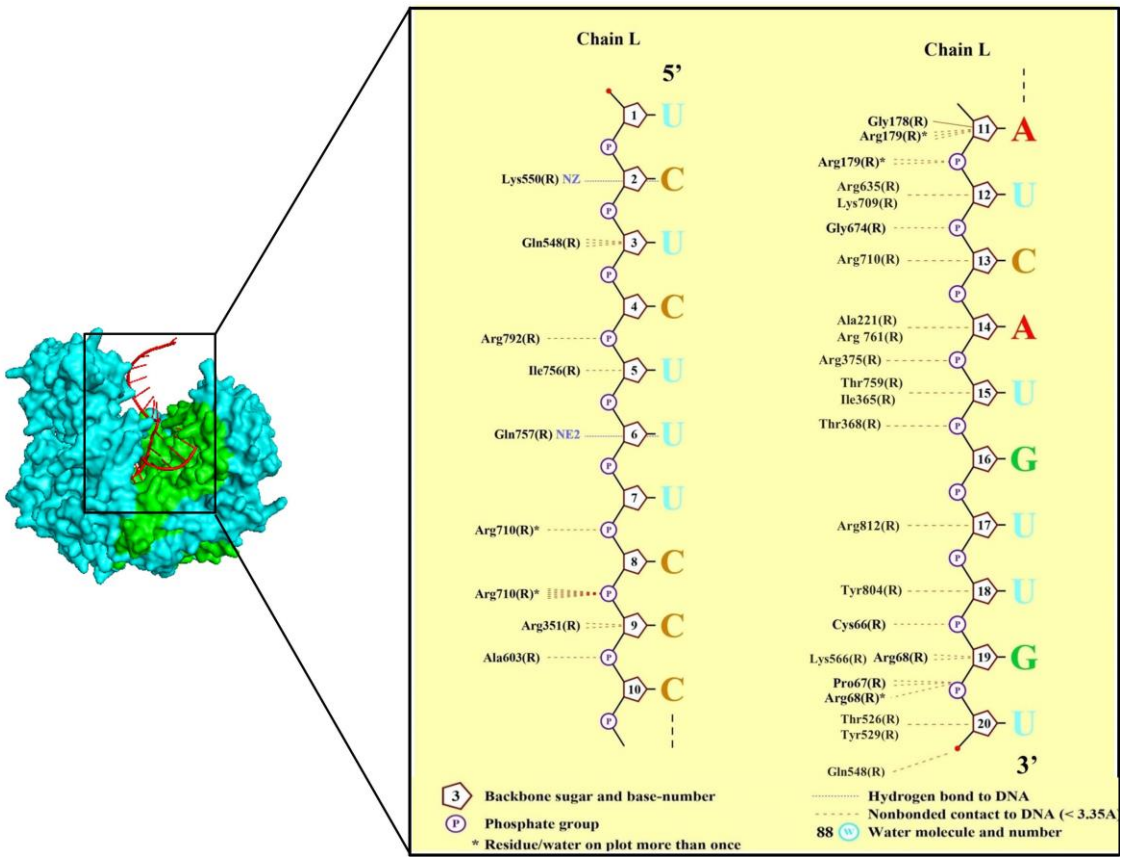

Figure 3. Docking interaction analysis of the siRNA complex S2-Argonaute-2 protein. Argonaute-2 protein is shown as a three dimensional surface structure (aqua) as well as the siRNA molecule S2 is shown as red. The interacting pocket is shown as green in the complex structure.

Ethical Statement

This project did not include any animal or human study.

Competing Interests

The authors declare that they have no competing interests.

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No specific grant was received for this study.

Data Availability

All data supporting the findings of this study are available within the article and its supplementary materials.

Authors Contribution

Rahatul Islam designed this study. Asif Shahriar and Rahatul Islam conducted this study. Asif Shahriar, Rahatul Islam, and Mohd Faijanur Rob Siddiquee wrote the manuscript. Mohd Faijanur Rob Siddiquee, Muhammad Ramiz Uddin, and Nour Fatema revised the manuscript and helped with writing and editing. Mrityunjoy Acharjee supervised this study and helped with editing. Khosnur Jahan Aurin, Md Mukhlesur Rahman Shepon, Ashikur Rahaman Bhuiyan, and Marline Gomes helped with writing and referencing.

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