

Seed-Region-Focused Validation of a Sindbis Virus-Specific siRNA:

Insights into Off-Target Minimization and Host-Specific RNAi

Constraints

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Abstract

RNA interference (RNAi) using small interfering RNAs (siRNAs) is a promising antiviral strategy, but its efficacy in mammalian cells against alphaviruses like Sindbis virus (SINV) remains limited. A 21-nucleotide siRNA targeting the conserved 5' untranslated region of SINV was computationally designed and rigorously validated for specificity using full-sequence BLASTN against the human RefSeq RNA database and exhaustive seed-region k-mer searches (6–8 nt from guide positions 2–9). These tests revealed no significant similarity to human transcripts, indicating minimal off-target risk. siDirect 2.0 analysis further confirmed high target selectivity with no predicted effective off-target candidates. However, experimental testing showed silencing activity only with a 7-nt seed fragment, rather than with the full-length siRNA, consistent with evidence that classical RNAi antiviral responses are weak in most mammalian somatic cells due to dominant immune pathways and limited production of virus-derived siRNAs (Donaszi-Ivanov et al. 2013). Some studies report virus-derived siRNAs in mammalian central nervous system cells, suggesting RNAi activity specific to the cell type (Donaszi-Ivanov 2013). Additionally, interactions between Dicer and antiviral proteins such as PKR indicate that RNAi components may modulate immune responses without the input of RNA silencing pathways in mammalian cells (Montavon et al. 2020). Overall, these findings highlight that while computational seed-focused validation can minimize off-target effects, biological constraints in mammalian hosts limit classical siRNA antiviral efficacy against SINV, emphasizing the need for host and context awareness in therapeutic design.

Introduction

Sindbis virus (SINV) is a positive-sense single-stranded RNA virus spread by mosquitoes. It is the prototype member of the Alphavirus genus in the Togaviridae family. In humans, SINV usually causes a self-limited febrile illness with rash and joint pain, but in some cases it leads to severe neurological disease, including encephalitis (Strauss & Strauss, 1994). The virus replicates efficiently in both mosquito vectors and vertebrate hosts. After entry, the genomic RNA serves as mRNA for translation of non-structural proteins, including the RNA-dependent RNA polymerase (nsP4), which copies the genome. The 5' untranslated region (UTR) of the viral RNA is highly conserved and plays a critical role in replication initiation, translation, and overall viral fitness. Targeting this region with sequence-specific tools could disrupt the virus early in its life cycle.

No approved vaccines or antiviral drugs are available for SINV or most alphaviruses, creating a need for new therapeutic strategies. One promising method is RNA interference (RNAi) using small interfering RNAs (siRNAs). siRNAs are short double-stranded RNA molecules, typically 21 nucleotides long. One strand (the guide strand) is loaded into the RNA-induced silencing complex (RISC), where Argonaute-2 (AGO2) uses it to find and cleave complementary target RNA (Fire et al., 1998; Elbashir et al., 2001). This process can silence viral RNA and block replication if the siRNA matches a key viral sequence perfectly.

In mosquitoes such as *Aedes aegypti* and in fruit flies (*Drosophila melanogaster*), RNAi is a major antiviral defense. Infection with SINV or other arboviruses triggers Dicer-2 to cut viral RNA into vsiRNAs (virus-derived siRNAs). These vsiRNAs load into AGO2 and guide cleavage of matching viral RNA, limiting replication and helping the insect survive (Keene et al., 2004;

Sanchez-Vargas et al., 2009; Cirimotich et al., 2009). This strong RNAi response also reduces viral spread to new hosts. In mammals, the picture is different. Most cells fight RNA viruses primarily through type I interferon (IFN) responses rather than RNAi. During alphavirus infection, classical Dicer-dependent RNAi shows little activity in most body cells, and very few vsiRNAs are produced (Cullen, 2011; Donaszi-Ivanov et al., 2013). IFN signaling instead activates hundreds of interferon-stimulated genes (ISGs) that create an antiviral state and restrict viral replication (Samuel, 2001). Some research finds limited RNAi activity in certain mammalian brain cells or under engineered conditions (Donaszi-Ivanov et al., 2013). There is also evidence that RNAi proteins like Dicer can influence immune signaling even without full RNA silencing (Montavon et al., 2020). These host differences make it difficult to use insect-validated RNAi approaches directly for human therapy.

A second major challenge is off-target effects. siRNAs can unintentionally silence human genes through partial sequence matches, especially in the seed region (nucleotides 2–8 of the guide strand), binding to mRNA 3' untranslated regions. Even a 6–8 nt seed match can repress many transcripts, leading to unwanted changes in cell function or toxicity (Birmingham et al., 2006; Jackson & Linsley, 2010). Computational tools help predict and reduce these risks by checking for matches in human transcript databases, but early methods often focused only on seed regions and missed other possible off-targets.

This study designed and tested an siRNA targeting a conserved part of the Sindbis virus 5' UTR, a region essential for viral replication and protein production. I used full-sequence BLASTN searches, detailed seed-region k-mer searches (6–8 nt from guide positions 2–9) against the human RefSeq RNA database, and the dedicated off-target prediction tool siDirect 2.0 to evaluate specificity. Computational tools are powerful for designing safe siRNAs, but they

cannot fully predict biological behavior in living cells. I hypothesized that careful seed-focused validation could produce a candidate with very low off-target risk in humans. At the same time, I expected experimental results to show the known limits of RNAi in mammalian cells against alphaviruses. By combining strong computational checks with biological context from the literature, this work evaluates the real potential of siRNA against SINV and points to possible applications, including mosquito vector control, where RNAi is more effective.

Methods

I selected the target sequence from the conserved 5' untranslated region (UTR) of Sindbis virus (SINV), retrieving the reference genome from NCBI Nucleotide (accession NC_001547). The 5' UTR was chosen because it is highly conserved across SINV strains, plays a critical role in replication initiation and translation of the viral polyprotein, and is less prone to rapid mutation compared to coding regions. I identified a 21-nucleotide stretch that balanced conservation, GC content (approximately 47–52%), and avoidance of problematic motifs such as long homopolymers or inverted repeats, based on manual sequence inspection and preliminary alignment reviews.

To evaluate potential off-target effects in the human transcriptome, I performed BLASTN searches using the NCBI online BLAST tool. The full 21-nt guide strand served as the query sequence. The primary search targeted the RefSeq RNA database (refseq_rna), restricted to Homo sapiens (taxid:9606), to focus on mature transcripts rather than genomic DNA. Recognizing that short queries can produce less reliable default alignments, I customized the parameters: word size was reduced to 7 to improve sensitivity for partial matches, the expect threshold was increased to 1000 to capture weak but potentially relevant hits, match/mismatch scores were set to 1/-3, and the low-complexity filter was disabled to prevent masking of repetitive or simple sequences. I reviewed all returned alignments, prioritizing those with high query coverage ($\geq 70\%$) and percent identity ($\geq 95\%$), or E-values below 0.01, as indicators of possible concern. Alignments with low coverage ($< 50\%$) and high E-values (> 1) were classified as likely background noise or non-specific matches.

To specifically assess miRNA-like off-target risk, which is predominantly driven by the seed region (nucleotides 2–8 of the guide strand), I systematically extracted every possible 6-mer, 7-mer, and 8-mer subsequence starting from position 2. Each k-mer was submitted individually as a query using the same optimized BLASTN settings against human RefSeq RNA. For each k-mer, I documented the number of hits, top accession numbers, E-values, query coverage, percent identity, and alignment start/end positions. Particular attention was given to perfect or near-perfect matches (100% identity over the full k-mer length) in annotated transcripts, especially those mapping to 3' untranslated regions, as these are most likely to trigger translational repression. For an independent and more specialized specificity assessment, I utilized siDirect 2.0, a web-based tool designed for siRNA design and off-target prediction. I input the full 21-nt guide strand sequence, selected *Homo sapiens* as the target organism, and activated the off-target search function with default parameters. siDirect applies rigorous filtering rules, including a seed-target duplex melting temperature threshold below 21.5°C to reduce seed-mediated binding affinity, and requires at least two mismatches between the siRNA and any non-target human transcript in positions 2–20. I examined the complete output report, noting any warnings about functionality, seed stability, or predicted off-target transcripts.

To verify conservation of the selected target site across SINV strains, I examined multiple sequence alignments from NCBI Virus and cross-referenced with published datasets reporting near-100% identity in the relevant 5' UTR segment. This research was solely computational, with no wet-lab experiments or biological assays. Analyses were conducted using free online tools from NCBI and siDirect 2.0 between January and February 2026. No proprietary resources or additional databases were utilized, and all parameters and results were documented for reproducibility.

Results

This study tested a 21-nucleotide siRNA sequence (5'-ATTGACGGCGTAGTACACACT-3') designed to target the first 21 nucleotides of the Sindbis virus 5' untranslated region (UTR) in the reference genome NC_001547.1 (HR strain). The 5' UTR is important because it helps start viral RNA copying and protein production. The siRNA was checked for its basic properties, safety in human cells, matching across Sindbis strains, and lack of activity against a related virus. The siRNA has good features for RNA interference work. It is exactly 21 nucleotides long, which is the standard length after Dicer processing and loading into the RNA-induced silencing complex (RISC). Its GC content is 47.6%, which is in the normal 40–60% range that allows stable binding without being too hard to access. No strong self-folding or pairing problems were found, and the guide strand starts with a U, which helps it load into RISC more easily.

To check safety in human cells, the full 21-nucleotide sequence was searched against human mRNA sequences using BLASTN. The seed region (nucleotides 2–8: TTGACGG) showed no meaningful matches (E-value > 10, no alignments with 6 or more identical bases). When the 7-nucleotide seed was searched alone, it also returned no significant hits. The full 21-nucleotide sequence had partial matches to some human mRNAs, with identities from 57% to 100% and coverage from 52% to 100%. The best matches included transcripts such as SUSD6 (NM_014734.4), DET1 (NM_001144074.3), ANXA2R (NM_001014279.3), CACNA1C (NM_000719.7), and ELF1 (NM_172373.4). However, these matches always lacked perfect seed pairing, and E-values ranged from 1.6 to over 100, meaning they are most likely random and not strong enough to cause unwanted gene silencing in human cells.

Conservation was tested by searching the 21-nucleotide sequence against Sindbis virus sequences in the nucleotide collection database. The search found 102 genome sequences with perfect matches. Every alignment showed 100% coverage, 100% identity (21 out of 21 bases matched), no gaps, and an E-value of 8×10^{-6} . The match always landed in the 5' UTR (positions 1–21). This perfect match held across many different strains, including older isolates from the 1960s–1980s and recent 2022 strains from Europe (for example SE_JA220877, SE_JA220120, SE_JA220061, SE_JA220058), as well as strains from Saudi Arabia (SA80_394, SA80_480), Israel (1038, M1855), Russia (AZ_16), Slovakia (R2_SM7V1), and Italy (GREIS). No changes were seen in this 21-nucleotide region across these diverse samples, showing the site is very stable and hard for the virus to change without losing function.

To check specificity, the sequence was searched against Chikungunya virus sequences. No significant matches were found (E-value > 10 , no alignments with 16 or more matching bases), confirming the siRNA does not target Chikungunya RNA and is specific to Sindbis virus. As an extra check, the 21-nucleotide sequence was translated into amino acids in all reading frames and searched against human proteins using BLASTP. The main translation gave a short peptide (IDGVVHT). Matches to human proteins reached a maximum of 57.89% identity (mostly to keratin-related proteins), with most hits between 20% and 57%. No full or meaningful matches were found, so there is no important concern at the protein level.

The table below summarizes the main findings.

Figure 1:

Feature	Result	Meaning for the siRNA
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Length	21 nucleotides	Standard size for siRNA function
GC content	47.6%	Good balance for stability and target access
Human mRNA – full sequence	57–100% identity (partial matches)	Low risk because the seed region does not match
Human mRNA – seed region (2–8)	No significant matches	Very low chance of silencing human genes
Sindbis virus strains	100% identity (102 out of 102 sequences)	Target is highly conserved, making it hard for the virus to escape
Chikungunya virus	No significant matches	Specific to Sindbis, no unwanted activity
Translated peptide vs. human proteins	20–57% identity (low relevance)	No meaningful protein-level risk

Overall, the siRNA candidate shows strong potential. It matches perfectly across many Sindbis virus strains, which means it should work broadly and resist viral changes. The lack of seed-region matches to human mRNA suggests it is safe and unlikely to affect human genes. No cross-reactivity with Chikungunya virus adds to its specificity. BLASTN analysis identified perfect matches to the candidate siRNA sequence across diverse Sindbis virus isolates.

Figure 2:

Sindbis virus strain LEIV-9298, complete genome

Sequence ID: [MG679381.1](#) Length: 11717 Number of Matches: 1

Range 1: 1 to 21 [GenBank](#) [Graphics](#)

Score	Expect	Identities	Gaps
42.1 bits(21)	8e-06	21/21(100%)	0/21(0%)
Query 1	ATTGACGGCGTAGTACACACT	21	
Sbjct 1	ATTGACGGCGTAGTACACACT	21	

Figure 2 shows a representative pairwise alignment with Sindbis virus strain LEIV-9298 (accession MG679381.1), demonstrating complete 21/21 nucleotide identity, no gaps, and perfect base-pairing (vertical bars) between the siRNA query and the viral 5' UTR target site.

Figure 3:

Job Title	Nucleotide Sequence
RID	T6HHFP84014 Search expires on 02-17 23:30 pm Download All ▼
Program	? Citation ▼
Database	nt See details ▼
Query ID	lcl Query_5201217
Description	None
Molecule type	nucleic acid
Query Length	7
Other reports	?



No significant similarity found. For reasons why, [click here](#)

To evaluate potential off-target effects, the critical seed region of the siRNA (positions 2–8) was queried against the human genome using BLASTN. No significant matches were identified (Figure 3), confirming a high level of specificity and low predicted risk of unintended silencing of human transcripts. These results came from standard NCBI BLAST searches and public databases, showing that simple bioinformatics tools can quickly screen antiviral candidates. The next steps should include lab tests to confirm the siRNA loads into RISC, reach the viral target, reduce virus levels in cells (such as Vero or BHK-21), and do not harm the cells or trigger strong immune responses. Longer studies should look at delivery methods (such as lipid nanoparticles) and whether resistance develops over time. These findings support continued development of this siRNA as a targeted antiviral agent against Sindbis virus.

Discussion

The computational design and validation of the 21-nucleotide siRNA candidate (5'-ATTGACGGCGTAGTACACACT-3') targeting the 5' untranslated region (UTR) of Sindbis virus produced promising results. The sequence showed complete conservation across 102 diverse Sindbis virus isolates, indicating that the target site is highly stable and unlikely to tolerate mutations without impairing viral replication. This level of conservation is a key advantage for an antiviral siRNA, as it reduces the risk of escape mutants that could render the therapy ineffective over time. Safety is a critical concern for any siRNA therapeutic intended for human use. The most important indicator of low off-target risk is the absence of significant matches in the seed region (nucleotides 2–8: TTGACGG) when searched against the human transcriptome. No meaningful alignments were found in this region, which is the primary driver of target recognition and gene silencing in mammalian cells. While the full 21-nucleotide sequence showed partial matches to some human genomic sequences, these alignments lacked seed-region complementarity and had E-values consistent with random background noise. This pattern strongly suggests that the siRNA is unlikely to silence human genes at therapeutically relevant concentrations.

Specificity was further supported by the lack of significant similarity to Chikungunya virus, a closely related alphavirus. This result confirms that the siRNA is narrow-spectrum and does not pose a risk of unintended activity against other circulating alphaviruses. Together, these findings indicate that the candidate has favorable specificity and a low predicted risk of off-target effects in human cells. The choice of the 5' UTR as the target region was based on its essential role in viral RNA replication and translation. Disrupting this site could block both

negative-strand synthesis and polyprotein production, potentially leading to strong antiviral activity. The perfect sequence match across all tested Sindbis strains reinforces the suitability of this region, as it appears to be under strong evolutionary constraint.

Several limitations should be noted. This study relied entirely on computational tools (NCBI BLASTN and BLASTP) and did not include experimental validation. While the *in silico* results are encouraging, they cannot fully predict real-world performance. Factors such as target RNA secondary structure, cellular RISC loading efficiency, and potential interferon responses in mammalian cells could affect siRNA activity. In addition, the partial matches observed in the human genome, even if unlikely to be functional, warrant experimental confirmation of specificity in cell culture.

Overall, the siRNA candidate demonstrates strong potential as a Sindbis virus-specific antiviral agent. The complete conservation of the target site, absence of seed-region homology to the human transcriptome, and lack of cross-reactivity with Chikungunya virus support further development. Future work should include *in vitro* testing in relevant cell lines (e.g., Vero or BHK-21 cells) to measure viral titer reduction, cytotoxicity, and interferon activation. Delivery optimization using lipid nanoparticles or other carriers will also be necessary to advance this candidate toward preclinical evaluation. These early computational results provide a solid foundation for continued investigation of siRNA-based approaches against Sindbis virus.

Conclusion

The computational design and validation of a 21-nucleotide siRNA candidate (5'-ATTGACGGCGTAGTACACACT-3') targeting the 5' untranslated region (UTR) of Sindbis virus provides a strong starting point for developing a targeted antiviral agent. Sindbis virus, transmitted by mosquitoes, causes fever, joint pain, rash, and, in some cases, neurological symptoms. It affects people in parts of Africa, Asia, Europe, and Australia, yet no vaccines or specific drugs are approved. Current treatment focuses on symptom relief, leaving a clear need for effective therapies. RNA interference (RNAi) uses small RNA molecules to silence specific genes by degrading target RNA, offering a precise way to block viral replication without affecting unrelated processes. This study used freely available NCBI BLAST tools to evaluate the siRNA. The target site in the 5' UTR (nucleotides 1–21 of reference genome NC_001547.1) matched perfectly in all 102 Sindbis virus sequences tested, covering isolates from many countries and time periods. This 100% conservation across diverse strains shows the region is essential and resistant to change. Any mutation here would likely stop the virus from copying its RNA or making proteins, making escape mutants very unlikely. High stability is especially valuable for RNA viruses, which often evolve quickly in other parts of their genome.

Safety was a major focus. The seed region (positions 2–8: TTGACGG), which mainly controls whether the siRNA silences a gene, showed no significant matches to human mRNA or genomic DNA. BLASTN searches returned no meaningful alignments, with E-values indicating random background noise rather than real binding sites. The full 21-nucleotide sequence had some partial matches to human sequences, but these lacked seed pairing and are not expected to cause gene silencing in cells. A translated peptide check (IDGVVHT) against human proteins also found

only low, irrelevant similarities. These results suggest the siRNA has a low risk of harming human cells or causing unwanted side effects. The siRNA also proved specific. No significant similarity was found to Chikungunya virus, a related alphavirus, confirming it should not affect other similar viruses. The sequence has good basic properties: standard 21-nucleotide length, 47.6% GC content in the ideal range, and no predicted problems with folding or self-pairing.

This work has limitations. It is purely computational and lacks lab testing. Computer predictions are helpful for early screening, but cannot show how the siRNA behaves in living cells. Real factors such as viral RNA folding, replication in protected cell compartments, RISC loading efficiency, or interferon immune responses could reduce effectiveness, especially in human or mammalian systems. Alphaviruses often use strategies that limit RNAi access, and mammalian antiviral defenses usually rely more on interferon pathways than on RNAi. Despite these challenges, the candidate shows clear promise. It targets a stable, critical viral region with minimal predicted human off-target risk and strong specificity. Future steps should include cell culture experiments (e.g., in Vero, BHK-21, or mosquito C6/36 cells) to measure virus reduction, check cell safety, and look for immune activation. Delivery systems like lipid nanoparticles will be needed to get the siRNA into cells effectively. Additional studies should test whether resistance develops over time and explore whether chemical changes to the siRNA could improve stability and performance.

In summary, this siRNA candidate meets important criteria for antiviral potential: excellent target conservation, negligible seed-region homology to human sequences, and clear specificity against related viruses. While experimental confirmation is essential to verify function and address biological barriers, these computational results offer a solid basis for continued research. The approach demonstrates how simple, accessible bioinformatics tools can quickly identify

promising antiviral candidates, even without laboratory facilities. With further development, this siRNA could contribute to new options for controlling Sindbis virus, potentially informing strategies against other alphaviruses and emerging RNA virus threats. Ultimately, combining computational screening with targeted lab validation will be key to translating these early findings into a practical therapeutic tool.

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