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siRNAs Targeting *icaD* Gene of *Staphylococcus aureus* to Inhibit Biofilm Formation: Structural Analysis and Efficacy

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Abstract

Antibiotic resistance in *Staphylococcus aureus* infections, especially those involving biofilm formation, is a global health issue. Biofilm protects bacteria from the immune system and antibiotic treatment, making them 10 to 1000 times more resistant. The *icaD* gene, part of the ica operon, is crucial for biofilm synthesis by enhancing the enzymes responsible for forming the biofilm matrix. The *icaD* gene sequence of *Staphylococcus aureus* was obtained from the GenBank NCBI database with the accession code CP140612.1, with a gene sequence length of 306 bp and employed several bioinformatics methods, including siDirect for designing and evaluating effective siRNA sequences to select the most promising candidates. Additionally, siRNA Scales, MaxExpect, Duplex Fold, and siPred were employed to analyze the siRNA sequence length, secondary structure, binding energy, and efficacy predictions of siRNAs targeting the icaD gene. The study found that out of 54 siRNA candidates, siRNA22, siRNA50, and siRNA25 achieved inhibition rates of 93.69%, 92.82%, and 92.52%, respectively. These results bioinformatically demonstrated their potential to suppress the expression of the *icaD* gene and highlight their promise as siRNA-based antibacterial therapies to combat biofilm-related infections. The designed siRNA computationally shows potential as an innovative therapy to combat biofilm infections caused by *Staphylococcus aureus*.

Keywords: *Staphylococcus aureus*; Biofilm; *icaD* Gene; siRNA; Antibiotic Resistance.

INTRODUCTION

Antimicrobial resistance (AMR) is one of the top global to public health and development. It is estimated that bacterial AMR was directly responsible for 1.27 million global deaths in 2019 and contributed to 4.95 million deaths (Murray et al., 2022). *Staphylococcus aureus* (*S. aureus*), which often forms biofilms, is becoming increasingly difficult to treat, especially strains resistant to methicillin (MRSA). Bacterial biofilms protect them from the immune system and antibiotics, making them 10-1000 times more resistant (Tran et al., 2023). Biofilm formation in *S. aureus* is encoded by several genes, including *icaA*, *icaB*, *icaC*, and *icaD*, which are involved in the biofilm matrix formation (Peng et al., 2023). One promising therapeutic approach is the use of small interfering RNA (siRNA) to inhibit the expression of the *icaD* gene, which plays a role in biofilm formation. siRNA can regulate gene expression through the RNA interference (RNAi) mechanism, which targets mRNA and inhibits the translation of specific proteins. With the advancement of bioinformatics technology, the analysis of siRNA structure and efficacy has become easier. Previously, siRNA has been used for antiviral therapy, such as for hepatitis C and Zika virus infections (Perez-Mendez et al., 2020). This study aimed to explore, analyze the structure, and predict the efficacy of siRNA to suppress the expression of the icaD gene in *S. aureus* as a strategy to combat infections caused by bacterial biofilms.

MATERIALS AND METHODS

Study area

The study took place in Faculty of Medicine Palangka Raya University between June and Desember 2024.

Procedures

Retrieval of the *icaD* gene sequence

The downloaded FASTA sequence of the *icaD* gene from *Staphylococcus aureus* (NCBI Accession Number: CP140612.1) was renamed accordingly and stored in a dedicated folder for further analysis. The sequence was then utilized to perform several computational analyses, including the design of siRNA molecules targeting the *icaD* gene, analysis of mRNA length percentage, prediction of the secondary structure of the designed

siRNAs, calculation of binding energies between siRNA and target mRNA, and prediction of siRNA efficacy in silencing the *icaD* transcript. These analyses were conducted using appropriate bioinformatics tools and databases to ensure the accuracy and reliability of the results.

Design of Gene-Silencing siRNA Molecules

The icaD gene sequence of Staphylococcus aureus was obtained from the GenBank NCBI database with the accession code CP140612.1, The gene sequence is 306 bp long and employs several bioinformatics methods. The design of siRNA to silence the S. aureus icaD gene was performed using siDirect (http://siDirect2.RNAi.jp/).

Percentage Analysis of mRNA Length

Percentage analysis of mRNA length was performed using siRNA Scales, a software that predicts the location of the remaining mRNA sequence in the cell after siRNA cleavage (http://gesteland.genetics.utah.edu/siRNA_scales/).

Analysis of the Secondary Structure of siRNA

Secondary structure analysis of siRNA was done using MaxExpect and could be accessed through a website https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/MaxExpect/MaxExpect.html. Subsequently, the 21nt guide RNA oligo sequences (5′→3′) obtained from siRNA design analysis using siDirect were submitted.

Analysis of the Binding Energy Between siRNA and Its Target mRNA

Analysis of the binding energy between siRNA and the S. aureus icaD gene target was done using DuplexFold (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/DuplexFold/DuplexFold.html). The software showed the analysis results, including the binding energy and a predicted secondary RNA structure diagram.

Efficacy Prediction of siRNA Molecules

Prediction of siRNA efficacy against the *icaD* target in *S. aureus* was performed using siPRED (http://predictor.nchu.edu.tw/siPRED/), then the software displayed predicted position and inhibitory effects of the siRNA strand on the *icaD* gene.

RESULTS AND DISCUSSION

Results and Discussion should be written as a series of connecting sentences, however, for manuscript with long discussion should be divided into subtitles. Results should be clear and concise.

Result of the E xploration of siRNA Targeting the *icaD* Antigen of *Staphylococcus aureus*

Fifty-four siRNA candidates were successfully designed based on the complete genome of the *icaD* gene from *S. aureus* (NCBI Accession Number: CP140612.1). The analysis of target locations, target region length, binding, and inhibition of the antigen sequences in these fifty-four siRNA candidates targeting the *icaD* antigen of Staphylococcus aureus resulted in data as shown in Table 1 below.

Table 1. The analysis of target siRNA.

No	Target Position	RNA oligo sequences 21nt guide (5′→3′)	RNA oligo sequences 21nt passenger (5′→3′)	siRNA Scales	Max Expect	Duplex Fold	siRNA Efficacy
1	10-32	UGGGUAUUCCCUCUGUCUGGG	CAGACAGAGGGAAUACCCAAC	14	1.4	-38.2	74.17
2	16-38	UAGCGUUGGGUAUUCCCUCUG	GAGGGAAUACCCAACGCUAAA	7	1.9	-37.4	74.54
3	18-40	UUUAGCGUUGGGUAUUCCCUC	GGGAAUACCCAACGCUAAAAU	11	2.0	-34.7	None
4	19-41	UUUUAGCGUUGGGUAUUCCCU	GGAAUACCCAACGCUAAAAUC	11	2.0	-31.7	None
5	20-42	AUUUUAGCGUUGGGUAUUCCC	GAAUACCCAACGCUAAAAUCA	25	1.8	-29.0	None
6	30-52	UUUAGCGAUGAUUUUAGCGUU	CGCUAAAAUCAUCGCUAAACA	6	1.5	-28.5	75.9
7	32-54	UGUUUAGCGAUGAUUUUAGCG	CUAAAAUCAUCGCUAAACAUU	17	1.9	-26.9	70.01
8	42-64	UCUCUUAUAAUGUUUAGCGAU	CGCUAAACAUUAUAAGAGAAA	10	1.6	-28.2	85.79
9	43-65	UUCUCUUAUAAUGUUUAGCGA	GCUAAACAUUAUAAGAGAAAC	2	1.7	-27.3	85.79
10	44-66	UUUCUCUUAUAAUGUUUAGCG	CUAAACAUUAUAAGAGAAACA	14	1.8	-23.2	None
11	49-71	UGCUGUUUCUCUUAUAAUGUU	CAUUAUAAGAGAAACAGCACU	12	1.7	-27.9	86.47
12	59-81	UAGCGAUAAGUGCUGUUUCUC	GAAACAGCACUUAUCGCUAUA	8	1.9	-32.5	82.54
13	65-87	ACGAUAUAGCGAUAAGUGCUG	GCACUUAUCGCUAUAUCGUGU	14	1.9	-32.5	80.71
14	73-95	AAAGACACACGAUAUAGCGAU	CGCUAUAUCGUGUGUCUUUUG	14	1.9	-31.2	83.94
15	74-96	AAAAGACACACGAUAUAGCGA	GCUAUAUCGUGUGUCUUUUGG	9	1.9	-30.9	83.94
16	81-103	UAUAUCCAAAAGACACACGAU	CGUGUGUCUUUUGGAUAUAUU	5	1.8	-30.1	80.02
17	82-104	AUAUAUCCAAAAGACACACGA	GUGUGUCUUUUGGAUAUAUUG	14	1.8	-28.9	80.02
18	86-108	AACAAUAUAUCCAAAAGACAC	GUCUUUUGGAUAUAUUGUUUA	26	1.9	-26.4	87.55
19	88-110	UAAACAAUAUAUCCAAAAGAC	CUUUUGGAUAUAUUGUUUAGU	15	1.9	-24.1	80.08
20	93-115	ACAACUAAACAAUAUAUCCAA	GGAUAUAUUGUUUAGUUGUUC	19	1.8	-26.6	85.78
21	94-116	AACAACUAAACAAUAUAUCCA	GAUAUAUUGUUUAGUUGUUCU	11	1.8	-23.3	84.44
22	**102-124**	**ACGAGUAGAACAACUAAACAA**	**GUUUAGUUGUUCUACUCGUUU**	**20**	**1.7**	**-30.0**	**93.69**
23	107-129	UAUAAACGAGUAGAACAACUA	GUUGUUCUACUCGUUUAUAUU	12	1.8	-28.1	83.92

No	Target Position	RNA oligo sequences 21nt guide (5′→3′)	RNA oligo sequences 21nt passenger (5′→3′)	siRNA Scales	Max Expect	Duplex Fold	siRNA Efficacy
24	113-135	UACCAAUAUAAACGAGUAGAA	CUACUCGUUUAUAUUGGUACU	16	1.8	-28.1	92.07
25	**116-138**	**UAGUACCAAUAUAAACGAGUA**	**CUCGUUUAUAUUGGUACUAUA**	**15**	**1.8**	**-27.7**	**92.52**
26	118-140	UAUAGUACCAAUAUAAACGAG	CGUUUAUAUUGGUACUAUAUU	-3	1.7	-26.1	82.05
27	119-141	AUAUAGUACCAAUAUAAACGA	GUUUAUAUUGGUACUAUAUUU	14	1.6	-24.9	82.05
28	129-151	UGAAUUUCAAAUAUAGUACCA	GUACUAUAUUUGAAAUUCAUG	15	1.8	-24.0	77.03
29	132-154	UCAUGAAUUUCAAAUAUAGUA	CUAUAUUUGAAAUUCAUGACG	16	1.8	-23.6	85.43
30	140-162	UACUUUCGUCAUGAAUUUCAA	GAAAUUCAUGACGAAAGUAUC	14	1.6	-28.4	91.93
31	146-168	UAUUGAUACUUUCGUCAUGAA	CAUGACGAAAGUAUCAAUACA	12	1.8	-28.0	82.7
32	149-171	UUGUAUUGAUACUUUCGUCAU	GACGAAAGUAUCAAUACAAUA	8	1.8	-28.6	88.71
33	151-173	UAUUGUAUUGAUACUUUCGUC	CGAAAGUAUCAAUACAAUACG	9	1.6	-25.2	None
34	156-178	ACACGUAUUGUAUUGAUACUU	GUAUCAAUACAAUACGUGUUG	23	1.9	-27.8	83.19
35	160-162	AGCAACACGUAUUGUAUUGAU	CAAUACAAUACGUGUUGCUUU	26	1.4	-29.2	88.89
36	165-187	UUUAAAGCAACACGUAUUGUA	CAAUACGUGUUGCUUUAAACA	5	1.8	-26.4	80.67
37	171-193	UCAAUGUUUAAAGCAACACGU	GUGUUGCUUUAAACAUUGAAA	9	1.5	-28.6	74.8
38	173-195	UUUCAAUGUUUAAAGCAACAC	GUUGCUUUAAACAUUGAAAAU	17	1.5	-26.1	73.85
39	176-198	UAUUUUCAAUGUUUAAAGCAA	GCUUUAAACAUUGAAAAUACU	9	1.8	-23.9	84.08
40	184-206	AAUUUCAGUAUUUUCAAUGUU	CAUUGAAAAUACUGAAAUUUU	6	1.8	-22.4	73.05
41	195-217	AAUAUAUCUAAAAUUUCAGUA	CUGAAAUUUUAGAUAUAUUUG	17	1.8	-20.9	71.95
42	224-246	UGAUAAUCGCGAAAAUGCCCA	GGCAUUUUCGCGAUUAUCAUU	16	1.9	-31.3	None
43	225-247	AUGAUAAUCGCGAAAAUGCCC	GCAUUUUCGCGAUUAUCAUUU	16	1.9	-28.6	None
44	226-248	AAUGAUAAUCGCGAAAAUGCC	CAUUUUCGCGAUUAUCAUUUU	18	1.9	-25.7	None
45	232-254	AACAAAAAUGAUAAUCGCGAA	CGCGAUUAUCAUUUUUGUUUU	18	1.8	-26.0	87.89
46	233-255	AAACAAAAAUGAUAAUCGCGA	GCGAUUAUCAUUUUUGUUUUU	12	1.8	-25.1	82.24
47	234-256	AAAACAAAAAUGAUAAUCGCG	CGAUUAUCAUUUUUGUUUUUU	4	1.8	-21.3	None
48	235-257	AAAAACAAAAAUGAUAAUCGC	GAUUAUCAUUUUUGUUUUUUU	14	1.8	-21.1	None
49	241-263	UGUAAAAAAAAACAAAAAUGAU	CAUUUUUGUUUUUUUUUACAAU	14	1.8	-20.3	85.58
50	**248-270**	**UGCUAAUUGUAAAAAAAACAA**	**GUUUUUUUUACAAUUAGCAUA**	**21**	**1.6**	**-23.6**	**92.82**
51	258-280	UGAAUCAAUAUGCUAAUUGUA	CAAUUAGCAUAUUGAUUCAAA	5	1.5	-26.1	84.7
52	271-293	UUUCUGCCAUUUUUGAAUCAA	GAUUCAAAAAUGGCAGAAAGG	9	1.7	-28.7	79.92
53	275-297	UUCCUUUCUGCCAUUUUUGAA	CAAAAAUGGCAGAAAGGAAGA	10	1.8	-30.0	87.94
54	284-306	ACGAUUCUCUUCCUUUCUGCC	CAGAAAGGAAGAGAAUCGUGA	28	1.8	-31.6	71.35

*Top 3 siRNAs with highest efficacy was highlighted with bold font.

Results of the Structural Analysis of the Optimal *icaD* siRNA

This structure was selected based on the parameters of siRNA scales, MaxExpert, Duplex fold, and siRNA Efficacy according to the optimal criteria. The 3D visualization of the best siRNA structure provides a detailed overview of the domain arrangement and the interactions between the siRNA binding and the target RNA, which in this case is *icaD* from the *S. Aureus* biofilm, as shown in Figure 1 below.

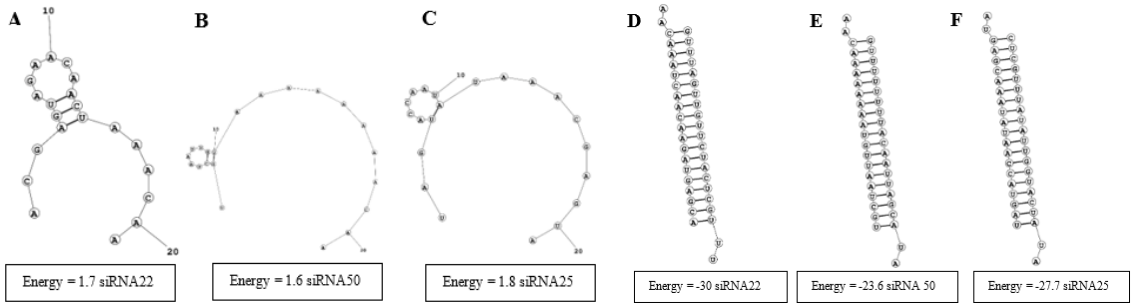

Figure 1. (A) The structure of siRNA22 (B) The structure of siRNA50 (C) The structure of siRNA25 (D) The structure of siRNA22 (E) The structure of siRNA50 (F) The structure of siRNA25.

siRNA candidates 22, 50, and 25 are some of the best targets for gene silencing based on binding strength and the highest predicted efficacy among all siRNA candidates, with potential to exert a suppressive effect on the icaD RNA.

Discussion

The siRNA molecules were designed using siDirect with a multi-step bioinformatics screening approach. siDirect provides functional siRNA designs by considering the relationship between siRNA sequences and RNAi activity, and it also calculates potential gene candidates that do not match the target. The rapid and sensitive homology search, with an updated algorithm, significantly reduces off-target silencing. The analysis results were implemented to select potential icaD gene siRNA candidates targeting the icaD RNA of *S. aureus*. Based on the exploration of siRNA design for icaD using

siDirect, 54 RNA oligo sequences were obtained, including 21nt guide (5′→3′) and 21nt passenger (5′→3′) RNA oligo sequences. The siRNA candidates were evaluated using siRNA scales to show the analysis results in the form of the percentage of mRNA remaining in the cells after siRNA-mediated cleavage. The range of predicted efficiency values represents the percentage of mRNA remaining after cleavage by siRNA, which refers to the gene knockdown efficiency by siRNA. This value is usually expressed as the percentage of mRNA that was not successfully degraded (remaining in the cell). SiRNAs with less than 30% remaining mRNA are considered efficient in suppressing the target gene expression. In this study, siRNAs with ≤10% remaining mRNA were categorized as highly effective because they resulted in nearly perfect knockdown. Meanwhile, siRNAs with 10–30% remaining mRNA are still quite effective for certain biological applications. On the other hand, siRNAs with >30% remaining mRNA are considered to have low knockdown efficiency and are typically avoided as candidates. The smaller the percentage of remaining mRNA, the higher the effectiveness of the siRNA in working according to its target without indirectly affecting other genes or processes (Angart et al., 2013; Dana et al., 2017). Based on guidelines from siRNA design, such as those from biotechnology companies (Thermo Fisher Scientific, Qiagen, or Dharmacon), high doses (>30%) may increase the risk of nonspecific effects, such as activation of the interferon pathway or off-target effects. A low percentage of remaining mRNA indicates a more effective mRNA cleavage by the RISC complex, thereby enhancing the knockdown efficiency of the target gene (Bartel & Sharp., 2004). The candidate siRNA was further analyzed using MaxExpect and DuplexFold to calculate the free binding energy of the siRNA and the free binding energy between the guide strand and the target. MaxExpect was tested on a database of siRNA sequences for the icaD antigen of *S. aureus* with known secondary structures. MaxExpect predicts the optimal structure (with the highest expected strand accuracy) and suboptimal structures as alternative hypotheses for that structure. The optimal structure, the maximum expected accuracy structure, is predicted and compared with the known structure in the database, and the prediction accuracy is reported as sensitivity and PPV (positive predictive value) (Lu et al., 2009). Target mRNA regions with MaxExpect values >1.5 indicate high accessibility to siRNA, reflecting greater sensitivity in identifying open target areas. This increases the chances of successful siRNA knockdown. Furthermore, higher predicted PPV supports the effectiveness of siRNA in reducing target gene expression during experimental tests. Therefore, MaxExpect values >1.5 can be considered optimal, especially when supported by other parameters such as knockdown prediction results showing remaining mRNA ≤30%, as analyzed through previous siRNA scales (Pan et al., 2011; Mysara & Garibaldi, 2011; Filhol et al., 2012). The DuplexFold analysis is conducted to find the structure with the Gibbs free energy (ΔG), which is a measure used to calculate the maximum work that can be done in a thermodynamic system when temperature and pressure are kept constant, indicating the most stable conformation under specific thermodynamic conditions. DuplexFold predicts the lowest free energy conformation of RNA hybrid duplexes based on intermolecular base pairing, while targetRNA identifies the complementarity of base strands and calculates the RRI score using the mean forecast error (MFE) model for RNA duplexes (Lybarger & Sandkvist, 2004). Two siRNA candidates that did not meet the criteria, with DuplexFold results ranging from -36.1 to -20.5 kcal/mol, which is the optimal binding free energy value generated for siRNA prediction, were eliminated. In the context of selecting the most effective siRNA, a lower (more negative) ΔG value typically indicates a stronger and more stable interaction between the siRNA and the target mRNA, which leads to more effective gene silencing. This more stable interaction is crucial because it allows the siRNA to more effectively guide the RNA interference (RNAi) process to reduce the expression of the target gene. The lower the ΔG value, the stronger the binding between the siRNA and mRNA, which increases the likelihood of forming a functional RISC complex, an essential factor in the effectiveness of gene silencing (Kajino et al., 2022). The inhibition ability of the selected remaining siRNA is predicted using siPred. Accurate predictions will facilitate the design of optimized siRNA by maximizing the success of the knockout of the siRNA candidate on the target (high sensitivity) and minimizing off-target effects (high specificity) (Chuai et al., 2018; Chuai et al., 2017). The 22nd, 50th, and 25th siRNA candidates are among the best targets for gene silencing based on binding strength and the highest efficacy predictions of all siRNA candidates, showing potential to suppress the icaD RNA of *S. aureus* effectively. SiRNA with a 90% inhibition rate demonstrates exceptional ability to reduce target gene expression efficiently. This ensures that the target mRNA is almost completely inhibited, which is crucial in therapeutic applications or research requiring highly effective gene knockdown (Liu et al., 2012; Caffrey et al., 2011). With high efficiency, the dose of siRNA required to achieve therapeutic effects can be minimized. This reduction in dose can decrease the risk of side effects, such as immune response activation or off-target effects, which are often associated with higher siRNA doses (Caffrey et al., 2011). SiRNA with high inhibition levels typically have a strong binding affinity to the target mRNA. This strong affinity ensures that, even with a smaller amount of siRNA, binding to the target remains efficient, allowing for optimal gene knockdown. In a clinical context, the ability to achieve near-perfect knockdown provides greater confidence in the

effectiveness of siRNA as a therapy. This is especially important for diseases that require drastic inhibition of the target gene, such as cancer or chronic infections (Liu et al., 2012). The application of gene silencing based on siRNA technology is a powerful strategy to limit bacterial infections by targeting and degrading bacterial mRNA, ensuring that its sequence matches the target (Hartawan et al., 2022). Bacteria like *S. aureus* have undergone many mutations over time. Therefore, siRNA for bacteria must be targeted at genes that are considered to have an impact on infection. The icaD gene is a promising target for gene silencing because it is located within the biofilm structure of *S. aureus*, which plays an important role in the virulence factors of an infectious disease.

CONCLUSIONS

Based on the results of the bioinformatics study on the exploration, structure, and efficacy prediction of siRNA icaD interacting with icaD RNA in *S. aureus* bacteria, this study found the binding energy and inhibition capability of siRNA in silencing the icaD gene. The best results were obtained with siRNA strands 22, 50, and 25, which resulted in inhibition of 93.69%, 92.82%, and 92.52%, respectively. SiRNA with high inhibition levels typically has a strong binding affinity to the target mRNA. This strong affinity ensures that, even with a smaller amount of siRNA, binding to the target remains efficient, leading to optimal gene knockdown. These three potential siRNA molecules can be used as an siRNA-based antibacterial therapy to suppress infections caused by *Staphylococcus aureus* with biofilms. However, the siRNA predictions in this study are important to validate through laboratory experiments.

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