# An Integrated Platform of siRNA Drug Discovery: A Comprehensive Study on Design, Modification, Activity and Off-Target Evaluations of C5-targeting siRNAs

WuXi Biology

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## Abstract

Small interfering RNA (siRNA) has emerged as a promising novel therapeutic modality. For enabling the discovery of siRNA agents, we have established an integrated platform covering siRNA sequence design, chemical synthesis and modification, delivery ligand exploration, and activity and off-target evaluations. Using this platform, we conducted a comprehensive study on complement component 5 (C5) siRNAs.

C5 plays important roles in the immune responses and involves in several autoimmune/inflammatory diseases. C5 is mainly expressed in human liver. We employed in-house bioinformatics tools for sequence design, and optimized our approach based on the C5-targeting siRNA potency. The activity of the synthesized C5-targeting siRNAs was evaluated in Huh-7 cells for their inhibition of C5 mRNA determined by RT-qPCR. More than half of the C5-targeting siRNAs tested had >50% knockdown efficiency at 10 nM in Huh-7 cells. To enhance stability and specificity of the C5-targeting siRNAs, we then conducted various siRNA modifications, including multiple placements of 2'-OMe, 2'-F, PS backbone, LNA, and other monomers. It was observed that that the knockdown efficiency of the C5-targeting siRNAs was increased with the LNA modification at the 3' end of antisense strand, but decreased with the LNA modification at the seed or middle region of antisense strand tested in Huh-7 cells. Furthermore, we performed a high-throughput transcriptome analysis coupled with bioinformatics prediction to identify potential off-target genes, and found that the siRNAs with high potency has lesser off-target effects.

In summary, we conducted a comprehensive research of C5-targeting siRNA using our integrated siRNA platform. We found that several chemically modified C5-targeting siRNAs exhibited potent inhibition of C5 mRNA in Huh-7 cells and had minimal off-target effects in Huh-7 cells. Using this case study, we evidently demonstrated that our siRNA platform can enable the discovery of siRNA therapeutic agents.

## Methods and Results

### ➢ 1. Sequence design of C5-targeting siRNAs

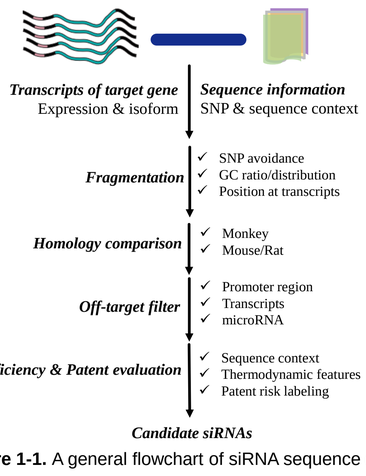


**Figure 1-1.** A general flowchart of siRNA sequence design

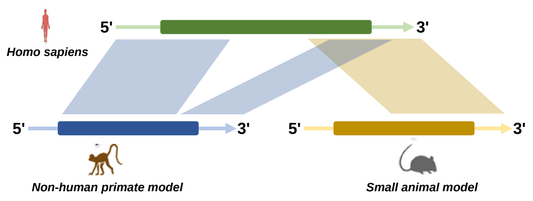


**Figure 1-2.** Cross-species comparison of candidate siRNAs

***Results-1***

- ✓ A platform for siRNA sequence design has been established.
- ✓ The siRNAs for further evaluations were selected based on multiple parameters, including off-targets, cross-species and efficacy.

### ➢ 2. Evaluation of C5-targeting siRNA activity in Huh-7 cells

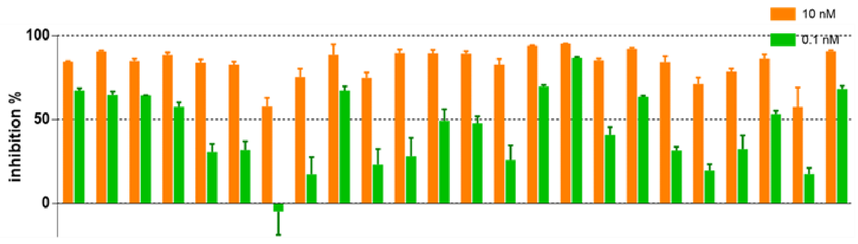


**Figure 2-1.** Primary screening for C5-targeting siRNAs in Huh-7 cells

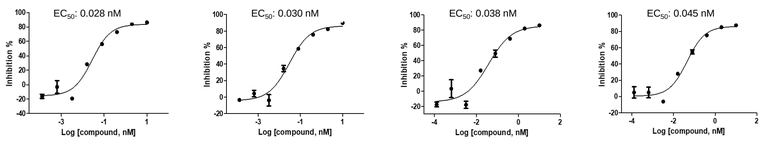


**Figure 2-2.** The activity of the C5-targeting siRNAs in Huh-7 cells

***Results-2***

- ✓ More than half of the 200 siRNAs tested had > 50% inhibition of C5 mRNA in Huh-7 cells at 10 nM in primary screening.
- ✓ The range of $EC_{50}$ values of the 34 siRNAs tested was 0.018 nM to 6.12 nM. 17/34 siRNAs had $EC_{50}$ values < 0.1 nM.

### ➢ 3. Chemical modifications of C5-targeting siRNAs

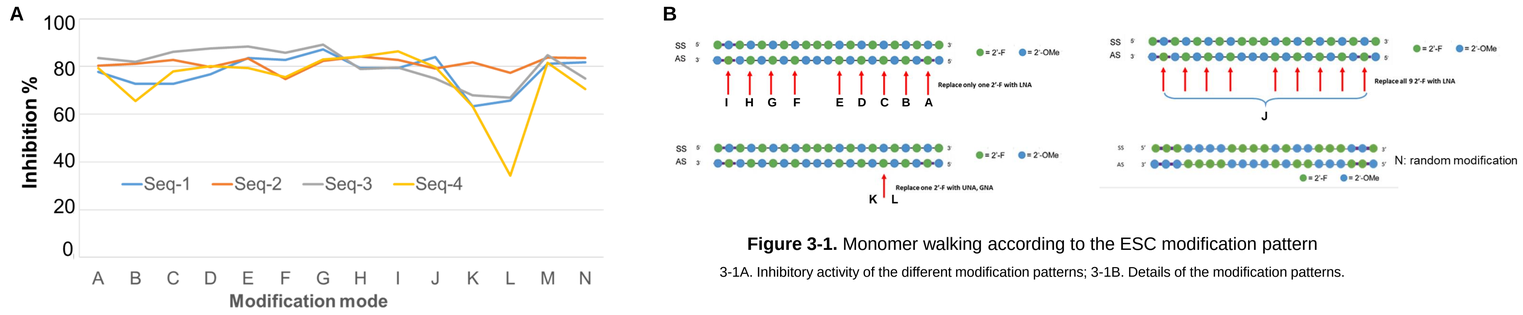


**Figure 3-1.** Monomer walking according to the ESC modification pattern

3-1A. Inhibitory activity of the different modification patterns; 3-1B. Details of the modification patterns.

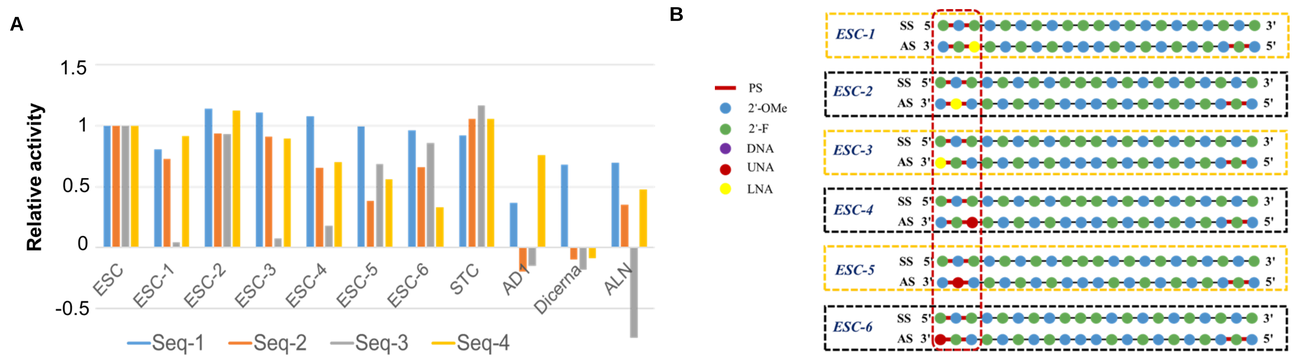


**Figure 3-2.** The specific monomer replacements at the 3' end of the antisense strand

3-2A. Relative activity for the different replacements; 3-2B. Details of the modification patterns.

***Results-3***

- ✓ The Comparison of A to I: The single LNA modification was tolerated with the siRNA activity. Furthermore, the LNA modification located closer to the 3' end of siRNA had the better effect on the siRNAs activity.
- ✓ Comparison of C, K, L: The LNA modification had superior effect on the activity to the UNA modification. However, the GNA modification had negative impact on the siRNA activity.
- ✓ The LNA modification was tolerated with the siRNA activity at the 3' end of antisense strand in the ESC modification pattern.
- ✓ Different modifications might have the distinct effects on different siRNA sequences.

### ➢ 4. Detection of off-target genes of C5-targeting siRNAs by the 3'-tag-seq in Huh-7 cells

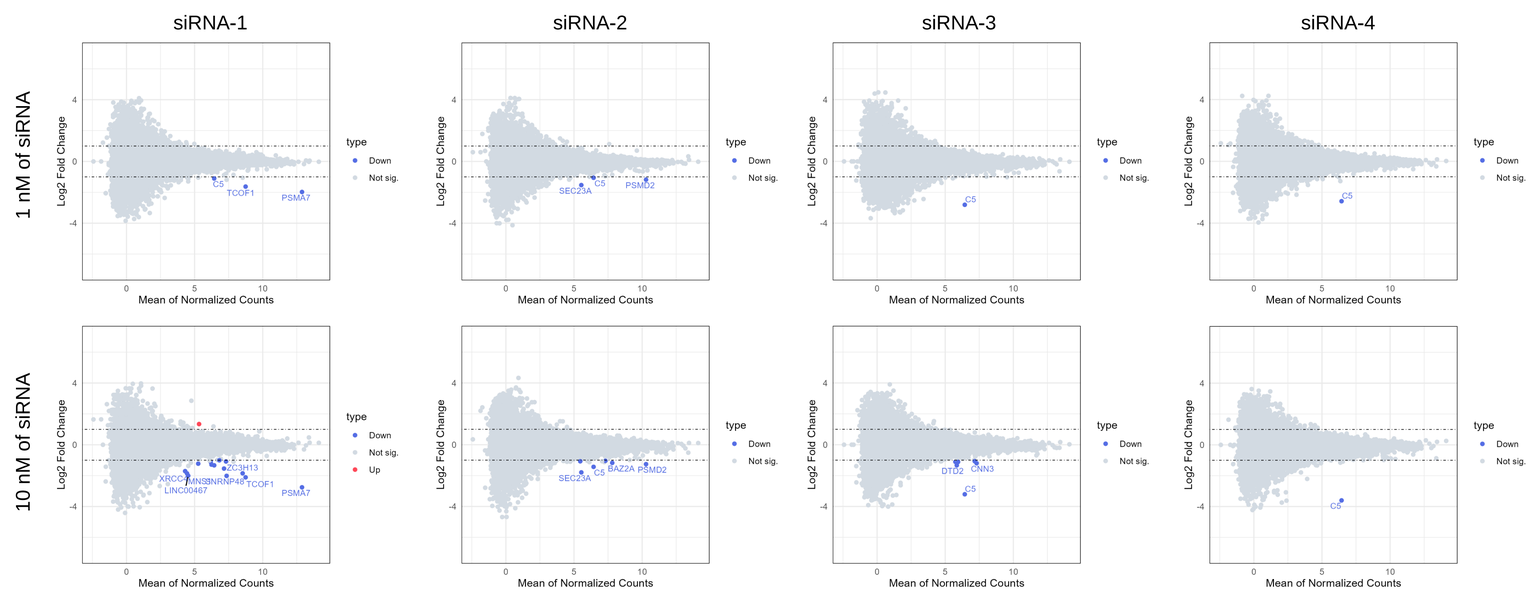


**Figure 4.** MA plot of gene expressions (treatment *vs.* non-treatment control)

Grey, not significant expression; Blue, down regulated genes; Red, up regulated genes.

**Table 1.** Alignment of differential expression genes and siRNAs

| siRNA ID | Strand | Off-target gene | Off-target score | Alignment (5'->3' from siRNA strand) |
|---|---|---|---|---|
| siRNA-1 | sense | ALCAM | 3 | \|\|\|\|\|\|*\|\|\|*\|\|\|\|\| |
| siRNA-1 | sense | ALCAM | 4 | *:\|\|\|\|\|\|\|\|\|\|\|\|:*\|*\|\| |
| siRNA-1 | sense | CAMLG | 4 | \|\|\|\|\|\|\|\|\|***\|\|\|\|\|:\|\| |
| siRNA-1 | sense | CBL | 3.5 | \|\|*\|\|\|\|\|\|\|\|\|\|\|\|**: |
| siRNA-1 | antisense | CNN3 | 3 | \|\|\|\|\|:\|\|:\|\|\|*\|\|\|\|\|*\| |
| siRNA-1 | antisense | DTD2 | 2.5 | \|\|\|\|\|:\|*:\|\|\|\|\|\|\|\|\|\|\|: |
| siRNA-1 | sense | LINC00467 | 3 | \|\|\|\|\|\|*\|\|\|*\|\|*\|\|\|\|\| |
| siRNA-1 | sense | LINC00467 | 3 | \|\|\|:\|:\|*\|\|:\|\|\|\|\|\|\|:\|\| |
| siRNA-1 | sense | LINC00467 | 3 | \|**\|\|\|\|\|:\|\|\|\|\|\|\|\|\|\|: |
| siRNA-1 | sense | MNS1 | 2.5 | \|\|\|\|\|:\|\|\|\|\|\|\|\|\|\|:*\| |
| siRNA-1 | sense | PSMA7 | 3 | \|\|\|:\|\|\|**\|\|\|\|\|\|\|\|\|\|: |
| siRNA-1 | antisense | SEC23A | 5 | \|\|\|\|*\|\|\|*\|*\|\|\|\|*\| |
| siRNA-1 | sense | SELENOK | 4 | \|\|\|*\|\|\|\|\|\|\|\|\|***\|\| |
| siRNA-1 | sense | SELENOK | 4 | \|\|\|::*\|:*:\|\|\|\|\|\|\|\|\|\| |
| siRNA-1 | sense | SNRNP48 | 2.5 | *\|\|\|\|\|\|\|:\|\|\|\|\|\|\|\|\|*\| |
| siRNA-1 | sense | TCOF1 | 1.5 | :\|\|\|\|\|\|\|:\|\|\|\|\|\|\|\|\|:\|\| |
| siRNA-1 | sense | TCOF1 | 2.5 | :\|\|:\|\|:*\|\|\|\|\|\|\|\|\|\|\|\| |
| siRNA-1 | antisense | VEZT | 4 | \|\|\|\|\|\|\|\|:\|\|\|**\|\|\|\|\|\|*: |
| siRNA-1 | sense | VEZT | 4 | *\|\|\|:\|:*\|\|:\|\|\|\|\|\|\|\|:\| |
| siRNA-1 | sense | XRCC4 | 3 | \|\|\|\|\|\|*\|\|\|*\|\|*\|\|\|\|\| |
| siRNA-1 | sense | XRCC4 | 3.5 | **\|\|\|\|\|\|\|\|\|\|\|\|\|\|:*\|\| |
| siRNA-1 | sense | ZC3H13 | 3.5 | \|\|\|\|:\|\|*\|\|:\|*\|:\|\|\|\|\|\| |
| siRNA-1 | sense | ZC3H13 | 3.5 | **\|\|\|\|\|\|:\|\|\|\|\|\|\|\|\|*\|\|\| |
| siRNA-2 | antisense | BAZ2A | 4 | \|*\|\|\|\|\|:\|\|\|\|\|\|\|\|*\|*\|:\| |
| siRNA-2 | sense | CNN3 | 6 | :*\|:\|\|\|**:\|\|:\|\|\|\|*\|\| |
| siRNA-2 | antisense | SEC23A | 1 | \|\|\|\|\|\|\|\|\|\|*\|\|\|\|\|\|\|\|\|\| |
| siRNA-3 | sense | DNAJC19 | 5.5 | \|*\|\|*\|\|\|*\|\|\|*\|*\|:\|\|\| |
| siRNA-3 | antisense | DTD2 | 4.5 | *\|\|\|\|\|\|\|\|\|\|:\|\|\|\|*\|*\|\|* |
| siRNA-3 | antisense | LGSN | 4 | :\|:*\|\|\|\|\|\|\|\|\|\|\|**\|\|\| |

- ➢ Alignment status: "**|**", Watson-Crick pairing; "**:**", GU pairing; "*****", Mismatch;
- ➢ Off-target score = 0.5* (GU count) + 1* (mismatch count).

***Results-4***

- ✓ The 3'-tag-seq had high repeatability and high throughput for detection of potential off-target genes.
- ✓ The majority of the differentially expressed genes observed by the 3'-tag-seq had nearly perfect match sites at different loci between the sense and antisense strands of the siRNAs.
- ✓ The siRNAs with higher potency are likely to have the less off-target effects by comparing the siRNAs evaluated.
- ✓ The strand exchange and nearly perfect pairing with the off-target genes are likely to be the main causes of the siRNA off-target effects.

## Summary

We setup an integrated platform for support of the siRNA drug discovery. Our platform covers siRNA sequence design, chemical synthesis and modification, delivery ligand exploration, and activity and off-target evaluations. We have applied the platform to the services to our world-wide clients. Here, we use C5-targeting siRNA as an example to demonstrate the usefulness of our siRNA platform for facilitating the discovery of siRNA drug.

We also developed a 3'-tag-seq method for identification of siRNA off-targets. The 3'-tag-seq method is highly sensitive, cost-effective and high-throughput, therefore it may provide an alternative solution to efficiently identify off-targets for the discovery of siRNA drugs.

*In addition, WuXi Biology provides an integrated service for support of the discovery of oligo drugs. Our services cover design and synthesis of oligos, conductance of in vitro and in vivo biological and PK/PD evaluations, and assessment of oligo off-targets.*