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4′-Guanidinium-modified siRNA: A molecular tool to control RNAi activity through RISC priming and selective antisense strand loading †

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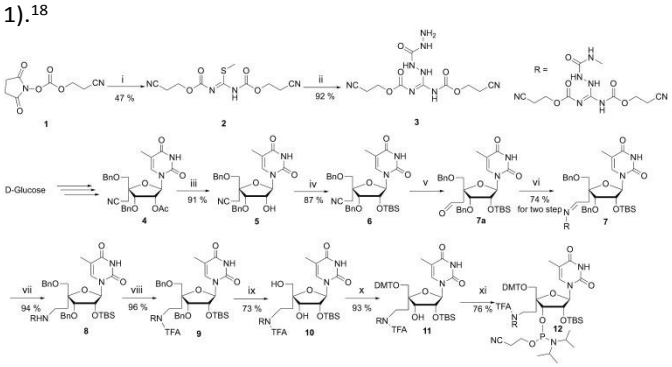
We designed novel 4′-C-guanidinocarbohydrazidomethyl-5-methyl uridine (GMU) modified small interfering RNA (siRNA) and evaluated their biophysical and biochemical properties. Incorporation of GMU units significantly increased thermodynamic stability as well as the enzymatic stability against nucleases in human serum. Gene silencing experiment indicated that GMU modfied siRNA (siRNA6) resulted in ≈4.9-folds more efficient knockdown than unmodified siRNA.

Since the discovery of synthetic siRNA as a potent gene-silencing molecule in mammalian cells, siRNA-based drugs have emerged as a promising technology for the treatment of a variety of diseases.[1] The RNA interference (RNAi) is an endogenous pathway for post-transcriptional gene silencing that uses sequence-specific knockdown of target mRNA. In this mechanism, short double-stranded RNA (21–23-mer) undergoes a natural antisense or guide strand selection process into the RNA-induced silencing complex (RISC) in the cytosol.[2-4] This activated RISC binds mRNA, resulting in gene silencing.[5, 6] Even though the RNAi based drug development platform looks promising, there are several challenges to overcome. These include challenges to enhance the stability of such drugs against nucleases,[7] minimize off-target effects,[8] immune activation,[9] and most importantly to enhance *in vivo* delivery.[10] We have recently developed the first non-cationic non-viral siRNA delivery method using hyaluronic acid, a natural biopolymer present in the extracellular matrix.[11]

In this communication, we present synthesis of a novel 4′-guanidinium modified siRNA and their systematic physicochemical, biochemical, computational and *in vitro* gene silencing analysis. The GMU modification at the 3′-end is expected to be protonated at the physiological condition,[12, 13] which could enhance nuclease stability,[14-16] and potentially improve immunocompatibility.[17] To develop GMU modification, we first synthesized guanidinocarbohydrazide ligand (**3**) by modifying our recently reported method (Scheme 1).[18]



Scheme 1. Synthesis of guanidinocarbohydrazide ligand **(3)** and 4′-*C*-guanidinicarbohydrazidomethyl-5-methyl uridine phosphoramidite (**12**)**.** *Reagents and conditions*: i) S-methylisothiourea hemisulfate, DCM/sat. $NaHCO_3$, 45 °C, 2 h; ii) carbohydrazide, MeOH, reflux, 12 h; iii) NaOMe/MeOH, room temperature (rt), 3 h; iv) TBDMS-Cl, imidazole, DMF, rt, 2 h; v) 1 M DIBAL/hex, DCM, -78 °C, 2 h; vi) Compound **3**, MeOH, reflux, 5 h; vii) $NaBH_4$, MeOH, rt, 3 h; viii) $EtOCOCF_3$, THF, rt, 4 h; ix) 20 % $Pd(OH)_2/C$, ammonium formate, EtOH, 70 °C, 48 h; x) DMTr-Cl, Py, DMAP, 45 °C, 3 h; xi) DIPEA, CEP-Cl, DCM, rt, 3 h.

The S-methylisothiourea hemisulfate and activated hydroxypropionitrile compound **1** was reacted to obtain compound **2**. The S-methyl group was further substituted with carbohydrazide to furnish guanidinocarbohydrazide ligand **3**. To prepare the GMU building block, we first synthesized the cyano nucleoside **4** from D-glucose using our previously reported procedure.[19] The 2′-acetate group of nucleoside **4** was hydrolysed using sodium methoxide to yield 2′-hydroxy nucleoside **5**. Compound **5** was further silylated using tert-butyldimethylsilyl chloride (TBDMS-Cl) to obtain 2′-*O*-silyl (TBS) nucleoside **6**.[20] We further reduced the cyano group of **6** to obtain crude aldehyde modified nucleoside **7a** which was subsequently converted to hydrazone nucleoside **7**.[21] The hydrazone derivative was further reduced to afford guanidino nucleoside **8.** We further protected the amino group of the hydrazine moiety of **8** using ethyltrifluoro acetate (CF_3COOEt) to obtain trifluoro acetate (TFA) protected nucleoside **9**. The debenzylation of **9** was performed using 20 % $Pd(OH)_2$ to

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obtain the deprotected diol nucleoside **10**. The 5′ hydroxyl of compound **10** was selectively protected using 4,4′-dimethoxytrityl chloride (DMTr-Cl) to furnish **11**.[22] Finally, phosphitylation of compound **11** was carried out to furnish the phosphoramidite **12**.[22] The unmodified and GMU modified siRNAs targeting *signal transducer and activator of transcription 3 (STAT3)* mRNA were synthesized using a solid phase RNA synthesizer (Table 1). The cleavage from the solid support and deprotection of guanidinium and phosphate protecting groups were carried out using 50 % piperidine.[23] Further nucleobase deprotection, 2′-*O*-TBDMS group deprotection was carried out and the product was purified by denaturing polyacrylamide gel electrophoresis (PAGE).[22] In order to evaluate the effect of chemical modification on the thermal stability of siRNA duplexes, we performed UV-melting studies (Table 1).

Table 1. The sequences of siRNA duplexes and T_m values

Name	Passenger (5′-3′, above) and guide strand (3′-5′, below)	T_m[a]	ΔT_m[b]/ mod.
siRNA1	GGAAGCUGCAGAAAGAUACTT TTCCUUCGACGUCUUUCUAUG	66.9 ± 0.1	
siRNA2	GGAAGCUGCAGAAAGAUACTT TTCCUUCGACGUCUUUCUAUG	67.8 ± 0.3	+0.9
siRNA3	GGAAGCUGCAGAAAGAUACTT TTCCUUCGACGUCUUUCUAUG	69.8 ± 0.1	+2.9
siRNA4	GGAAGCUGCAGAAAGAUACTT TTCCUUCGACGUCUUUCUAUG	69.5 ± 0.2	+2.6
siRNA5	GGAAGCUGCAGAAAGAUACTT TTCCUUCGACGUCUUUCUAUG	69.7 ± 0.3	+2.8
siRNA6	GGAAGCUGCAGAAAGAUACTT TTCCUUCGACGUCUUUCUAUG	70.3 ± 0.2	+3.4

[a]T_m represents melting temperatures for unmodified and GMU modified siRNA duplexes (red colour indicates modification) in °C. [b]ΔT_m represents the [T_m (RNA mod.) – T_m (RNA unmod.)]. The T_m values were determined using 1 µM of siRNA in buffer containing 50 mM NaCl, 10 mM Na_2PO_4, pH 7.4. All experiments were triplicated, and the T_m values have reported an average of 3 measurements with the estimated standard deviation.

Incorporation of single GMU modification at the 3′-terminal of the passenger strand of siRNA (**siRNA2)** showed a change in melting temperature (ΔT_m) of +0.9 °C per modification as compared to the unmodified duplex. Incorporation of GMU modification on both the overhang nucleotides of siRNA in either passenger (**siRNA3)** or guide (**siRNA4)** strand demonstrated a similar increase in the melting temperature (ΔT_m = 2.6–2.9 °C). When both the strands of siRNA were modified together, an increase in ΔT_m of 2.8–3.4 °C was observed (**siRNA5** and **siRNA6)**. In general, GMU modifications resulted in a significant thermal stabilization of all the modified siRNA duplexes (Table 1).

To elucidate the role of guanidinium modifications in thermal stability of siRNA duplexes, we performed *in silico* molecular modelling and simulation of the modified and unmodified siRNA duplexes (Fig. S1-S2). These studies implied that the guanidinium linkers near 3′- ribonucleotides (C4′ of ribose sugar) and penultimate thymidine residues (deoxyribose sugar) at **siRNA6** termini conferred additional non-covalent interactions to protect the fraying ends of siRNA duplex and impart thermodynamic stability. In **siRNA6**, dual GMU modifications at 3′-overhang amplified the network of electrostatic interactions with adjacent residues (Fig. S1B). We further analyzed the energetics of siRNA conformers, by performing MM-PBSA calculations, which decomposes the interaction energies for the modified residues by considering molecular mechanics and solvation energies (Table S1).

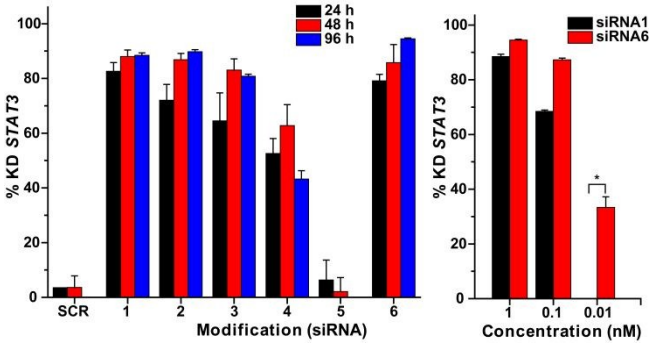

Fig. 1. The RNAi activity of unmodified and GMU modified siRNAs. (A) Time-dependent RNAi activity after 24 h, 48 h and 96 h of transfection with 1 nM siRNA. (B) Concentration-dependent RNAi activity post 96 h of transfection. Experiments were performed in triplicate for three times. The Student's *t*-test was used to determine statistical differences between pairs of groups (* = $p \leq 0.05$).

The modified siRNAs were further evaluated for their RNAi efficacy in human colon cancer cell line (HCT116). HCT116 cells were transfected with different concentrations of siRNA, and the expression levels of *STAT3* mRNA was analyzed after 24 h, 48 h or 96 h (Fig. 1). The scrambled siRNA sequence (SCR) was used as a negative control in these experiments. The gene knockdown (KD) results of unmodified and GMU modified siRNAs are summarized in Fig. 1. These results indicate that the modifications of overhang are well tolerated in passenger strand as well as guide strand of siRNA (Fig. 1A). The modifications in passenger strand of siRNA duplexes such as **siRNA2** and **siRNA3** have shown no loss of RNAi activity when compared to the unmodified **siRNA1** (in 48 or 96 h 2-5 % less KD). The effect of modifications in the guide strand (**siRNA4)**, however, was found to be sensitive with 20-40 % less KD efficiency as compared to **siRNA1**. Surprisingly, when both the strand of siRNA were modified in an asymmetric manner with single modification on passenger and two modifications on guide strand (**siRNA5**), there was a complete loss of RNAi activity. Interestingly, in the case of **siRNA6** where both the overhangs were modified with GMU in a symmetrical manner, the RNAi activity was restored and was in fact was superior (7 % more KD for 96 h) to the unmodified siRNA (Fig. 1A). We further performed concentration-dependent study to validate the actual difference in RNAi activity between **siRNA1** and **siRNA6**. Gratifyingly, these experiments suggested that **siRNA6** with completely modified overhangs were ≈4.9 folds more efficient as compared to the **siRNA1** control (IC50 for **siRNA1** ≈311 pM, while for **siRNA6** ≈63 pM, Fig. S3). Specifically, the **siRNA6** demonstrated 20–40 % higher activity as compared to the native siRNA after 96 h (Fig. 1B). One of the key aspects of siRNA duplex that dictates its activity is RISC priming, which enable correct orientation of the duplex for specific loading of the antisense strand.[24] The 3′-overhang plays an important role in this step as it binds to the PAZ domain of the

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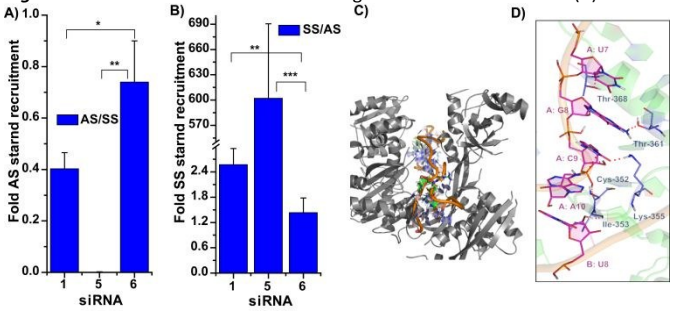
Argonaute-2 protein within the RISC.[25] Intrigued by the difference in bioactivity between **siRNA5** and **siRNA6**, we were prompted to investigate the differences in actual loading of sense and antisense strands. For this purpose, we performed recruitment assay using stem-loop qPCR.[26] This experiment clearly indicated enhanced recruitment of guide strand in case of modified **siRNA6** (≈1.8-fold enhancement as compared to the **siRNA1**; Fig. 2A) while the recruitment of guide strand in **siRNA5** was not observed (Fig. 2A). Interestingly, we observed exclusive recruitment of sense strand in case of **siRNA5** (≈234-fold enhancement as compared to the **siRNA1**) and significantly less in **siRNA6** (0.5 fold less compared to the **siRNA1** (Fig. 2B).

Fig. 2. Strand recruitment and RISC binding of GMU modified siRNA. (A) Effect of

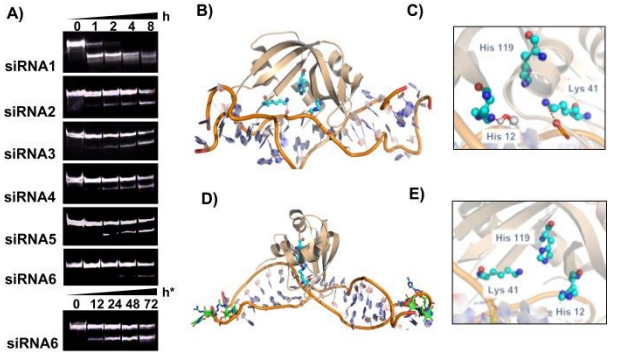


modification on guide (AS) strand recruitment, and (B) on passenger (SS) strand recruitment within RISC as analyzed by stem-loop qPCR with the scrambled sequence as a control. (C) Recruitment of **siRNA6** into the PAZ domain of Argonaute protein. (D) Principal non-covalent intermolecular interactions between the PAZ domain of Argonaute protein and **siRNA6**. Experiments were performed in triplicate for three times. The Student's *t*-test was used to determine statistical differences between pairs of groups (* = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$).

To further investigate the role of guanidinium modifications in guide strand recruitment, we carried out molecular docking studies between Argonaute protein and siRNA duplexes (**siRNA1** and **siRNA6**) and compared the intermolecular interactions. The PAZ domain of Argonaute, which is regarded as the principal RNA binding module to recognize the 3' overhang of siRNA is observed to make stable non-covalent interactions with **siRNA6**, consistent with our experimental evidence (Fig. 2C). Symmetric guanidinium modifications at both ends favoured spatial positioning of siRNA into the binding pocket of PAZ domain of RISC complex. Thr 361, Ile-353, Lys-355 allow the passage of **siRNA1** and **siRNA6** into RISC complex (Fig. S4B and 3D). In **siRNA6**, Cys-352 and Thr-368 additionally play important roles to constitute electrostatic interactions with A10, G8 and U7 and enable improved strand recruitment into RISC complex (Fig. 2D). Structural dynamics of **siRNA1** evidenced stable Watson-Crick base pairing in the central domain, which spanned the binding pocket of PAZ. We observed strong pairing between G11:C9, stabilized by neighbouring G8, which projected a π–π stacking interaction with C9 in the same strand. This sequestered the hydrogen-bond interaction between Lys 355 and C9, which might play a critical role to compromise its recruitment into RISC complex (Fig. S4A and S4B). Further structural investigations confirmed that the sugar puckering of **siRNA6** remained in *C3'-endo* (N-type sugar) sugar conformation (Fig. S5), which is essential to maintain the integrity of A-type RNA.

One of the major challenges of the nucleic acid drug is the stability towards endo and exonucleases present in blood plasma and within the cytosol.[7] We have previously shown that a single guanidinium modified locked nucleoside at the 3'-terminal can impart significant enzymatic stability for over 24 h in human serum.[18] This prompted us to evaluate the stability of the GMU modified siRNAs in human serum. Incubation of modified and unmodified siRNAs with 70 % human serum at various time points followed by PAGE analysis of the nuclease digested product indicated that all modified sequences (**siRNA2**-**6**) showed a significant improvement in nuclease stability (Fig. 3A).

Fig. 3. Serum stability for modified siRNA. (A) 15 % Native PAGE denoting



stability of siRNA duplexes (30 μM) after incubating in 70 % human serum (t = 0, 1, 2, 4 and 8 hours). h* indicates serum stability study for up to 72 h for **siRNA6**. The intensity of the full-length siRNA (intact overhang) bands decreases with an increase in incubation time. (B) RNase A-**siRNA1** complex. (C) The catalytic core of RNase A (His119, Cys41, and His12) providing in-line attack in a **siRNA1** duplex. (D) RNase A-**siRNA6** complex. (E) The catalytic core (His119, Cys41, and His12) of RNase A is spatially placed far apart from **siRNA6** to invade the double-stranded RNA.

The siRNA duplex with single or double GMU modifications at the 3'-end of the passenger or guide strand showed stability for over 4 h in human serum. The dual modification on both guide and passenger strands (**siRNA6**) further enhanced nuclease stability with nearly intact duplex after 12 h and almost 50 % intact siRNA after 72 h (h*, Fig. 3A). The unmodified siRNA duplex (**siRNA1**) on the other hand was completely degraded within one hour of incubation time. We reasoned that the stabilized non-covalent interactions by GMU modifications at 3'-termini sequester siRNA molecules from the catalytic pockets of RNase A-type endonucleases, which enhance nuclease resistance of the siRNA molecules in the sera. Structural analyses of *in silico* RNase A-siRNA complexes further underscored that RNase A catalytic triad (His12, His119, and Lys41) invades **siRNA1** to promote RNA cleavage (Fig. 3B and 3C). However, the Watson-Crick pairing in **siRNA6** is perturbed due to the end modifications rendering reduced efficiency of RNase A catalytic triad to remain in the spatial proximity of the ribonucleotides, required for the nucleophilic attack towards 2'-OH of the ribose sugars. Therefore, **siRNA6** is

not as vulnerable as **siRNA1** and is able to evade nuclease cleavage (Fig. 3D and 3E).

In summary, we have synthesized novel 4′-guanidinium-modified amidite to obtain several chemically modified siRNAs. These modified siRNAs not only increase thermal stability but also significantly improved stability in human serum. The incorporation of GMU nucleotides at the two terminal 3′-overhangs (**siRNA6)** also resulted in sustained gene silencing activity at significantly lower concentration (picomolar) after 96 h of transfection. The stem-loop qPCR experiments confirmed that such sustained activity was due to enhanced guide strand recruitment within the RISC complex. Structural investigations of the Argonaute protein and **siRNA6** also confirmed altered spatial positioning of siRNA into PAZ domain resulting in successful recruitment of the desired guide strand into RISC complex and enhanced activity. We believe that the 4′-guanidino modifications at the 3′-overhangs of siRNA will allow development of next generation of RNAi drugs with enhanced potency and minimal sense-strand mediated off-target effects. Since such modifications are incorporated at the overhang region, it could be adapted to other siRNA sequences, without losing its bioactivity.

Conflicts of interest

There are no conflicts to declare.

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