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Systematic chemical modifications of single stranded siRNAs significantly improved CTNNB1 mRNA silencing

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ABSTRACT

Single-stranded silencing RNAs (ss siRNA), while not as potent as duplex RNAs, have the potential to become a novel platform technology in RNA interference based gene silencing by virtue of their simplicity and plausibly favorable characteristics in pharmacokinetics and biodistribution. Like other therapeutic pharmaceutical agents, ss siRNA can be optimized to achieve higher potency through a structure–activity based approach. Systematic chemical modification at each position of a 21-mer oligonucleotide identified 2′,5′-linked 3′-deoxythymidine (3dT) at position 1 and locked nucleic acids (LNAs) at the seed region as key components to afford significant enhancement in knockdown activity both in vitro and in vivo. Further optimization by additional chemical modifications should enable ss siRNA as an alternative gene silencing modality.

RNA interference (RNAi) is an evolutionarily conserved cellular mechanism of post-transcriptional gene silencing found in fungi, plants and animals utilizing small RNA molecules to inhibit gene expression in a sequence-specific manner.[1] RNAi is controlled by the RNA-induced silencing complex (RISC), which is initiated by double-stranded short interfering RNA (ds siRNA) molecules in a cell's cytoplasm. The siRNA interacts with argonaute 2 (Ago2), the catalytic component of RISC, which cleaves target mRNA that is complementary to the bound RNA.[2] One of the two RNA strands, known as the guide strand, binds to the Ago2 protein and directs gene silencing, while the other strand, known as the passenger strand, is degraded during RISC activation.

The initial discovery of the RNAi gene-silencing mechanism and recent advances in the clinic as therapeutic medicines were primarily based on double-stranded siRNAs. However, single-stranded RNAs (ss RNAs) have also been known to silence complementary mRNA in cells through an RNAi-based mechanism[3] and have the potential to become a therapeutic agent considering the current status of antisense oligonucleotides (ASOs) as FDA-approved treatments.[4] Since it is single stranded, ss siRNA can simplify oligonucleotide synthesis reducing the cost of goods significantly and potentially lead to therapeutic agents with more desirable biodistribution and pharmacokinetic profiles.[5] The gene silencing mediated by ss RNA has been reported to be dose-dependent, but the potencies are substantially lower compared to ds siRNA in both cell based assays and animal models.[6] The metabolic instability of ss RNA has been implicated in the inferior silencing activity.[7]

Chemical modification of nucleotides incorporated into RNAi molecules leads to improved physical and biological properties, such as stability towards ribonucleases,[8] reduced immune stimulation,[9] diminished off-target activity,[10] higher knockdown activity,[11] and enhanced lipophilic character to improve cellular uptake and delivery to the cytoplasm.[7] Thus, chemical modifications have the potential to further increase fundamental potency of RNA compounds, allowing lower doses of administration, reducing the potential for toxicity, and decreasing the overall cost of therapy. However, except for the recently reported investigation

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on metabolically stable 5′-end modifications by Prakash et al.,[12] the scope of chemical modifications applied to ss siRNAs reported in the literature have been limited mostly to conventional nucleotide analogs, such as 2′-OMe, 2′-F, and 2′-MOE.[6] In this study we have employed a wide range of modified nucleotides and systematically investigated the structure activity relationship of each nucleotide unit in a 21-mer ss RNA in order to efficiently and comprehensively optimize the intrinsic silencing activity of the single stranded oligonucleotide.

As described in Figure 1, we introduced various chemically-modified nucleotides into the 5′-position 1 of antisense single-stranded siRNA targeting CTNNB1 (1797) where an alternating 2′-F/2′-OMe pattern[11] was utilized as the benchmark sequence. The ss siRNA described here contained a phosphate or an equivalent (such as a phosphonate) at the 5′-end, because the 5′-phosphate (or its surrogate) is required for the single stranded RNA to demonstrate any measurable silencing activity.[13] In addition to conventionally used 3′,5′-linked nucleotides such as omeU (2′-O-methyluracil) and dT (2′-deoxythymidine), 2′,5′-linked 3dNs (3′-deoxynucleotides; N = A, T, C, and G) were incorporated into oligonucleotides using commercially available phosphoramidite precursors. Although not used for biological information storage, 2′,5′-linked oligonucleotides are compatible with the Watson–Crick base pairing and are known to form naturally during intron splicing.[14] There has been interest in using 2′,5′-linked oligonucleotides in antisense RNA applications as they preferred to selectively hybridize with their RNA complements, rather than DNA complements,[15] and displayed improved resistance toward several types of nucleases.[16] However, only limited studies describing the ability of 2′,5′-linked ribonucleotides to efficiently degrade target gene expression through an Ago2-mediated RNAi pathway have been reported.[17]

The ss siRNA molecules were initially screened for their in vitro silencing activity in Hepa1-6 cell cultures transfected with RNAiMax. Table 1 summarizes the knockdown activities of regular 3′,5′-linked single-stranded siRNA molecules as well as the new 2′,5′-linked single-stranded siRNA molecules. The name of each siRNA molecule (bold) provided in Table 1 corresponds to the com-

Table 1
Reduction of CTNNB1 levels in cell culture transfected with modified single-stranded siRNA; comparison of ss siRNAs with chemical modification at 5′-position one

ss siRNA	CTNNB1 reduction	
	At 10 nM	At 1 nM
omeU	42%	11%
omeUs	64%	16%
dT	86%	50%
dTs	77%	36%
3dT	91%	80%
3dTs	91%	80%
3dA	53%	9.2%
3dAs	60%	16%
3dC	63%	17%
3dCs	65%	17%
3dG	12%	−3.0%
3dGs	15%	0.3%
3omeU	30%	3.5%
3omeUs	33%	−1.7%
3fU	44%	1.4%
3fUs	23%	8.4%
3daraT	37%	4.7%
3daraTs	5.0%	2.7%
3rT	47%	8.9%
3rTs	35%	-6.8%
v3dT	43%	11%
v3dTs	34%	10%

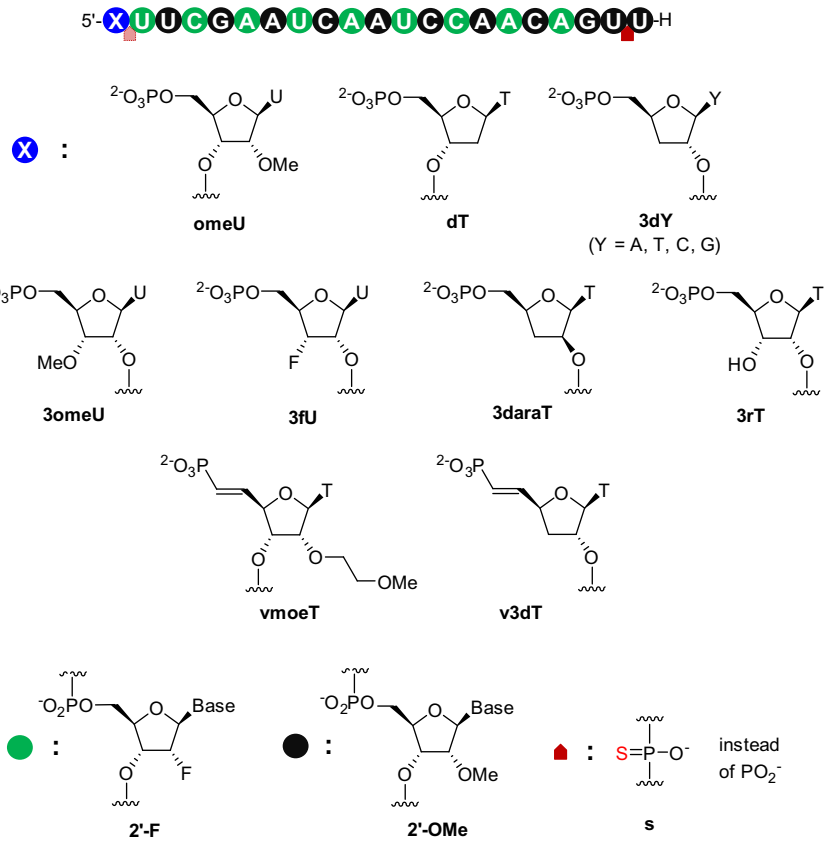

Figure 1. CTNNB1 (1797) single-stranded siRNA sequences and chemical modifications.