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# Circular RNA
## and gene knockdown

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# Introduction

New functional roles for RNA have been discovered in what is now recognized as the emerging RNA renaissance. The discovery of RNA interference (RNAi) led to the use of short double-stranded synthetic RNAs, commonly referred to as small interfering RNA (siRNA) to silence genes for investigating gene functions and for gene knockdown therapy. ONPATTRO® (Patisiran), a double-stranded small interfering RNA, has FDA approval for treatment of hereditary transthyretin-mediated (hATTR) amyloidosis, a rare and often fatal nerve disease attributed to the transthyretin gene (TTR). This infusion combines the drug delivery technology of lipid nanoparticle (LNP) complex formation with the targeting of the hepatic asialoglycoprotein receptor (ASGPR). More recently, GIVLAARI® (givosiran) was approved for treatment of acute hepatic porphyria (AHP). Givosiral relies on an N-acetylgalactosamine-siRNA (GalNAc-siRNA) covalent conjugate to target 5'-aminolevulinate synthase 1 (ALAS1). Both of these are door-openers for future therapeutic siRNA drugs. Messenger RNA (mRNA) as a therapeutic or preventive medicine has reached general public awareness with the success of encapsulated mRNA LNP vaccines used during the COVID-19 pandemic. Guide RNA (gRNA) used for CRISPR-Cas-based sequence editing has provided researchers with readily accessible tools for gene editing research in vitro, and potentially in vivo, to correct diseases resulting from DNA sequence errors. This note will skim over some of the types and functions of additional important non-canonical classes of RNA, and then introduce a relative newcomer, circular RNA (circRNA). The aim of this note is to outline the synthetic chemistry and gene knockdown effects captured in a recent study where researchers describe the design, synthesis, properties, and use of a new gene silencing platform (Figure 1) they call small circular interfering RNA (sciRNA).[1]

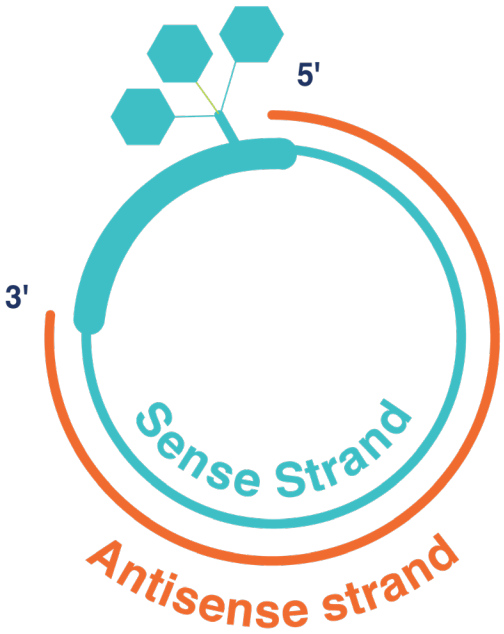



Figure 1. Hepatic Targeted Small Circular Interfering RNA

# Background

## Linear RNA

The remarkable variety in RNA functions ascribes to the linear nucleotide sequence that confers base-paired stem and loop structures with intrinsic flexibility and intramolecular interactions resulting in a folded 3-dimensional form and functionality. Many of these individual RNA molecules are present at very low abundance and so previously have been difficult to detect. The renaissance in RNA research has been greatly facilitated by high-throughput sequencing and optimized commercial RNA-Seq kits. These kits are compatible with custom RNA adapter sequences and offer greatly expanded RNA synthesis capabilities Additional RNAs present in the cell may be further subdivided by size into long non-coding RNA (lncRNA) and short (chain) non-coding RNAs (sncRNAs such as microRNA (miRNA), piwi RNA (piRNA), and siRNA, among others).

LncRNAs, which are generally longer than 200 nt, are in the nucleus or cytoplasm and rarely encode proteins. Recent investigations have begun to reveal the functions of lncRNAS, which appear to mainly use four archetypal mechanisms for regulating gene expression: as signals, decoys, guides, and scaffolds. Studies of genomic imprinting and X chromosome inactivation were the first to reveal a role for lncRNAs in epigenetic regulation. Two lncRNAs, H19 RNA and Xist RNA, respectively, induce histone methylation and methylation of gene DNA, in the latter case with X chromosome-wide repression of gene expression. The functions of lncRNA remain actively researched and the progress in elucidating the roles of linear forms of lncRNA is outside the scope of this note. Key functions also are being identified for short RNAs; for example, many miRNAs (typically 21-25 nt) are highly conserved across species and are found to inhibit gene expression by targeting mRNA stability, affecting translation. piRNA is a large class of small non-coding RNA molecules (21-35 nt) that in animals can form RNA-protein complexes with piwi-subfamily Argonaute proteins. In addition to that participation in targeted RNA cleavage, they are involved in regulation through participation in DNA methylation and heterochromatin assembly. Double-stranded siRNAs, containing typically ~20-25 base pair oligonucleotide components with a two-nucleotide 3’-terminus overhang, direct gene silencing and will be a focal point of this note below. This and other short linear RNA types are a dynamic area of research where significant progress has been made.

## Circular RNA Significance in Eukaryotic Cells

In 2012 the discovery of the widespread presence of circRNA in eukaryotic cells[2] elevated researchers’ attention to understanding their distribution and functions[3]. Viral circRNAs were observed as early as 1976 by electron microscopy among plant RNA viruses, as viroids, small circular RNA molecules, lacking the protein coat of a virus and that are infectious agents of some plants. Many years later, endogenous circRNA was discovered in the deleted in colon cancer (DCC) gene in humans. We now have found significant changes in the occurrence of circRNAs by cell type, during organismal development and in disease. These closed-loop circular RNAs are formed by backsplicing pre-mRNA via the spliceosome through three proposed circularization mechanisms (Figure 2) where the upstream 3'-acceptor is joined to a downstream 5'-acceptor.[4] In the leftmost mechanism an RNA binding protein (RBP) may recognize a sequence motif or alternatively (center), inverted complementary sequences will

bring flanking intronic regions into proximity to promote circularization. On the figure's right side, circularization begins with canonical splicing, producing linear mRNA with a long intron-containing lariat sequence. An internal splicing between sequence donor (SD) and acceptor (SA) completes the circularization.

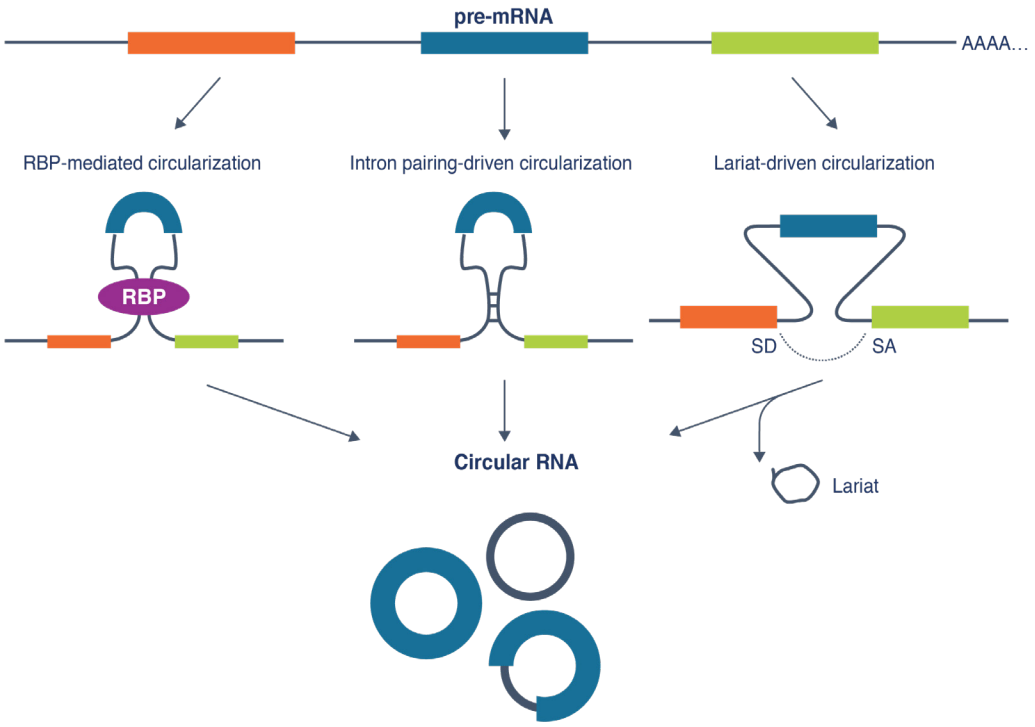



**Figure 2. Biogenesis of circular RNA**

Some circRNAs accumulate to higher levels than their associated linear mRNAs, an aspect especially evident in the nervous system, and have clear regulatory functions that result in organismal phenotypes. Quite soon after recognition of their omnipresence, it was apparent that circRNAs are not accidental by-products of splicing. Different circRNA regulatory roles are proposed to operate through alternative mechanisms: regulating transcription, acting as miRNA sponges, regulating other protein activities (through binding), and encoding proteins (Figure 3).

The importance of circular RNA in normal biology and diseased states can be appreciated from the following sampling of research findings. circRNAs are abundant in skeletal muscle tissue, and their expression levels are regulated during muscle development and aging.[5] They are implicated in cardiovascular diseases and play a role in adipocyte metabolism and obesity by regulation of adipogenesis and lipolysis in adipose tissue and the liver. RNA seq profiling of young (1-month-old) and aged (22-month-old) mouse cortex, hippocampus, and heart samples was performed and showed increased circRNAs accumulated in aged mouse cortex and hippocampus, but not in the heart revealing strong circRNA upregulation during aging in neural tissues.[6] A circRNA generated from the AGO2 gene for the Argonaute 2 protein (circAGO2) is critical to early development and is a novel regulator of AGO2-miRNA complexes and cancer progression. In addition, circAGO2 is up-regulated in gastric, colon,

prostate, and neuroblastoma cancers, and is associated with poor prognosis of patients; it promotes the growth, invasion, and metastasis of cancer cells in vitro and in vivo.[7]

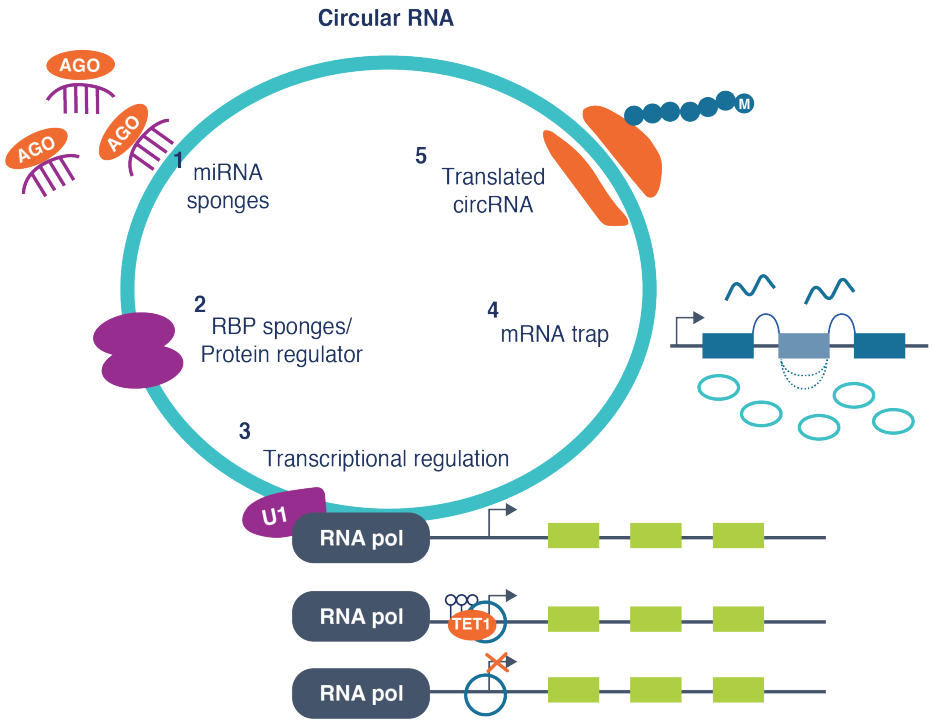



**Figure 3. Regulatory Mechanisms of circRNAs**

Although the possibility of in vivo circRNA translation from eukaryotic organisms long has been speculated upon, there is now strong evidence for a translational function of circRNAs in the initiation and treatment resistance of glioma.[8] Although circRNA does not have the 5'-adenyl cap structure and 3'-polyadenlyation used classically for mRNA translation, highly structured RNA sequences known as internal ribosome entry sites (IRES) are present that facilitate translation initiation in the absence of these former features. The closed circular architecture permits the possible translation of multiple copies from a single circRNA or large concatenated peptides/proteins depending on the presence of a stop codon, the absence of a stop with a continuous open reading frame (ORF), or other factors. It is clear now that some eukaryotic circRNAs are translated[9] and their translation can be mediated by IRES-like sequences.[10] Furthermore, N6-methyladenosine (m6A) promotes efficient initiation of protein translation from circRNAs in human cells[11] and it is a matter of current research whether the strategic placement of m6A in circRNA can prevent innate immune system activation by the pattern receptors RIG-I and TLR.

These and many other findings have led to efforts to engineer circular RNA to efficiently achieve therapeutic translational goals[12]; key design elements recently have been summarized.[13] Commercial development of circular RNA to treat human diseases is underway with the development of fully engineered molecules of circular RNA and endless RNA.

# Circular RNA-Based Gene Silencing

Researchers recently carried out a drug delivery study that targets liver cell asialoglycoprotein receptors (ASGPRs) via GalNAc-conjugated siRNA and LNP encapsulation but takes a further technological step to introduce circRNA as the delivery form for the siRNA.[14] They coined the term small circular interfering RNA (sciRNA) for their new platform and chose to evaluate sciRNA potency using synthetic sciRNAs for the knockdown of two well-characterized gene targets for the Transthyretin (Ttr) and Complement Component 5 (Complement C5) protein. Particularly interesting is the manner of preparing the circular RNA by closing the circle using high-yield copper-catalyzed "click" chemistry described below.

## sciRNA Design and Oligonucleotide Synthesis

The double-stranded circRNA was prepared by hybridization of individually synthesized linear antisense oligonucleotide with a circular sense oligo containing the GalNAc receptor ligand (Figure 4). Both the antisense strand and the circular RNA strand were modified with strategically placed 2'-OMe, 2'-F, and phosphorothioate linkages, while the antisense strand included 5'-vinylphosphonate (VP) to resist phosphatase and exonuclease activities and enhance silencing activity.[15] The circular sense strand was synthesized as a linear precursor oligonucleotide and then cyclized following solid-phase synthesis. Trivalent GalNAc CPG support was used to synthesize the linear precursor RNA; the alkyne was placed adjacent to the 3'-end using alkyne hydroxyprolinol phosphoramidite, followed by oligonucleotide synthesis; the 5'-end was terminated with 5'-bromohexyl phosphoramidite.

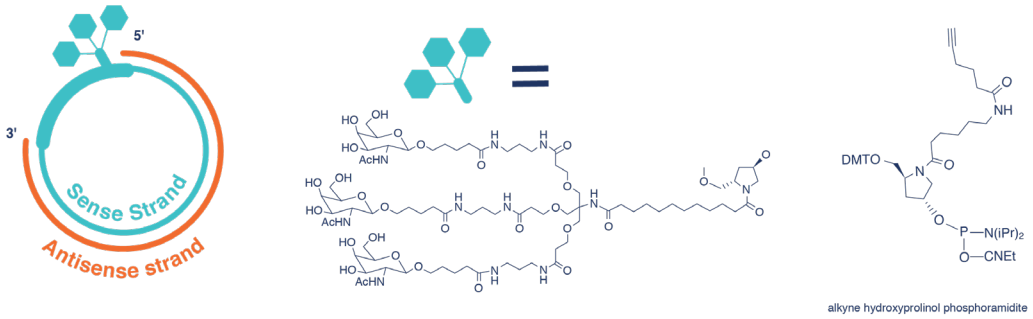



**Figure 4. Design of sciRNA**

The bromohexyl-modified oligonucleotide is converted to 5-azidohexyl oligo by shaking the CPG in dry DMF with excess 1:1 sodium azide/sodium iodide at 65 °C.[16, 17] The reader is referred to the publication for additional synthesis details.

## Cyclization of Linear Precursor to Circular RNA

The closure of the oligonucleotide circle is a key step. Historically, the chemical synthesis of small circular DNA molecules, e.g., 13 to 105 nt, was achieved in the 1990s, and both enzymatic ligation and chemical coupling in the presence of a "splint" oligonucleotide have proven useful. Similar strategies more recently have been applied to circular RNAs.[18] The most-used chemical methods for intramolecular 5'-hydroxyl and 3'-phosphate group cyclization suffer from competing side-reactions, like intermolecular coupling and formation of the unnatural 2', 5'-phosphodiester bonds.

The researchers selected to use the copper-catalyzed click chemistry reaction (Figure 5). Initially, a published procedure using a methanol:water solvent system and a microwave reactor adapted to obtain cyclization in 40 minutes at 60 °C, with some hydrolysis byproducts (<10%), and no intermolecular linearized or cross-polymerization products.

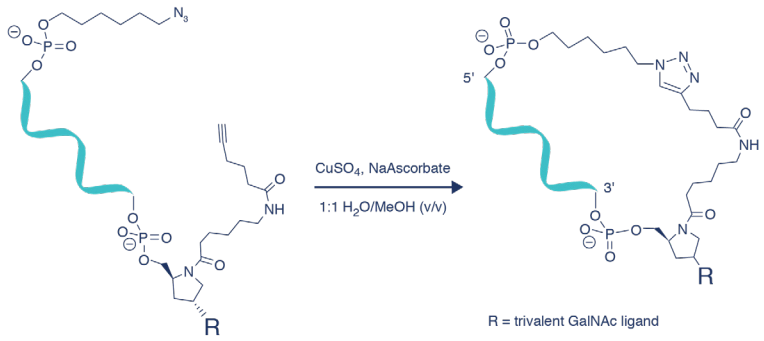



**Figure 5. "Click" Cyclization of a Linear Sense Strand**

A preferred alternative “click” cyclization method was optimized at room temperature to avoid the use of a microwave reactor. In a typical experiment oligonucleotide (9 mg, 0.9 mL) was suspended in methanol:water (1.8 ml:0.4 ml) under argon. A 0.5 ml mixture of 1:1 (v/v) 0.1 mM sodium L-ascorbate and 20 mM copper sulfate is added to initiate the reaction. The representative time course of this reaction is shown in Figure 6 using anion exchange HPLC analysis immediately after mixing (0 hr), after 4 hours (4 hr), and after 17 hours (17 hr). The maximal cyclic product (92.5%, with a retention time of just over 9 minutes) observed at 4 hr had declined to 79% at 17 hr, demonstrating that high yield/purity product can be obtained within several hours using a simple, scalable process. The decline in yield at long incubations was attributed to slow copper-mediated phosphodiester backbone degradation that is minimized by product purification immediately following cyclization, typically resulting in a very satisfactory 30% isolated yield.

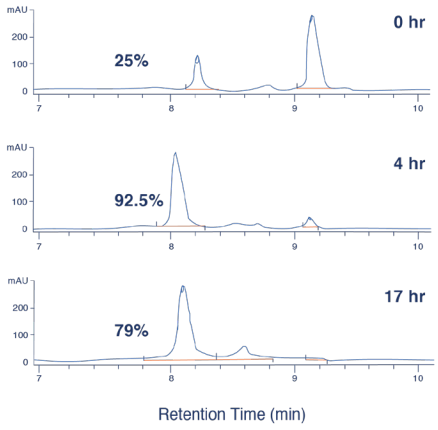



**Figure 6. HPLC of Cyclization Progress**

## Characterizing Cyclic sciRNA

The final sciRNA designs were prepared by annealing cyclic RNAs with their corresponding antisense strand. Duplex stability of the sciRNA was compared with corresponding linear siRNAs (Table 1). The melting temperatures were reduced by 20.5 °C and 15.4 °C for the cyclic forms of the C5 and Ttr targeted gene constructs compared with their corresponding linear counterparts (si-7 vs si-6; si 5 vs si-2; si-4 vs si-1), respectively.

Proton NMR ($^1$H NMR) of the cyclic sciRNA duplexes (not shown) revealed fewer and broadened $^1$H-NMR signals in the 11.2 to 14.4 ppm region where hydrogen-bonded imino proton resonances are found. The researchers suggested that strain of the cyclic sense strand in si-4 and si-5 duplexes may cause disrupted base-pairing of 21 nt circular RNA with its complement and that only a subset of the nucleobases forms Watson–Crick base pairs.

| siRNA | Target | Sequences (5'-3')[a] | $T_m$ (°C) |
|---|---|---|---|
| **si-1** | *Ttr* | *A* • a • *Ca*G*u*G*u*U*C*U*u*G*c*U*c*U*a*U*a*A**L**<br>u • *U* • a*U*a*G*a*G*c*A*aga*A*c*A*c*U*g*U*u • u • u | 68.2 |
| **si-2** | *Ttr* | *A* • a • *Ca*G*u*G*u*U*C*U*u*G*c*U*c*U*a*U*a*A**L**<br>**VP**u • *U* • a*U*a*G*a*G*c*A*aga*A*c*A*c*U*g*U*u • u • u | 67.8 |
| **si-3 (linear sense)** | *Ttr* | **Q***A* • a • *Ca*G*u*G*u*U*C*U*u*G*c*U*c*U*a*U*a*A***YL**<br>u • *U* • a*U*a*G*a*G*c*A*aga*A*c*A*c*U*g*U*u • u • u | 67.3 |
| **si-4 (cyclic sense)** | *Ttr* | **Z***A* • a • *Ca*G*u*G*u*U*C*U*u*G*c*U*c*U*a*U*a*A**L**<br>u • *U* • a*U*a*G*a*G*c*A*aga*A*c*A*c*U*g*U*u • u • u | 52.4 |
| **si-5 (cyclic sense)** | *Ttr* | **Z***A* • a • *Ca*G*u*G*u*U*C*U*u*G*c*U*c*U*a*U*a*A**L**<br>**VP**u • *U* • a*U*a*G*a*G*c*A*aga*A*c*A*c*U*g*U*u • u • u | 52.5 |
| **si-6** | *C5* | *G* • a • *Ca*A*a*A*u*A*A*C*u*C*a*C*u*A*u*A*a*U*L*<br>a • *U* • u*A*u*A*g*U*g*A*guu*A*u*U*u*U*g*U*c • a • a | 62.3 |
| **si-7 (cyclic sense)** | *C5* | **Z***G* • a • *Ca*A*a*A*u*A*A*C*u*C*a*C*u*A*u*A*a*U*L*<br>a • *U* • u*A*u*A*g*U*g*A*guu*A*u*U*u*U*g*U*c • a • a | 41.8 |

[a]Sense strand – top rows; antisense strand – bottom rows
•, PS linkage; lower case nucleotides, 2'-OMe; upper case nucleotides in italics, 2'-F;
**Q**, 6-azidohexyl-phosphate; **VP**, 5'-(*E*)-vinylphosphonate; **L**, triantenarry GalNAc; **Y**, hydroxyproline alkyne and **Z**, click cyclized form

**Table 1. sciRNA and siRNA Designs**

## In Vitro Stability of sciRNA

The in vitro stability of sciRNAs si-4 and si-5 were evaluated in rat plasma and rat liver homogenate using LCMS. In plasma, no degradation of either the circular sciRNA strands or the linear siRNA strands was detected for up to 8 hours. However, after 24 hours a ring opening product formed (addition of water) and several additional components were observed indicating base excision from both strands (data not shown).

In rat liver homogenate after 24 hours there was no degradation of cyclic si-4 or si-5, while at this point only ~55% of the linear full-length sense strand si-3 was detected (Figure 7). For additional detail, review the total ion chromatograms and metabolite identification tables in the Supplementary Data provided in the Jahns publication.

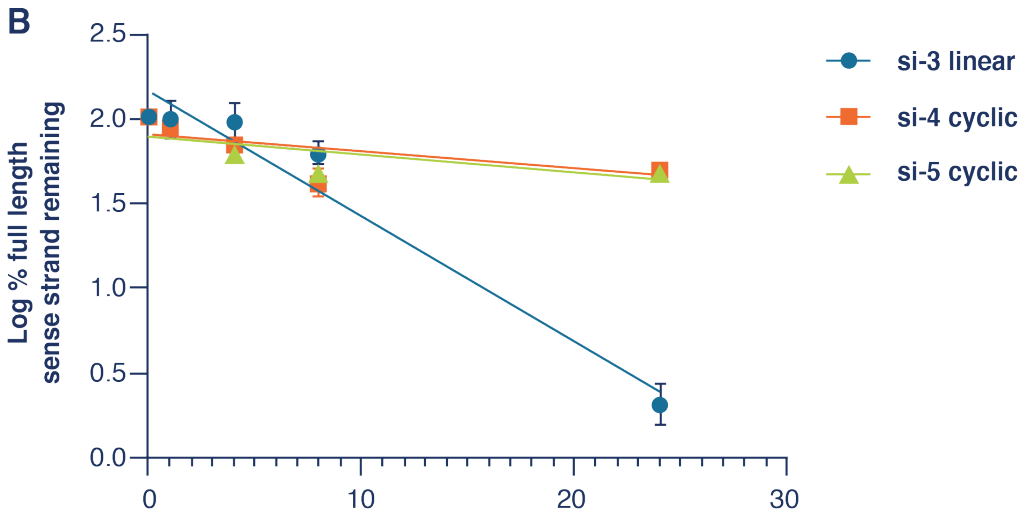



Figure 7. Stability of sciRNA in Liver Homogenate

# Gene Silencing by GalNac-sciRNA

In vitro gene silencing studies were carried out via cell transfection and in vivo mouse studies using the new sciRNAs targeting Ttr and C5 genes.

## Cell Transfection

Cell transfection was evaluated in two ways 1) using Lipofectamine RNAiMAX (Invitrogen) and 2) via free uptake using primary mouse hepatocytes. mRNA levels were quantified after 24 hours using qPCR for Ttr, C5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping control (see the Jahns publication for details). While the transfections were successful, the potency (based on $IC_{50}$ values) of Ttr-targeted si-5 sciRNA was 3-fold less than its linear control and the C5-targeted cyclic sciRNA si-7 was 14-fold less than its control.

## Mouse Studies

In vivo, sciRNA activity was evaluated by 3mg/kg subcutaneous injection on Day 0, and the circulating serum TTR or C5 protein was determined over time using a prealbumin-specific ELISA and a C5-specific ELISA, respectively. Results were compared with pre-bleed values (Figure 8).

The Ttr sciRNAs si-4 and si-5 were long-lasting while considerably less potent than the linear si-1 and si-2 (Figure 8A). Note, that the presence of VP modification on si-5 significantly improved the silencing compared with si-4. In contrast, the C5 targeting duplex RNAs—the siRNAs with linear (si-6) and circular (si-7) sense strands—had similar silencing potency (Figure 8B). These results show that sciRNAs are effective at pharmacologically and therapeutically relevant doses.

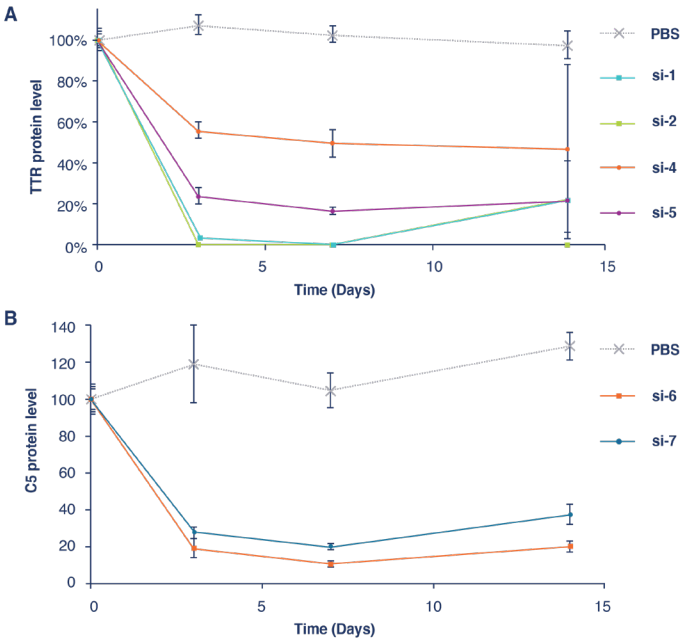



**Figure 8. Gene silencing by sciRNAs and siRNAs**

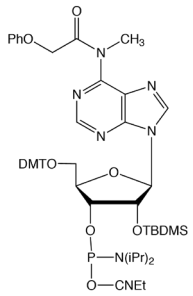
# Summary

These researchers showed how to synthesize a new class of GalNAc-conjugated sciRNAs composed of a cyclic sense strand annealed to a complementary linear antisense strand. These small circular interfering RNA duplexes were shown to have good potency in vivo, equivalent to or only slightly less than corresponding linear siRNA controls. Melting temperature and $^{1}$H-NMR data suggest that Watson-Crick pairing in these small circular structures is not ideal and suggests further optimization of the structure is possible.[19] The authors suggested that additional attention to engineering 3-dimensional RNA structure into sciRNA designs could benefit metabolic stabilization, reduce off-target binding, and possibly affect tissue distribution and uptake.

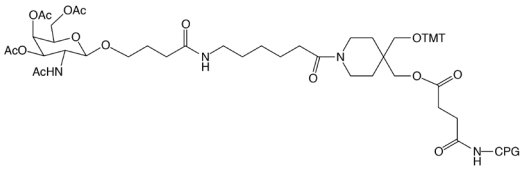
Glen Research provides phosphoramidites for the synthesis of fully modified sciRNA duplexes with 2'-deoxy, 2'-fluoro (2'-F) and 2'-O-methyl (2'-OMe) sugar modification, and reagents for backbone modification with phosphorothioate (PS). We also offer N6-methyl-A-CE phosphoramidite to prepare 6-methyl adenosine (m6A) modified RNA, found to be important to avoid activation of innate immune system pattern receptors. A triantennary 3'-GalNAc ligand cluster may be mimicked using GalNAc C3 CPG or using a universal support and corresponding sequential GalNAc C3 phosphoramidite couplings.[20] Alkyne-Modifier Serinol Phosphoramidite may then be attached with or without a spacer to provide the 3'-alkyne click partner. Then, following placement of the RNA oligo sense sequence, our 5'-Bromohexyl Phosphoramidite is attached and subsequently modified to 5'-azidohexyl as described above, thus placing both click chemistry partners on the oligo. 3-Propargyloxypropanoic acid N-hydroxysuccinimidyl ester (Alkyne-NHS Ester) is also available for placement of the alkyne group on an amino-modification on your oligo. Details for synthesis and deprotection using these items may be found on our website.

## Highlighted Glen Products

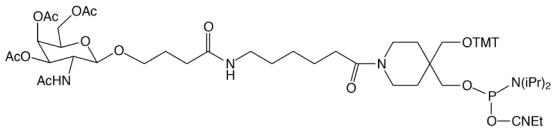
**N6-Methyl-A-CE Phosphoramidite (10-3005)**



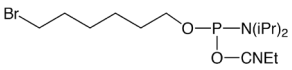

**GalNAc C3 CPG (20-2974)**



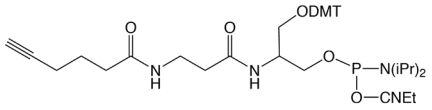

**5'-GalNAc C3 Phosphoramidite (10-1974)**



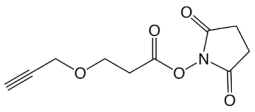

**5'-Bromohexyl Phosphoramidite (10-1946)**




**Alkyne-Modifier Serinol Phosphoramidite (10-1992)**




**Alkyne-NHS Ester (50-1905)**



## Table 1. Ordering Information

| Item | Pack Size | Catalog No. |
|---|---|---|
| N6-Methyl-A-CE Phosphoramidite | 0.25g | 10-3005-02 |
| | 100µmol | 10-3005-90 |
| | 50µmol | 10-3005-95 |
| GalNAc C3 CPG | 0.1g | 20-2974-01 |
| | 1.0g | 20-2974-10 |
| | 1x10µmol | 20-2974-13 |
| | 1x15µmol | 20-2974-14 |
| | 4x1.0µmol | 20-2974-41 |
| | 4x0.2µmol | 20-2974-42 |
| 5'-GalNAc C3 Phosphoramidite | 0.25g | 10-1974-02 |
| | 100µmol | 10-1974-90 |
| | 50µmol | 10-1974-95 |
| 5'-Bromohexyl Phosphoramidite | 0.25g | 10-1946-02 |
| | 100µmol | 10-1946-90 |
| Alkyne-Modifier Serinol Phosphoramidite | 0.25g | 10-1992-02 |
| | 100µmol | 10-1992-90 |
| | 50µmol | 10-1992-95 |
| Alkyne-NHS Ester | 2.3mg | 50-1905-23 |
| | 23mg | 50-1905-24 |

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