Bioconjugate Chemistry

pubs.acs.org/bc

Review

Ongoing Clinical Trials of Nonviral siRNA Therapeutics

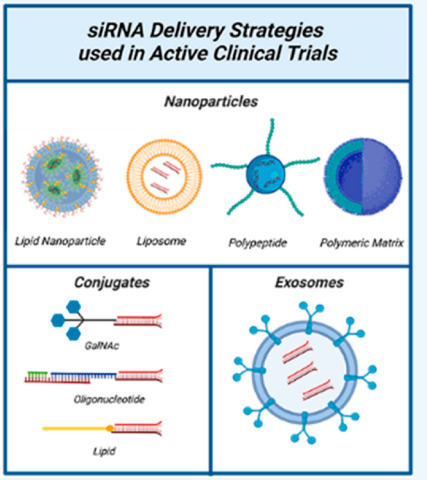
Eshan A. Narasipura, Rachel VanKeulen-Miller, Yutian Ma, and Owen S. Fenton*

Cite This: *Bioconjugate Chem.* 2023, 34, 1177–1197

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Short interfering RNAs (siRNA) are a powerful class of genetic medicines whose clinical translation can be hindered by their suboptimal delivery properties *in vivo*. Here, we provide a clinically focused overview that summarizes ongoing siRNA clinical trials from the perspective of innovations in nonviral delivery strategies. More specifically, our review begins by highlighting the delivery barriers and physiochemical properties of siRNA that make it challenging to deliver it *in vivo*. We then provide commentary on specific delivery strategies, including sequence modification, siRNA ligand conjugation, and nanoparticle and exosomal packaging, each of which can be used to control the delivery of siRNA therapies in living systems. Last, we provide a summary table of ongoing siRNA clinical trials which also highlights the indication of use, target, and National Clinical Trial (NCT) number associated with each entry. In writing this review, our work aims to highlight the key challenges and strategies for effective nonviral siRNA delivery *in vivo*, while simultaneously summarizing information on ongoing clinical trials for siRNA therapy in humans.



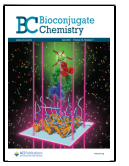
INTRODUCTION

The underlying cause of many diseases is the overexpression of native and aberrant proteins. Small molecules and biologics can treat some diseases via competitive inhibition and targeted degradation. However, estimates suspect that 75% of the human genome is "undruggable".[1] Challenges with some small molecules and biologics also include low solubility, low permeability, limited targeting capability, and limited shelf life, among other limitations. This can impede cellular uptake, present complex formulation considerations, and elicit off-target effects. Additionally, the paradigm of targeting active sites to block protein function cannot be generalized to every disease-causing protein as not all active sites of proteins can be readily accessed by active pharmaceutical ingredients. Notable examples of "undruggable" targets include MYC, STAT3, and RAS, key oncogenes attributed to several forms of cancer.[2−4] This knowledge of "undruggable" targets has highlighted the need for new approaches for treating diseases.

Gene therapies, including RNA-based therapeutics, exploit the body's own code to modulate protein function. Accordingly, RNA has an array of therapeutic applications. For example, messenger RNA (mRNA) therapy can increase the expression of a target protein, while microRNA therapy can decrease the expression of various proteins. While each of these are powerful tools, this review focuses on short interfering RNA (siRNA). siRNA is a double-stranded form of RNA and has proven to be a viable therapeutic option for treating various diseases. In essence, siRNA silences genes through an RNA interference mechanism. This occurs by targeted degradation of complementary mRNA, thus preventing translation by virtue of eliminating the "instructions" for the synthesis of the protein. Degradation of mRNA via siRNA can thus mitigate the progression of a host of genetic diseases, particularly those caused by protein overexpression. For example, five FDA-approved siRNA therapies (Patisiran, Lamsiran, Inclisiran, Givosiran, Vutrisiran), and several in-progress clinical trials indicate the exciting potential of siRNA therapy in the treatment of diseases.[5] This is promising, but there are still many challenges to consider in optimizing the delivery, uptake, and efficiency of siRNA therapy using strategies such as sequence modification, direct ligation, and carrier methods, among other strategies.

This review provides an overview of siRNA medicines, particularly from the lens of clinically approved and active clinical-trial therapies. We first provide a background of the RNA interference mechanism and the key proteins involved in mRNA degradation. We then investigate the structural properties and delivery challenges associated with siRNA. We review siRNA sequence modifications, conjugation, and

Received: May 7, 2023
Revised: June 21, 2023
Published: July 11, 2023



https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177–1197

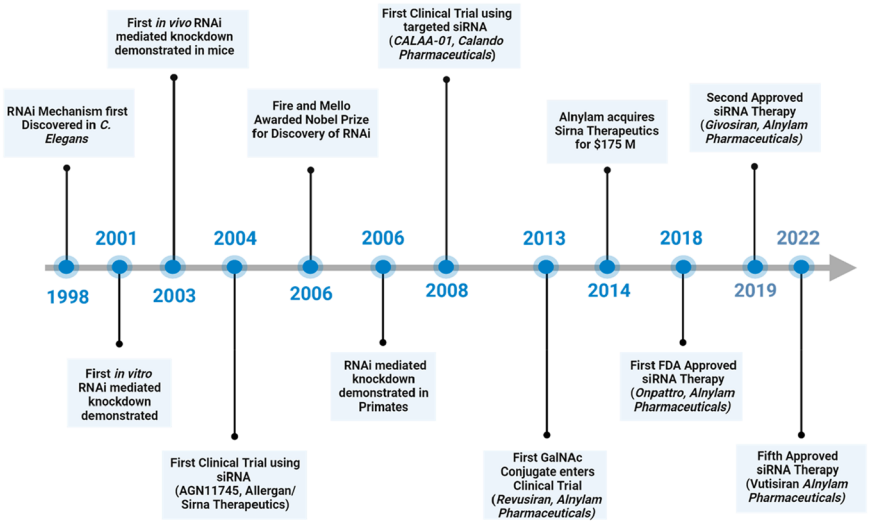


Figure 1. Timeline of the select siRNA breakthroughs.

packaging strategies actively employed in siRNA clinical trials. Finally, we provide a tabularized summary of all of the active clinical trials using siRNA and provide an outlook of siRNA as a future treatment. In writing this review, our goal is to establish the fundamental groundwork regarding delivery challenges within the siRNA field while more broadly highlighting the potential of siRNA therapies for treating human disease.

1. SIRNA MECHANISM

siRNA is an endogenous macromolecule that is responsible for various cell-regulating functions. More specifically, this type of RNA functions under the mechanism known as RNA interference (RNAi), a pathway that was discovered when studying gene expression defense mechanisms against nucleic acids (Figure 1).[6] Since its discovery, the siRNA mechanism has been extensively studied to develop therapeutic siRNA to knock down specific genes that are responsible for disease. The RNA interference mechanism is a unique inhibiting mechanism because siRNA leverages endogenous processes to degrade mRNA before translation, thereby silencing genes at the pretranslational level (Figure 2).

The structure of therapeutic siRNA is essential to the efficacy of a gene knockdown and an overall understanding of the RNA interference mechanism. Two unique structures of RNA are used for the RNAi mechanism: double-stranded RNA (dsRNA) and small interfering RNA (siRNA). dsRNA contains more nucleotides and is a precursor to siRNA. Both types can be used for therapy; however, siRNA is more commonly used in clinical trial therapies as the shorter nucleotide sequence of siRNA avoids a potential shutdown of cellular protein expression caused by the interaction of intracellular RNA receptors and RNA molecules greater than 30 nucleotides long.[7] Additionally, shorter sequences of RNA lower the likelihood of an innate interferon immune response.[8] Endogenously, dsRNA is made in the nucleus and translocated into the cytosol. It is then cleaved by the ribonuclease III Dicer into a 21−23 nucleotide siRNA. This step, often called the "initiation step", functionalizes the siRNA and is essential for effective gene knockdown.[9] The dsRNA is cleaved to create a 2-nucleotide overhang on the 3′ end of the RNA.[10] After this step, the RNA-induced silencing complex (RISC) is recruited onto the RNA. The RISC is a multiprotein complex and contains the proteins Argonaute RISC Catalytic Component 2 (ago2), Staphylococcal Nuclease Domain-Containing Protein 1 (SND1), Astrocyte Elevated Gene-1 (AEG-1), Fragile X Mental Retardation 1 (FMRR1), VIG (vasa intronic gene), R2D2 (dsRNA binding protein), Aubergine, and Armitage-RNA helicase.[11] Once bound to the siRNA, this complex opens up the double-stranded RNA and degrades the passenger strand not involved with binding to the mRNA. The remaining single strand complementary to the target mRNA (referred to as the guide strand) is then matched with the mRNA of interest.[12] Therapeutically, this mechanism is leveraged to transiently silence disease-causing proteins; synthetic siRNA also operates via this mechanism but does not need to first be cleaved by Dicer.[8] Clinical trials using synthetic siRNA are typically more common, an observation that may be in part due to powerful advancements in chemical synthesis routes toward siRNA therapeutics.[13] For example, siRNA therapies may often be produced using solid phase synthesis approaches, a strategy that enables simple, high-yielding, and potentially automated routes to siRNA drugs.[14,15] This synthesis approach can therefore lead to the rapid development and therapeutic evaluation of multiple siRNAs against a given target which can improve the odds of advancing a given siRNA therapy through clinical trials.[16]

2. DELIVERY BARRIERS

Although siRNA can selectively target genes, controlling the dose, location, and uptake of siRNA *in vivo* is a therapeutic challenge. Controlling for a specific dose can be difficult as endonucleases can degrade free siRNA and enable rapid renal clearance.[17] As a result, siRNA delivered without a platform has low circulation time with a half-life ranging from 15 min to 1 h.[18] Targeting location and promoting effective cellular uptake can also be challenging due to siRNA's sizable molecular weight and negative charge. The large molecular weight (~13 000 Da) hampers its ability to passively diffuse through the cell membrane, and the negative charge of siRNA's phosphate backbone makes cellular uptake inefficient.

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

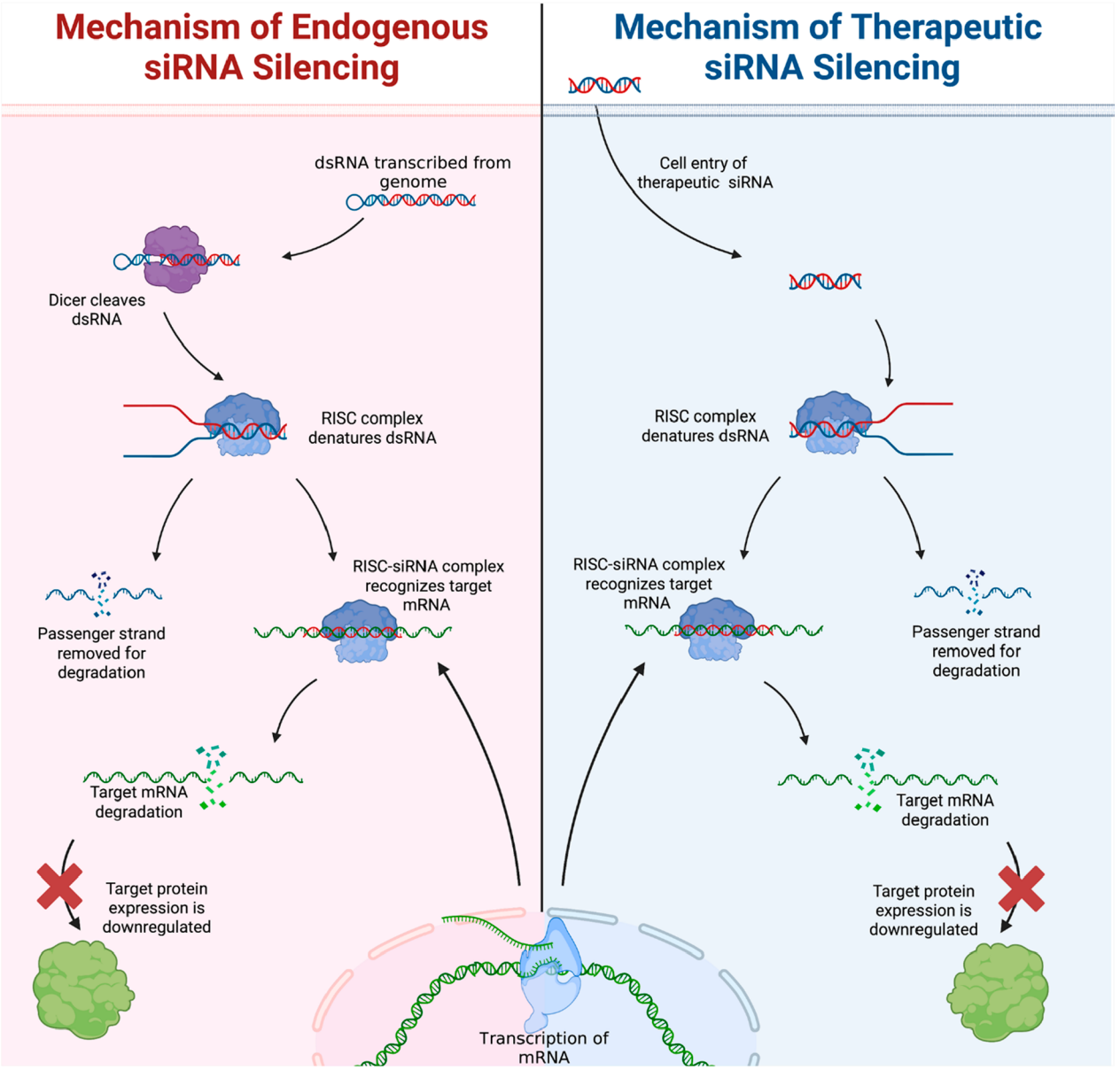


Figure 2. Mechanism of siRNA-mediated gene silencing.

Aside from degradation and cellular uptake concerns, the delivery of naked siRNA has been shown to activate the innate immune system.[19] Innate immunity is the branch of the immune system that functions as the first defense barrier against pathogens. In contrast to the adaptive immune system, cells involved in the innate immune system are not pathogen-specific and, therefore, do not need to be presented with an antigen to be activated. Instead, the innate immune system is a collection of different cells that can recognize conserved aspects of a pathogen and be activated to eliminate the pathogen quickly.[20] One key player in the innate immune system is Toll Like Receptors (TLRs). TLRs are receptor proteins found on many mammalian cells responsible for recognizing foreign agents and are then activated to produce downstream effects to eliminate the foreign agent.[21] TLRs have been shown to be readily activated by synthetic siRNA administered both *in vitro* and *in vivo*. Out of the 12 TLRs that have been identified in humans, TLR1, 2, 3, 7, and 8 have been shown to be activated by siRNA. TLR 7 and 8 are activated by siRNA in a sequence dependent manner, whereas TLR 3 recognize in a sequence independent manner.[22] To avoid the sequence-dependent recognition of TLR 7 and 8, synthetic siRNA typically avoids using uridine/guanosine and adenosine/uridine-rich regions as these two TLRs are activated by such motifs.[23]

Overcoming these delivery challenges is a central goal of the siRNA therapeutic research field. Below, we provide an overview of clinically relevant delivery strategies that aim to overcome these limitations.

3. DELIVERY STRATEGIES FOR SIRNA THERAPY CURRENTLY USED CLINICAL TRIALS

As described above, there are barriers within the body that make controlling the dose, location, and uptake of siRNA difficult. Strategies have been employed to enable better and more efficient delivery of siRNA. Below, we overview sequence

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

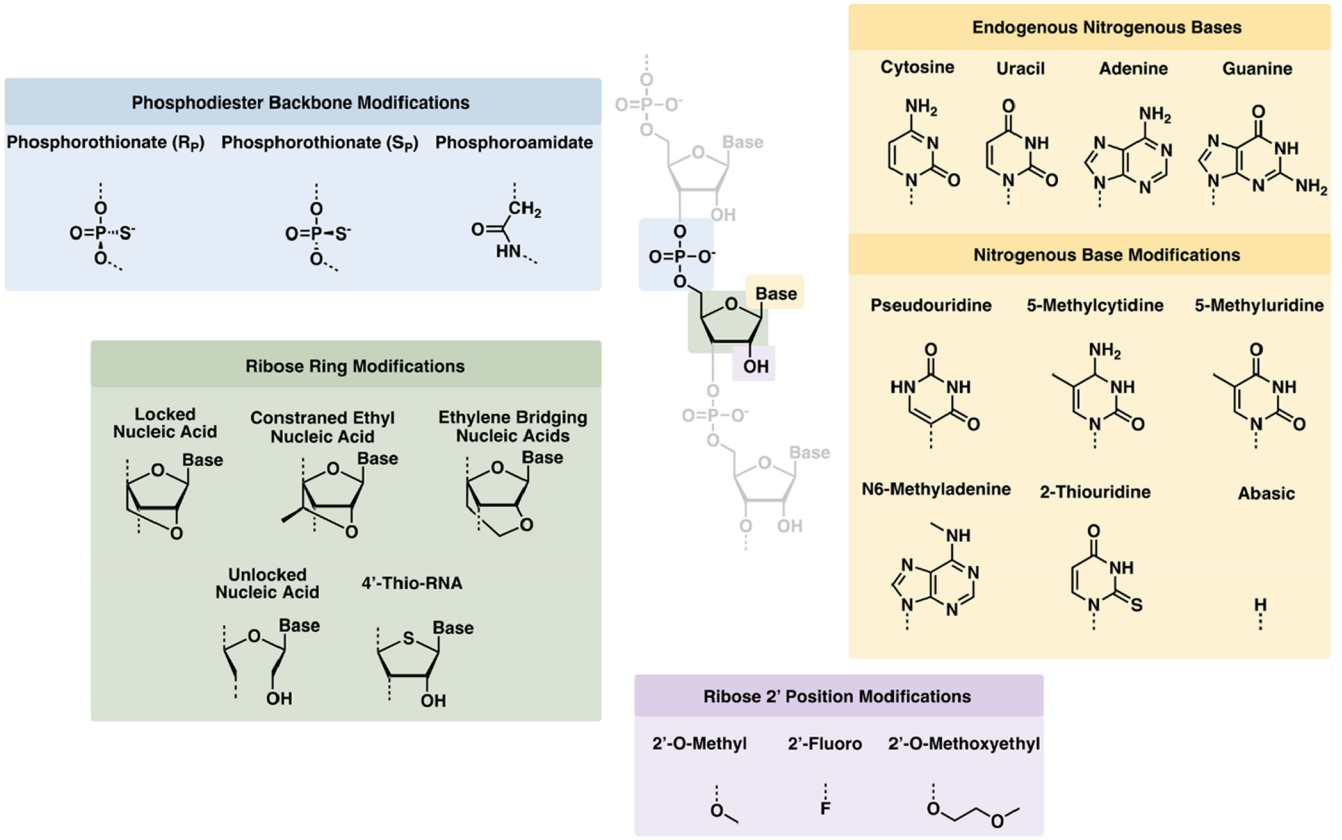


Figure 3. Common ribose, phosphate backbone, and nucleobase modifications used in synthetic siRNA.

modification and delivery platform strategies for clinically relevant and clinically approved nonviral siRNA therapies.

3a. Sequence Modifications. An effective siRNA therapy must be effectively delivered to the target cell of interest, enter the cytoplasm, engage the RISC complex, and target a specific mRNA. As a result, siRNA must have sufficient stability *in vitro* and *in vivo*, affinity for the RISC complex, and low immunogenicity. To optimize these characteristics, the chemical composition of the siRNA sequence can be manipulated by using synthetic chemistry methodologies. Three categories of RNA modifications used in clinically relevant and clinically approved therapies include ribose sugar modifications, phosphodiester backbone modifications, and nitrogen base modifications. The specific structural modifications, the ratio of modified to endogenous nucleotides, and the targeted area within the siRNA can all be modulated—this causes virtually limitless variations of possible siRNA therapeutics, each of which may have diverse immunogenicity, stability, and target mRNA affinity. Currently approved siRNA therapeutic drugs have included modifications of each of these classes—due to the vast number of sequence permutations in siRNA therapies, this review will cover representative modifications from each group (Figure 3).

3a.i. Ribose Sugar Ring Modifications. The building blocks of nucleic acids are five-membered ribose sugars that act as scaffolds for the genetic code. Modulating the chemical structures of these ribose sugars has led to increased stability and efficacy of siRNA therapies. One of these modifications involves forming a bridge between the 2′-hydroxyl oxygen and the 4′-carbon, forcing the nucleotide into a constrained conformation. Locked nucleic acids, also called bridged nucleic acids, have been introduced as a ribose modification that increases stability.[24,25] Constrained ethyl nucleic acids and ethylene bridging nucleic acids similarly showed increased resistance to nucleases.[26−28] Unlocked nucleic acids have also been synthesized, allowing for modulation of stability and specificity toward target mRNA.[29] Alternatively, utilizing a sulfur atom in place of the endogenous oxygen within the ring has been well tolerated with increased potency and stability.[30,31]

3a.ii. Ribose Sugar 2′ Hydroxyl Modifications. Modification at the 2′ position of the ribose sugar can be used to prevent ribonuclease attack. In its native structure, the 2′ OH group of ribose is a handle for enzymatic hydrolysis. To prevent hydrolysis, a 2′ O-methyl group or other moiety such as 2′ fluorine can be added to prevent this hydrolysis from occurring.[32] Ribose sugar modifications in currently approved siRNA therapeutics include 2′ O-methyl (2′-OMe) and 2′ deoxy-fluoro (2′-F) (Onpattro, Givosiran, Inclisiran, Lumasiran [Alnylam]). It has also been shown that these ribose modifications help to prevent TLR recognition, thus reducing innate immune response.[33]

3a.iii. Phosphodiester Backbone Modifications. In tandem with the ribose sugar, phosphodiester bonds comprise the backbone of the nucleic acids. These phosphate derivatives connect ribose monomers to longer polymers that house genetic information. Phosphate groups on the siRNA, similarly to the ribose sugars, have been targeted with chemical modifications to prevent nuclease degradation and increase the affinity between the RISC and siRNA.[34] Generally, in these modifications, one of the nonbridging oxygens of the ribose sugars of siRNA is replaced with a different group. Changes in

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

the hydrophilicity of the phosphate group have been shown to enhance hydrophobic protein-binding interactions and prevent endonuclease degradation.[35] Phosphodithionate is a common phosphate modification in siRNA. Here, a sulfur atom replaces one of the nonbridging oxygens which can enhance resistance to phosphatases.[36] Amide bonds can also substitute the phosphodiester linker and exhibit increased resistance to nucleases with similar or increased affinity for the target mRNA.[37,38] Similarly, incorporating boranophosphates into the phosphodiester bonds near the ends of the antisense strand of the siRNA have led to a nearly 10-fold resistance to nucleases and an increase in potency in some cases.[39] The native 5′ to 3′ phosphodiester linkage can be replaced with a 5′ to 2′ phosphodiester bond to yield a decreased immune response without negatively affecting target mRNA affinity.[40] Of the five currently approved siRNA therapeutics, three (Givosiran, Inclisiran, Lumasiran) incorporate phosphodiester modifications (Alnylam Pharmaceuticals).

3a.iv. Nitrogenous Base Modifications. Another component of nucleotides is nitrogenous bases; for endogenous RNA, these are cytosine, guanine, adenine, and uracil. Patterns of these nucleotides contain the genetic information that governs cellular function. The overall benefits and drawbacks of replacing nucleotides with analogs must be evaluated with considerations about how the metabolites of modified nucleotides could potentially be incorporated into the genome. Nonetheless, it has been shown that the replacement of uridine with pseudouridine, 2-thiouridine, 5-methylcytidine, 5-methyluridine, and 6-methyladenine in RNA has been shown to reduce innate immune activation.[41−44] Additionally, the incorporation of an abasic siRNA—the altogether removal of the nucleobase—has been shown to minimize off-target mRNA silencing while maintaining on-target activity.[45,46] Overall, these chemical modifications have exhibited a decrease in the activation of the innate immune system, an improvement of overall structural stability, and a decrease in off-target effects.[47]

3b. Delivery Platforms for siRNA Therapy. Sequence modifications of siRNA are not always sufficient for effective therapy, as naked siRNAs are still prone to degradation and short circulation times. To combat this, platform-based approaches for siRNA delivery have been developed. Generally, platforms provide diverse solutions that aim to stabilize siRNA, promote cellular uptake, escape the endosome, and promote activity within cells. Among active siRNA clinical trials, three delivery platforms that are actively being explored include conjugates (GalNAc, lipid-based, oligonucleotide), nanoparticles (lipid-based, polypeptide, or polymer-based), and exosomes. Here, we describe these delivery platform approaches and specific examples of their use in clinically relevant and active trials.

3b.i. siRNA Conjugates. One way siRNA therapies can be improved is by directly conjugating ligands onto the sequence by using chemistry approaches. This structural modification has been shown to increase the circulation time by preventing the degradation of siRNA and improving site specificity. Several types of conjugates have been explored in the literature; however, three currently being evaluated in various stages of clinical trials include *N*-acetylgalactosamine (GalNAc) conjugates, lipid-based conjugates, and CpG oligodeoxynucleotide conjugates (Figure 4).

GalNAc conjugates are one type of conjugate that have experienced success in the clinic. Alnylam Pharmaceuticals has

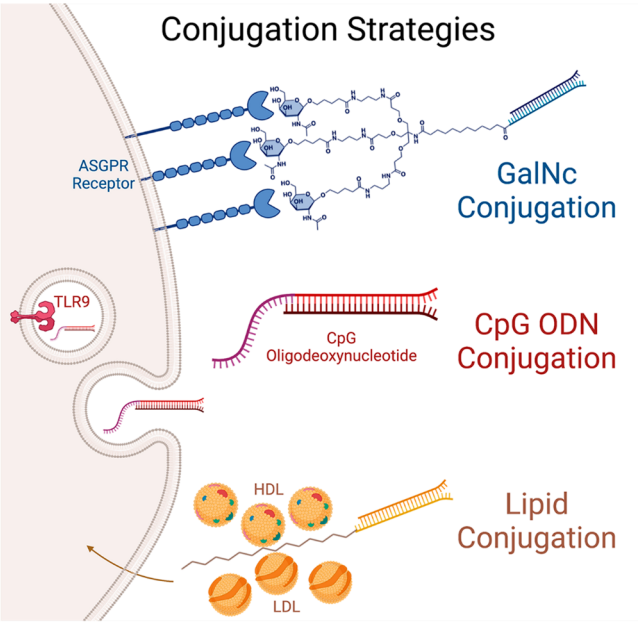


Figure 4. siRNA conjugate materials currently being evaluated in active clinical trials.

developed 4 FDA approved GalNAc-siRNA drugs for the treatment of acute hepatic porphyria (Givosiran), primary hyperoxaluria type 1 (Lumasiran), amyloidosis (Vutisiran), and hypercholesterolemia (Inclisiran). Sixteen active clinical trials using GalNAc for siRNA delivery are also being evaluated at various stages. GalNAc is a sugar amino derivative of galactose and has a high binding affinity to the asialoglycoprotein receptor (ASGPR), a cell membrane receptor highly expressed on liver hepatocytes.[48] siRNA conjugates of GalNAc typically have three *N*-acetylgalactosamine ligands resulting in a trivalent ligand covalently bound to the 3′ end of siRNA's sense strand. Several studies have leveraged the ASGPR receptor GalNAc ligand pair to target siRNA-mediated gene knockdown in hepatocytes.[49,50] Once bound to ASGPR, GalNAc-siRNA conjugates are internalized through clathrin-mediated endocytosis.[51] The acidic pH in the endosome then cleaves the linkage between GalNAc and siRNA, thereby releasing the siRNA. The specific endosomal escape mechanism of siRNA is complex; however, it is known that recycling endosomes return ASGPR onto the cell surface once the siRNA is released.[52,53] The recycling mechanism enables prolonged uptake of GalNAc-siRNA conjugates. The endocytosis pathway of GalNAc has been well studied, and key genes have been identified that regulate cellular uptake. Lu and colleagues found that the gene RAB18 is an important regulator in the uptake mechanism of GalNAc-siRNA conjugates. CRISPR-Cas-9 knockout of RAB18 of Hep3B cell lines further showed a 20-fold increase in gene silencing of GalNAc-HPRT1siRNA conjugates but not in Lipofectamine transfection of siRNA, indicating the uptake mechanism of ASGPR is regulated by additional genes that may be further leveraged to improve siRNA uptake and efficacy.[54]

Several adjustments on the linker chemistry, placement, and binding affinity of GalNAc-siRNA conjugates have been explored to optimize uptake by liver hepatocytes. Schmidt et al. showed that the valency of GalNAc is important for binding to ASGPR and promoting uptake. It was found that a monovalent GalNAc conjugate had a 2-fold reduction in gene

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

knockdown activity *in vivo* as compared to a trivalent conjugate, suggesting that the number of GalNAc ligands may contribute to overall knockdown efficacy.[55] To study the effects of GalNAc placement on the siRNA, Matsuda and colleagues used copper-assisted azide−alkyne cycloaddition click chemistry to site-specifically conjugate monovalent GalNAc onto various moieties of the siRNA sense strands. It was found that dispersing three singular GalNAc ligands spaced by two or more nucleotides on the siRNA's sense strand resulted in less gene knockdown than clustering the GalNAc ligands. Researchers demonstrated that the proximity of the GalNAc ligands to each other was critical for GalNAc binding capability to ASGPR. It was also shown that the clustered conjugates had similar gene knockdown efficacy compared to typical triantennary GalNAc conjugates.[56] Although highly effective at silencing its target gene, it has been reported that GalNAc conjugates may have off-target silencing leading to hepatotoxicity.[57,58] Janas et al. studied potential mechanisms of RNAi-mediated hepatotoxicity of GalNAc conjugates and found that toxicity is primarily caused by the seed region of the antisense strand rather than the chemical modifications of siRNA or the disturbance of the endogenous RNAi pathway.[59] To mitigate such toxicities, researchers introduced a destabilizing glycol nucleic acid (GNA) nucleotide into the seed region, which resulted in an improved safety profile in rats.[59] To date, four of the five FDA-approved siRNA therapies are GalNAc conjugates—these successes are due to a number of beneficial properties of the GalNAc system including, but not limited to, its ability to actively target the liver via the asiaglycoprotein receptor, its modular nature which enables its direct ligation to siRNAs of virtually any therapeutic sequence, and its *in vivo* tolerability properties.[60] Of note, these properties have led to clinically approved siRNA therapies with low toxicity and high efficacy, two crucial end points for FDA approval. Further, these results have also highlighted not only the power of the GalNAc system for translational siRNA therapy but, more generally, the power of utilizing chemistry strategies to afford conjugate molecules that may translate siRNA therapies from the benchtop to the clinic.

Conjugation of lipids to siRNA has also been explored as a strategy to control biodistribution and improve overall cellular uptake of the siRNA. Several studies have shown that lipids of varying alkyl tail lengths enable siRNA delivery to extrahepatic tissues.[61,62] Brown and colleagues found that conjugating a C16 carbon chain on the 2′ sense end of siRNA achieved effective delivery and gene knockdown in mice to the central nervous system via intrathecal administration, the eye via intravitreal injection, and the lungs via intranasal injection.[63] There has been extensive effort to understand the mechanism in which these siRNA lipid conjugates are taken up to predict their organ distribution. Multiple studies have shown that the endogenous proteins high-density lipoprotein (HDL) and low-density lipoprotein (LDL) bind onto conjugates when administered *in vivo*.[64] To understand the role of HDL and LDL in cellular uptake of siRNA−lipid conjugates, Wolfrum et al. conjugated lipids with varying degrees of lipophilicity and found that higher hydrophobicities of conjugates increased association with LDL and HDL. The greater association with these lipoproteins correlated to higher intracellular uptake, suggesting that HDL and LDL are involved in the uptake of siRNA−lipid conjugates.[65] Osborn and colleagues further demonstrated that changing the lipophilicity of siRNA conjugates changes HDL and LDL association, affecting tissue distribution. It was found that lipophilic conjugates such as cholesterol associate with LDL and are preferentially taken up by LDL-enriched tissues such as the liver and lung, whereas less lipophilic conjugates such as DHA associate with HDL and are preferentially taken up by SR BI-enriched tissues such as the ovaries and adrenal gland. It was thus determined that lipophilicity can influence the binding of serum proteins which can determine tissue distribution profile.[66]

Within the literature, cholesterol-based siRNA conjugates have been explored as potential therapeutics for multiple disease states. Chernikov et al. used a cholesterol-siRNA conjugate to knockdown Multidrug Resistant Gene 1 in xenografted SCID mice.[67] However, biodistribution studies showed that fluorescently labeled cholesterol-siRNA accumulated 10% in the tumor site and 60−80% in the liver, indicating limited tumor targeting. Interestingly, up to 60% of MDR1 in the tumor was suppressed after a single IV injection in SCID mice.[67] Additionally, cholesterol siRNA conjugates have been used to silence Myostatin in skeletal muscle and showed prolonged (>21 days) knockdown (85−95%) of the Mstn gene in a single IV injection on female CD-1 mice.[68] Lipid conjugates have had limited clinical success, as no FDA-approved drugs exist. However, there is one Phase I Clinical Trial (NCT05231785) for treating Alzheimer's Disease and Cerebral Amyloid Angiopathy. Sponsored by Alnylam Pharmaceuticals and Regeneron, this siRNA targets the knockdown of the amyloid precursor protein (APP) and is conjugated to a C16 tail, which has been shown to deliver into the Central Nervous system.

An additional conjugate-based method that has shown promise in reaching the clinic uses CpG oligodeoxynucleotide conjugates (CpG ODNs). This oligonucleotide has been used as a synthetic immunoadjuvant, specifically as an agonist for the Toll-like Receptor 9.[69−71] Within the literature, TLR-9 stimulation using CpG ODN triggers Type I interferon secretion and upregulation of IL-6 and TNFα in both B cells and plasmacytoid dendritic cells.[72,73] TLR-based activation using CpG ODNs has been leveraged therapeutically by conjugating the oligonucleotide onto siRNA and using it as a TLR-targeting ligand. These conjugates have gained interest in cancer, as TLRs are often suppressed within the tumor microenvironment. A CpG ODN targeting ligand delivered to a tumor site can stimulate suppressed TLRs and recruit proinflammatory cytokines, promoting the activation of cytotoxic $CD8^+$ T cells.[74,75] Furthermore, siRNA conjugation to CpG ODN can silence specific genes involved in tumor proliferation. This two-pronged approach for cancer treatment has been explored in the literature and is currently being investigated in a Clinical Trial. Huang et al. developed a CpG-MLAA-34 siRNA conjugate to silence the gene MLAA34, a key gene found in acute monocytic leukemia involved in the progression of the JAK2/STAT3 pathway, a key pathway involved in tumor survival and signaling within the tumor microenvironment.[76] *In vivo* studies performed on AML-M5 nude mice demonstrated that the CpG-MLAA-34 siRNA conjugates silenced MLAA34 mRNA levels and had antitumor effects.[77] Several studies have used a CpG(A)-STAT-3 siRNA conjugate to treat acute myeloid leukemia and multiple myeloma. The siRNA in this case silences the transcription factor STAT-3.[78,79] Zhang and colleagues found that this CpG conjugate allowed for effective targeting of TLR9+ immune and blood cancer cells while also having significant antitumor

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

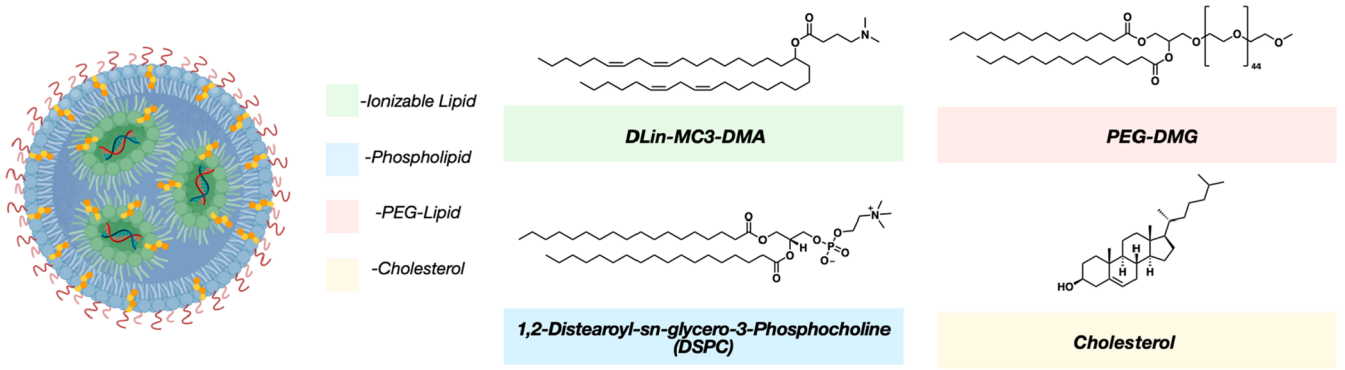


Figure 5. Lipid nanoparticle components of onpattro.

effects.[80] Houssain et al. also showed the ability of CpG(A)-STAT3 siRNA to effectively disrupt Myeloid-derived suppressor cell-mediated immunosuppression and restore T cell activity in prostate cancer.[81] The versatility of CpG(A)-STAT3 siRNA in treating multiple cancer types is an exciting prospect in cancer treatment. Currently, this conjugate is being evaluated in a phase I Clinical Trial by the City of Hope Medical Center to treat non-Hodgkin's Lymphoma (NCT:04995536).

3b.ii. Nanoparticles. Nanoparticles, in a general sense, are used to encapsulate cargo and are extensively used for drug delivery. Some key advantages of nanoparticle drug delivery are their large surface-to-mass ratio, modularity of the platform, and the ability to entrap drug cargo and deliver larger doses efficiently.[82,83] Within nanoparticles, three distinct types being used as a carrier method in active clinical trials for siRNA therapy include lipid nanoparticles, polymeric nanoparticles, and polypeptide nanoparticles.

3b.iii. Lipid Nanoparticles. Lipid nanoparticles have gained significant attention as a carrier for RNA. Used as the carrier material for the mRNA in the COVID-19 vaccine by Moderna and Pfizer/BioNTech, lipid nanoparticles are a highly effective method for encapsulating and delivering RNA.[84] Lipid nanoparticles comprise four components: an ionizable lipid (IL), phospholipid, cholesterol, and polyethylene glycol (PEG). Each component has unique characteristics that contribute to the stability and efficacy of RNA delivery.

The ionizable lipid is a long lipid chain linked to an ionizable headgroup.[85] In most ILs, the headgroup is a tertiary amine which can modulate the net charge of the IL at different pHs.[86] In acidic conditions, the lipid is positively charged as the amine is protonated. This positive charge enables the nanoparticle to condense around negatively charged RNA during formulation and stabilize the negatively charged RNA. At physiological pH, this lipid is at a neutral charge, limiting toxicity while circulating in the body. Once in the acidic environment of the endosome, the amine is once again protonated.[87] The positively charged IL can then interact with the phospholipids of the endosome, forming an ion pair. As previously described in the literature, this lipid ion pair can drive the formation of the inverted hexagonal H_{II} phase, which disrupts the lipid bilayer and promotes endosomal escape.[88,89] The fatty lipid tail of ionizable lipids also greatly affects the overall efficacy of a lipid nanoparticle. Several variations of the tail such as degree of tail saturation, branched tail lipids, and multitail lipids have been studied to investigate how structure variations affect overall performance.[7,90,91] Finally, the linkage between the headgroup and lipid tails has been explored, and degradable analogs have been developed to mitigate toxicity due to the accumulation of lipids after the repeat administration of LNPs.[92,93] The phospholipid of the lipid nanoparticle functions as a "helper lipid" and aids with endosomal escape as well as overall membrane structure and stability.[94] Additionally, phospholipids assist with stability due to their high phase transition temperature.[95] Cholesterol serves to fluidize the lipid membrane to a more liquid-ordered phase.[96,97] It has been shown that cholesterol can also aid in nanoparticle biodistribution by interacting with serum proteins such as apolipoprotein-E and facilitating the encapsulation of RNA.[98] Finally, polyethylene glycol (PEG) is used to improve circulation time and aid the overall stability of the nanoparticle. Specifically, PEG may reduce serum protein opsonization of the nanoparticle and prevent particle aggregation.[99] Taken together, these four components have been central to advancing lipid nanoparticles to the clinic.

Within the literature, several studies have employed lipid nanoparticles for nonviral siRNA therapy. Mitchell and colleagues covalently conjugated doxorubicin onto lipid nanoparticles encapsulating Bcl-2 siRNA and effectively knocked down the Bcl-2 gene while also exhibiting tumor killing in mice bearing lymphoma.[100] For treating KRAS mutant pancreatic cancer, it was found that tLyp-1-tagged anti-KRAS siRNA LNPs in combination with gemcitabine had enhanced pancreatic tumor accumulation compared with untagged LNPs with gemcitabine. This increase in accumulation led to an improvement in tumor reduction in mice bearing CFPAC1 KRAS mutant pancreatic cells compared to nontargeted LNPs.[101] These cotreatment approaches highlight the modularity of lipid nanoparticles and hold promise in new strategies in cancer treatment. As a platform for pulmonary delivery, Merkel et al. successfully spray-dried siRNA lipid nanoparticles that could pass through an *in vitro* mucus layer modeled after the mucus layer found in human lungs. These spray-dried particles also exhibited gene silencing on the gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) *ex vivo* in human lung tissues.[102] In treating nonalcoholic steatohepatitis, Yan and colleagues formulated a mannose-modified lipid nanoparticle encapsulating anti-HMGB1 siRNA to inhibit liver inflammation. The mannose-conjugated LNPs targeted liver macrophages via mannose receptors and knockdown the HMGB1 gene, which reduced inflammation and restored liver function in a NASH mouse model.[103] As shown by the variety of uses within the literature, siRNA lipid nanoparticles show

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

significant promise to be a clinically successful way to treat a range of diseases.

Evidence of a clinically successful siRNA LNP can be shown by the drug Patisiran. Approved by the FDA in 2018, Patisiran (Onpattro) is a siRNA therapy that uses lipid nanoparticles as a carrier to treat polyneuropathy and cardiomyopathy caused by hereditary transthyretin-mediated amyloidosis (hATTR amyloidosis).[104] The formulation of this LNP uses DLin-MC3-DMA as the ionizable lipid, distearoylphosphatidylcholine (DSPC) as the phospholipid, DMG-PEG-2000 as the PEG, and cholesterol (Figure 5).[105] DLin-MC3-DMA contains a lipid tail derived from linoleic acid, is linked to a tertiary amine via a degradable ester, and is one of three ionizable lipids approved for clinical use. In the actively recruiting clinical trials for siRNA therapy, two trials use lipid nanoparticles as the carrier for siRNA. The first trial (currently in phase I) is a siRNA silencing EphA2 receptor, a tyrosine kinase receptor highly overexpressed in several types of cancer.[106] The lipid nanoparticle consists of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC). The second trial using lipid nanoparticles as a carrier is currently in phase I and uses siRNA to target Glutathione S-Transferase P (GSTP) for treating nonsmall cell lung cancer (NCT03819387). GSTP is a detoxification enzyme that regulates the MAP kinase pathway.[107] This siRNA aims to inhibit GSTP, which has been hypothesized to disrupt the cellular function of KRAS mutant nonsmall cell lung cancer (NSCLC). The specific nanoparticle formulation for delivering siRNA has yet to be revealed. However, the ionizable lipid is said to be a "novel ionizable, non-immunogenic, biodegradable material".[108] Taken in tandem, each of these studies highlights the translational potential of LNPs as an enabling technology for clinically relevant nucleic acid therapies. Specifically, the modularity, tolerability, targeting properties, scalability, and the ability to produce LNPs using good manufacturing practices make LNPs an ideal platform for realizing the full clinical potential of siRNA-based drugs.[109,110] To date, these properties have best been exemplified in Alnylam Pharmaceutical's siRNA LNP therapeutic Onpattro which treats polyneuropathy in people with hereditary transthyretin-mediated amyloidosis.[111] Further, the broad clinical potential of LNP platforms has also been realized with the clinical approval of the Moderna and Pfizer/BioNTech COVID-19 vaccines.[112,113] Taken together, these seminal FDA-approved LNP therapies may pave the way toward the generation of additional nucleic acid therapies for the treatment of human disease, highlighting the generality and translational potential of LNP technologies for use in the clinic.

3b.iv. Polymeric Nanoparticles and Matrices. There are many applications for polymers in the field of drug delivery such as encapsulating drugs in nanoparticles or as implantable drug depots. Polymeric nanoparticles (PNPs) are one type of carrier that is commonly used to encapsulate siRNA.[114] These particles consist of a polymer that interacts with the negatively charged nucleic acid cargo.[115]

Generally speaking, polymers are large molecules consisting of many small repeating units. Physiologically, proteins, nucleic acids, and even some sugars are biological polymers with diverse structures and functions. Thus, unlike most lipid- and polypeptide-based nanoparticle components, polymers do not have discrete molecular weights. Consequently, when working with PNPs, it is important to consider the polydispersity index (PDI) of the polymers and the assembled nanoparticles' PDI, as this can significantly influence the structure and function of the nanoparticles. PNPs can be synthesized to be biocompatible, stable during storage, and biodegradable, making them ideal candidates for drug delivery.[116] An ideal PNP must provide extracellular stability, facilitate cellular uptake and cytosolic delivery, and eventually dissociate from and release the siRNA cargo.[117] The specificity of PNPs can be increased and modulated by covalent modification of the polymers to ligands specific to a target tissue.[118,119] However, PNPs can encounter toxicity issues and face difficulties in scaling up production.[120]

Many polymers have shown promise for *in vivo* delivery of siRNA. Nitrogen-based polymers are one such material and are ideal candidates for drug delivery because of their ability to be protonated. Protonated amines are positively charged and can complex with the negatively charged backbone of siRNA. Polymeric amine particles have shown success in siRNA delivery. Low molecular weight polyethylenimine (PEI) has efficiently delivered siRNA to endothelial cells *in vivo* without significantly affecting gene expression in immune cells or hepatocytes.[121] PEI can also be used as a polymer for nanoparticle drug delivery. PEI has been coupled with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, polylactic acid, and PEG to increase efficacy.[121−124] Fluorinated PEI cation has been identified as a polymeric nanoparticle that targets the liver and displays lower off-target toxicity.[125] Consideration must be given to the toxicity associated with PNPs with low biodegradability of polymers.

Poly-β-amino-esters (PBAEs) are another class of nitrogen-containing polymers that often exhibit low cytotoxicity due to their incorporation of a biodegradable ester bond.[126] Structurally, PBAEs can vary in different ways. They can form dendrimeric PNPs that branch into siRNA binding regions or via quaternization of PBAEs, which increases the ionization capacity and thus affinity for siRNA.[127,128] Peptide modifications of PBAEs have also helped to increase the specificity of tissue delivery.[129] Specifically, the modulation of the nanoparticle zeta potential by different peptide modifications can alter the function and specificity of these PNPs.[130] PBAEs have also been modified to include a peptide-derived cystamine functional group at the terminal end of the polymer that transiently increased siRNA affinity until the cellular environment induced release.[131] Additionally, the complexation with guanidinylated-*O*-carboxymethyl-chitosan or aliphatic diols can increase silencing efficacy.[132,133] PBAEs have also been engineered with functional groups that release nucleic acid cargo after exposure to pH changes.[134] PNPs consisting of PBAEs have even been incorporated into hydrogels to act as a drug depot for prolonged delivery and improved transfection.[135] Additionally, various amine-containing glycopolymers have seen success in siRNA delivery by utilizing carbohydrate moieties to optimize efficacy while still incorporating biodegradable functional groups to enable biodegradation.[136−138]

Poly-lysine-based PNPs have successfully delivered siRNA to Non-Hodgkin's lymphoma cell lines.[139] A small metalloprotease peptide conjugated to polyethylene glycol has also been successful in the delivery of siRNA.[140] Chitosan-based nanoparticles have also been successful in delivering siRNA.[141−143]

There are some examples of classical polymeric nanoparticles for drug delivery that do not contain nitrogen. siRNA-loaded polymeric nanoparticles consisting of poly(lactic-*co*-glycolytic) acid (PLGA) have been synthesized, and the

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

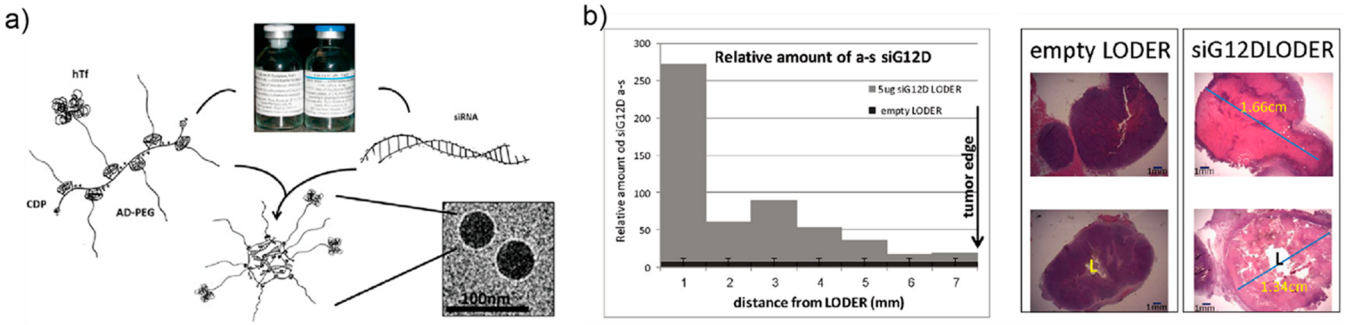


Figure 6. (a) CALAA-01 nanoparticle assembly. Cyclodextrin-based polymers (CDP) with hydrophilic adamantane polyethylene glycols (AD-PEG) and human transferrin protein targeting ligands (hTf) are contained in one vial, while the therapeutic siRNA targeting the M2 subunit of ribonucleotide reductase is contained in the second vial. In the pharmacy, the vials are combined, and the nanoparticles self-assemble, as shown in the cryoelectron micrograph. Reprinted with permission from ref 146, Copyright 2014 PNAS. (b) siG12D drug delivery across the pancreatic (Panc02) tumor. On the left, relative qPCR levels of siG12D across the tumor. On the right, hematoxylin and eosin-stained tumor tissue 7 days after implantation. Reprinted with permission from ref 147, Copyright 2015 Oncotarget.

functionalization with transferrin peptides have assisted the crossing of the blood-brain barrier, while conjugates to poly(vinyl alcohol) effectively delivered siRNA to pulmonary cells.[119,144] Additionally, intravaginal injection of PLGA PNPs loaded with siRNA has proven effective for treating HSV.[145]

Polymeric encapsulation has also been achieved via matrices that physically entrap the nucleic acid cargo. Unlike nanoparticles (where the payload is encapsulated in an inner region and surrounded by the polymer), a matrix is a dense web of polymers, throughout which the cargo is dispersed. Polylactic acid and poly-*ortho*-esters have been used in this application because they can be emulsified and precipitated to form the biodegradable polymeric matrix encapsulating the cargo. This preparation technique is easily scalable; however, encapsulation and nucleic acid stability are essential considerations for optimization.[115]

Though many of these studies displayed promising results, to our knowledge there are currently no FDA-approved siRNA drugs or ongoing clinical trials that utilize polymeric nanoparticles as delivery vehicles for siRNA. To our knowledge, the only clinical trial that has utilized PNP delivery vehicles was an siRNA therapy that sought to silence the RRM2 protein using a combination of cyclodextrin polymers, PEG, and polypeptide conjugates that targeted the transferrin receptor (NCT00689065) (Figure 6a).[146] This trial was not continued into phase 2 trials. Currently, one polymeric matrix is being evaluated for siRNA therapy in treating pancreatic ductal carcinoma (Figure 6b).[147] Developed by Silenseed Ltd., the polymeric matrix is named LOcal Drug EluteR (LODER). The delivery platform is an implantable drug depot for siRNA meant to be administered into solid tumors that are typically considered difficult to treat by the usual standard of care. The LODER platform enables the prolonged release of siRNA into the tumor.[147] The siRNA here targets the mutant gene KRAS, a significant oncogene in pancreatic cancer.[148] Currently, this platform is being evaluated in phase II clinical trials in combination with a chemotherapeutic. Polymeric nanoparticles present a platform for siRNA delivery that can be tailored through modifications in the composition, size, zeta potential, and functional group additions for many different applications. The ongoing clinical trial utilizing PNPs represents a promising direction in the field of siRNA delivery.

3b.v. Polypeptide Nanoparticles. Within nanoparticle delivery of siRNA, one clinical trial evaluates a polypeptide nanoparticle as a siRNA carrier platform. As the name suggests, a polypeptide nanoparticle (PNP) encapsulates siRNA by using peptides. The use of amino acids (monomers within peptides) as drug carriers is advantageous as there are virtually an infinite number of permutations of amino acid sequences to create unique peptides.[149] Varying the amino acid sequence can fine-tune the peptide chain's physiochemical properties, making for a modular carrier. The synthesis of novel peptide chains is straightforward and can be done at a low cost, making them an attractive delivery platform for many drugs.[149] As an added benefit, the amino acids used to synthesize peptides are naturally occurring and have been shown to have low immunogenicity and cell toxicity.[150] In the context of siRNA, it is helpful for these peptide sequences to have positively charged amino acids to complex the negatively charged phosphate backbone of siRNA to form the polypeptide nanoparticle.[151] Furthermore, hydrophobic amino acids within the sequence can aid with fusion into the cell membrane for efficient cellular uptake and endosomal escape.[152] The specific peptides that constitute polypeptide nanoparticles are known as cell-penetrating peptides (CPPs) and are usually 15−30 amino acids in length. Although very broadly categorized and derived differently (synthetic, humans, mice), CPPs are either cationic or amphipathic.[153] These amino acid sequences are unique because they can readily transport a cargo of interest across a cell membrane and can be engineered to actively target specific cell receptors.[154] It has been reported that CPP nanoparticles take many different endocytosis mechanisms, specifically clathrin and caveolae-mediated endocytosis.[155] This effective uptake mechanism and active targeting of CPP nanoparticles make them promising candidates for the siRNA carrier platform. A typical formulation strategy for these nanoparticles is mixing siRNA and the peptides in an aqueous media.[156] Self-assembly of particles occurs based on electrostatic and hydrophobic interactions between the peptides and the siRNA. As a result, the primary and secondary structure of the CPP itself is significant in particle formation. Specifically, it has been reported that the formation of particles is driven by positively charged amino acids (lysine, histidine, and arginine) complexing with the negative charge of siRNA's phosphate backbone. Adding hydrophobic amino acids, including tryptophan, has been shown to improve the particle stability. It has been found that the hydrophobic interaction with tryptophan and the cellular membrane may be important for

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

effective cell internalization and nanoparticle stabilization, specifically its ability to interact with double-stranded oligonucleotides.[157,158]

Different CPP nanoparticle combinations or "families" have been shown to encapsulate and deliver siRNA efficiently. Li et al. conjugated various fatty acids onto CPP Octa-Arginine (R8) to form FattyAcid-R8-siRNA nanoparticles. They indicated that the hydrophobic tails on R8 derivatives enabled higher stability, membrane permeability, and *survivin*-mRNA silencing on A549 and HEPG2 cell lines compared to native Octa-Arginine siRNA nanoparticles.[159] The RALA family of peptides is a 30-mer helical peptide sequence containing the amino acids arginine, leucine, and alanine.[160] siRNA delivery of FKBL siRNA using RALA-based nanoparticles showed slightly lower knockdown of FKBPL mRNA than the transfecting agent Lipofectamine in ZR-75-1 cell lines, albeit with significantly higher cell viability.[161] *In vivo* delivery of FKBPL siRNA RALA nanoparticles via a bilayer wound patch on a murine wound model showed pro-angiogenic effects and a 325.8% increase in vessel number at the wound site as compared to untreated mice.[162] C6 peptides, a 16-amino-acid sequence containing arginine and leucine, are another peptide-based nanoparticle able to transfect siRNA into cells.[163] Histidine-modified C6 nanoparticles carrying Bcl-2 siRNA enabled for delivery into tumor cells and significantly reduced tumor growth in A549 tumor-bearing mice.[164] Additionally, tryptophan-modified C6 peptides (C6-M1) showed similar cellular uptake in Chinese hamster ovary (CHO) cells as compared to native C6, but higher levels of gene knockdown indicated that tryptophan has a role in the endosomal escape mechanism for these peptide nanoparticles. C6-M1 Bcl-2 siRNA treatment on A549 tumor-bearing mice showed significant tumor growth inhibition compared to the untreated groups.[165] Tryptophan and arginine-rich amphipathic peptides (WRAP) have been explored for siRNA delivery. WRAPs are a 15- to 16-amino-acid sequence containing tryptophan, arginine, and leucine residues, forming an α-helical structure able to encapsulate siRNA.[166,167] Compared to naked siRNA, it was demonstrated that WRAP siRNAs significantly silenced firefly luciferase in U87 cells.[168]

Further optimizations on CPP nanoparticles have been developed for enhanced cellular uptake and tumor targeting capability. Ren et al. developed a CPP conjugated to a LyP-1 ligand and a membrane translocating domain (TP) to selectively bind and be taken up by ovarian tumor cells expressing the surface receptor p32. *Anti* ID4 siRNA encapsulated within the Lyp-1-CPP nanoparticle was administered intravenously into nude mice bearing OVCAR-4 tumors and showed penetration into the tumor site and significant silencing of the ID4 gene leading to tumor growth suppression and overall improvement in mice survival time.[169] Asai et al. encapsulated an *anti*-luciferase siRNA into a hybrid cell-penetrating peptide lipid nanoparticle and found enhanced gene knockdown compared with a native siRNA lipid nanoparticle when dosed on B16-F10-luc2 cell lines. The hybrid nanoparticles were observed to be taken up by heparan sulfate-mediated endocytosis, micropinocytosis, and clathrin-mediated endocytosis.[170] Pun and colleagues conjugated an HIV gp4−1 derived peptide to the polymer polyethylenimine (PEI) to form a PEI-peptide siRNA nanoparticle to increase cellular uptake. *In vitro* studies indicated that the peptide-modified PEI nanoparticle had significantly higher siRNA-mediated knockdown in HeLa cells than unmodified PEI. Interestingly, increased gene knockdown of the PEI-peptide nanoparticle was not caused by an increase in cellular uptake but rather a more efficient endosomal escape mechanism.[171] "Envelope-type" siRNA nanoparticles developed by Farokhzad and colleagues showed effective targeting and gene knockdown of the prostate tumor growth factor prohibitin 1 when delivered IV in LNCaP tumor-bearing mice. These "envelope-type" siRNA nanoparticles contained an arginine-PEG copolymer and a ligand specific to the prostate-specific membrane antigen (PSMA), which is overexpressed in advanced pancreatic cancer.[172] As shown by the range of studies, innovative strategies have been employed to further enhance the polypeptide nanoparticle delivery of siRNA.

Several challenges still exist for CPP nanoparticles *in vivo* as a delivery material for siRNA. Some of these include but are not limited to stability in serum due to proteasomal degradation, control of size and dispersity of nanoparticles, and short circulation time within the bloodstream.[173] Attachment of PEG or polysaccharides onto the surface of the particles has reduced overall protein opsonization of the particle leading to longer circulation times.[174] Aldrian and colleagues PEGylated a siRNA CPP nanoparticle containing the amino acids arginine, isoleucine, cysteine, and lysine (RICK) and found PEGylation did not alter nanoparticle formation or siRNA silencing while also showing significantly higher circulation time in mice as compared to a non-PEGylated RICK-siRNA formulation.[175]

Clinical trial development of CPPs as a carrier for siRNA is currently being evaluated in a Phase II study (NCT:04844983). Specifically, a polypeptide-based nanoparticle for delivering STP705 siRNA (a TGF-B1 and COX mRNA silencer) for treating squamous cell carcinoma is currently being explored. The COX mRNA is frequently overexpressed in several forms of cancer, while TGF-B1 is a key growth factor for cells and is often dysregulated in cancers.[176,177] This trial is currently assessing the tolerability and efficacy of different doses. As indicated by the vast development within the literature and clinical trial evaluation, polypeptide nanoparticles are another innovative platform for the delivery of siRNA that has the potential to achieve clinical success.

3c.vi. Exosomes. Exosomes are another carrier platform being evaluated for siRNA therapy in Clinical Trial settings. Exosomes are approximately 30−90 nm vesicles released by many cell types into the extracellular environment.[178] These vesicles have been indicated with a host of specific functions and enable intercellular communication.[179] One facet of exosome function is that they transport RNA between cells.[180] This unique characteristic of exosomes has been leveraged to deliver therapeutically relevant siRNAs. Typically, exogenous siRNA can be loaded into exosomes using electroporation or transfection agents such as Lipofectamine and Exo-Fect.[181] Exosomes carrying siRNA can resist degrading factors in the body, and the exosome's lipid membrane enables efficient cellular uptake.[182] An additional advantage of exosome-mediated siRNA delivery is its ability for active targeting. Exosomes can be generated from several different cell types and then can be further engineered to express specific membrane proteins that are ligands for receptors on target cells. This active targeting capability has been demonstrated within the literature, and it has been shown that exosomes carrying siRNA can pass through complex

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

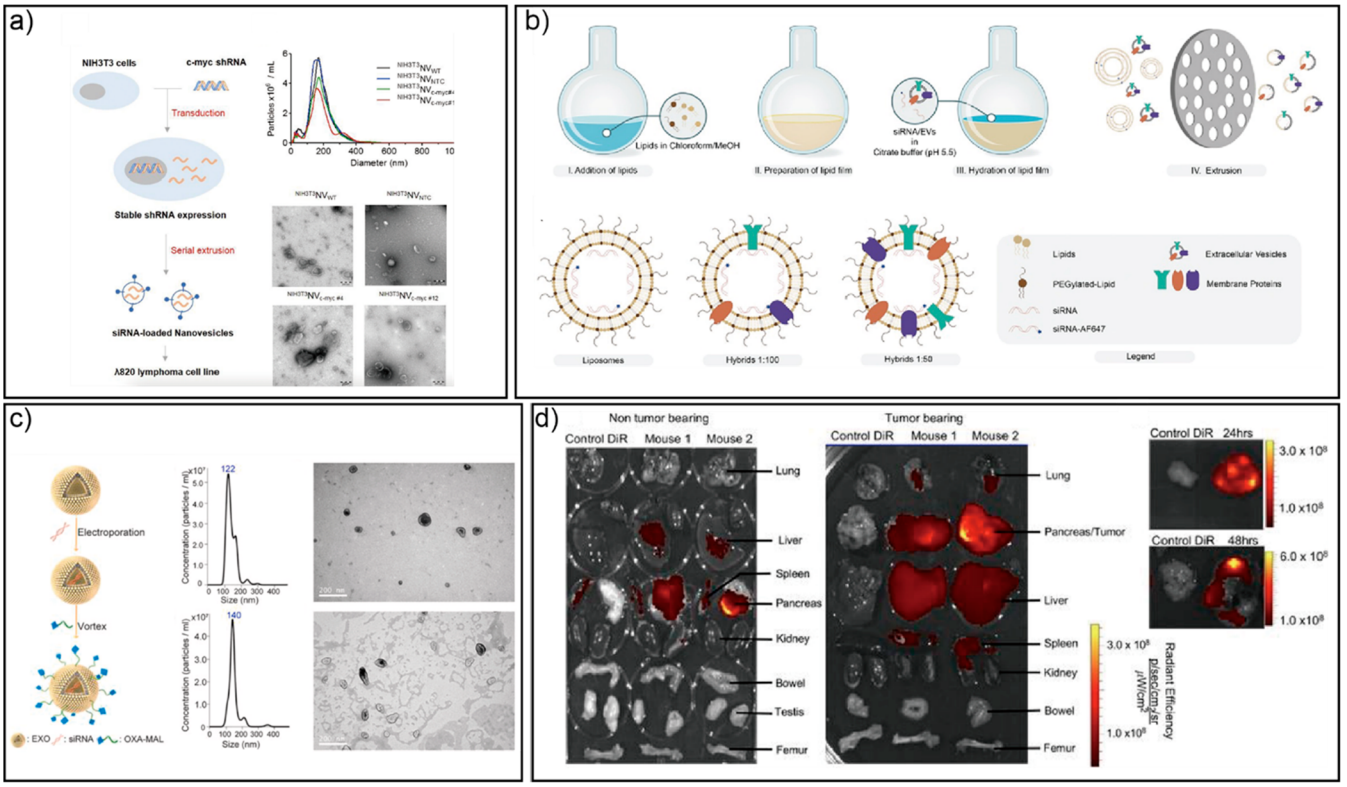


Figure 7. (a) Graphical overview and characterization of λ820 derived exosomes carrying siRNA against *c-Myc* oncogene. Reprinted with permission from ref 186, Copyright 2016 Elsevier. (b) Preparation of an extracellular vesicle−liposome hybrid nanoparticle using lipid-film hydration and extrusion. Reprinted with permission from ref 188, Copyright 2022 Wiley. (c) Graphical overview and characterization of BM-MSC derived exosomes carrying Gal-9 siRNA and Oxaliplatin for treating pancreatic ductal adenocarcinoma (PDAC). Reprinted with permission from ref 194, Copyright 2021 Elsevier. (d) Biodistribution data on non-tumor-bearing and tumor-bearing (KPC689) mice for DiR labeled mesenchymal stem/stromal cells (MSCs) derived exosomes. Reprinted with permission from ref 195, Copyright 2018 The Journal of Clinical Investigation.

barriers such as the Blood Brain Barrier (BBB) and the Blood-Spinal Cord barrier.[183,184]

Exosome-mediated delivery proved to be a promising strategy for the delivery of siRNA. For example, *in vitro* studies demonstrate that exogenous siRNA can be loaded into human plasma cell-derived exosomes using electroporation and chemical transfection to deliver to human mononuclear blood cells and effectively downregulate specific genes.[185] Additionally, anti-c-Myc-siRNA has been loaded via electroporation onto Human U937 derived exosomes, and *in vitro* studies on λ820 (murine lymphoma) cells showed significant RNAi-mediated knockdown of the oncogene and an overall decrease in λ820 cell viability (Figure 7a).[186] Further *in vitro* studies have demonstrated that HeLa and HT1080 human fibrosarcoma cell-derived exosomes can effectively load and deliver siRNA against *RAD51* and *RAD52* into HeLa and HT1080 human fibrosarcoma cells.[187] Evers and colleagues developed an extracellular vesicle−liposome hybrid nanoparticle for highly efficient siRNA loading and showed these particles deliver to multiple cell types (SKOV3, HEK293T, U87-MG) with dose-dependent silencing of luciferase on luciferase-expressing cell lines. This hybrid system was formulated by combining a SKOV3 exosome with a lipid nanoparticle containing DLin-MC3-DMA, DPPC, cholesterol, and DMG-PEG using lipid thin film hydration, followed by extrusion. The combination of the two systems enables the active targeting capabilities of exosomes and high siRNA loading efficiency typically found in lipid nanoparticle siRNA nanoparticles (Figure 7b).[188]

Within the literature, siRNA-loaded exosomes have moved into *in vivo* disease models, including those used to study neurodegenerative disorders and different forms of cancer. Exosomes hold great potential in treating neurodegenerative disorders, because they efficiently pass through the Blood Brain Barrier and the Blood-Spinal Cord Barrier. To treat Huntington's disease, Didiot, and colleagues loaded cholesterol-conjugated siRNA into U87-derived exosomes using coincubation to silence the *huntingtin* (HTT) gene. Direct injection into the right striata of mouse brain showed that exosomal delivery of *anti*-HTT siRNA induces a statistically significant knockdown of HTT mRNA levels in the ipsilateral and contralateral striata of the brain compared to free *anti*-HTT siRNA.[189] For the treatment of spinal cord injury (SCI), mesenchymal stem cell-derived exosomes containing *anti*-connective tissue growth factor (CTGF)-siRNA were administered intravenously into SCI rats. Treatment resulted in significant silencing of the CTGF gene, reduced inflammation/upregulated anti-inflammatory cytokines, and prevented neuronal apoptosis, reactive astrocytes, and glial scar formation, thus increasing overall motor functions in SCI rats.[190] IV administration indicated that the exosomes could pass through the blood-spinal cord barrier, highlighting the advantages of using exosomes as a delivery platform for siRNA therapy. To develop a treatment for Alzheimer's disease, exosomes derived from murine dendritic cells were engineered to express the endosomal membrane protein Lamp2b fused to the neuron-specific rabies viral glycol protein (RVG) peptide to pass through the BBB. It has been shown that the RVG peptide

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

Table 1. Active Clinical Trials Using siRNA

siRNA	Indication of Use	Target	Delivery Strategy	Developer	Cotreatment	Stage	NCT	Additional References
			Conjugate Based Delivery Strategies					
CpG-STAT3 siRNA Cas3/	Relapse/Refractory B-cell Non-Hodgkin's Lymphoma	TLR9 receptor, STAT3	Oligonucleotide Conjugate	City of Hope Medical Center	Radiation Therapy	I	NCT04995536	
ALN-APP	Early Onset Alzheimer's Disease	APP	C16 Conjugate	Alnylam Pharmaceuticals/ Regeneron	N/A	I	NCT05231785	206
SLN124	Myelodysplastic Syndrome Polycythemia Vera	TMPRSS6	GalNAc conjugate	Silence Therapeutics	N/A	I/II	NCT05499013 NCT04718844	207
SLN360	Atherosclerotic Cardiovascular Disease	Lipoprotein (A)	GalNAc conjugate	Silence Therapeutics	N/A	II	NCT05537571	208
VIR-2218 **VIR-3434**	Hepatic Impairment, Cirrhosis	HBV gene	GalNAc conjugate	Vir Biotechnology, Inc.	N/A	I	NCT05484206	208,209
Zilebesiran (ALN-AGT01)	Hypertension	AGT	GalNAc conjugate	Alnylam Pharmaceuticals	Olmesartan AmlodipineIndapamide	II	NCT05103332 NCT04936035	210
Fitusiran	Hemophilia	AT	GalNAc Conjugate	Genzyme	N/A	II/ III	NCT03974113	211
ALN-HSD	Nonalcoholic Steatohepatitis	HSD17B13	GalNAc Conjugate	Regeneron Pharmaceuticals	N/A	II	NCT05519475	211
Belcesiran (DCR-A1AT)	Alpha 1-Antitrypsin Deficiency	AAT	GalNAc Conjugate	Dicerna Pharmaceuticals	N/A	I/II	NCT04174118 NCT04764448	211
Nedosiran (DCR-PHXC)	Primary Hyperoxaluria	LDHA	GalNAc Conjugate	Dicerna Pharmaceuticals	N/A	II/ III	NCT05001269 NCT04580420 NCT04042402	212
Cemdisiran	Complement Mediated Diseases	C5	GalNAc Conjugate	Regeneron Pharmaceuticals Alnylam Pharmaceuticals	Pozelimab EculizumabRavulizumab	II/ III	NCT05131204 NCT05070858 NCT05133531 NCT04811716 NCT04888507 NCT03841448	213
Fazirsiran (ARO-AAT)	Alpha 1-Antitrypsin Deficiency	AAT	GalNAc Conjugate	Arrowhead Pharmaceuticals	N/A	II	NCT03946449 NCT03945292	214
ARO-APOC3	Dyslipidemia, Familial Chylomicronemia, Severe Hypertriglyceridemia	ApoC3	GalNAc Conjugate	Arrowhead Pharmaceuticals	N/A	II/ III	NCT05413135 NCT04998201 NCT05089084 NCT04720534	214
ARO-ANG3	Homozygous Familial HypercholesteremiaMixed Dyslipidemia	ANGPTL3	GalNAc Conjugate	Arrowhead Pharmaceuticals	N/A	II	NCT05217667 NCT04832971	214
Olpasiran (AMG 890)	Cardiovascular Disease Renal Impairment Hepatic Impairment	Lipoprotein (A)	GalNAc Conjugate	Arrowhead Pharmaceuticals/ Amgen	N/A	I/III	NCT03626662 NCT05489614 NCT05481411 NCT05581303	215
RBD1016	Hepatitis B	HBV gene	GalNAc Conjugate	Suzhou Ribo Life Science Co. Ltd.	Entecavir	I	NCT05017116	216

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

Table 1. continued

siRNA	Indication of Use	Target	Delivery Strategy	Developer	Cotreatment	Stage	NCT	Additional References
			Nanoparticle Based Delivery Strategy					
EphA2 siRNA	Advanced Malignant Solid Neoplasm	EphA2	Liposome	M.D. Anderson Cancer Center	N/A	I	NCT01591356	217
NBF-006	Non-Small Cell Lung Cancer Pancreatic Cancer Colorectal Cancer	Glutathione S-Transferase-P (GSTP)	Lipid Nanoparticle	Nitto BioPharma Inc.	N/A	I	NCT03819387	108
siG12D-LODER	Pancreatic Ductal Adenocarcinoma Pancreatic Cancer	KRAS G12D	Polymeric Matrix	Silenseed Ltd.	Gemcitabine+nab-Paclitaxel or Folfirinox or Modified Folfirinox	II	NCT01676259	147
STP705	Cutaneous Squamous Cell Carcinoma	TGF-β1 and COX-2 mRNA	Polypeptide nanoparticle	Sirnaomics	N/A	II	NCT04844983	218
			Exosome Based Delivery Strategy					
KRAS G12D siRNA	Pancreatic Cancer	KRAS G12D	Exosome	M.D. Anderson Cancer Center	N/A	I	NCT03608631	
			siRNA alone					
OLX10212	Neovascular Age-Related Macular Degeneration	Undisclosed	None	Olix Pharmaceuticals	N/A	I	NCT05643118	
OLX10010	Hypertrophic Scar	CTGF	None	Olix Pharmaceuticals	N/A	II	NCT04877756	219
Tivanisiran	Dry Eye Disease Sjögren Syndrome	TRPV1	None	Sylentis	N/A	III	NCT04819269	219
BMT101	Hypertrophic Scar	CTGF	None	Hugel	N/A	II	NCT04012099	219
SYL1801	Macular Degeneration	NRARP	None	Sylentis	N/A	II	NCT05637255	219
MBS-COV	COVID-19	RdRp region SARS-CoV-2	None	Oneness Biotech Co.	N/A	I	NCT05677893	220
			Undisclosed					
ADX-324	Hereditary Angioedema	PKK	Undisclosed	ADARx Pharmaceuticals Inc.	N/A	I	NCT05691361	221
ALN-PNP	Non-Alcoholic Steatohepatitis	PNPLA3	Undisclosed	Regeneron/Alnylam Pharmaceuticals	N/A	I	NCT05648214	211

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

binds to the acetylcholine receptor on neuronal cells to allow passage through the BBB.[191] These RVG exosomes were loaded with GAPDH siRNA to knock down protein BACE1, a key target in treating Alzheimer's disease. Intravenous administration of GAPH-loaded exosomes in mice showed a 60% knockdown of BACE1 mRNA in the brain, indicating the targeting capability and therapeutic potential of siRNA RVG exosomes.[192] Additionally, Liu et al. engineered human embryonic kidney 293T (HEK293T) derived exosomes carrying MOR siRNA. Expression of the neuron-specific rabies viral glycol protein (RVG) peptide on the exosome enabled effective delivery through the blood brain barrier (BBB). It was further demonstrated *in vivo* that these siRNA-carrying RVG exosomes could knock down the opioid receptor Mu (MOR) to treat morphine relapse.[193] siRNA therapy in cancer treatment has also been explored due to the tumor-targeting potential of an exosome carrier. BM-MSC-derived exosomes carrying Gal-9 siRNA have been used to treat pancreatic ductal adenocarcinoma (PDAC) to reverse the immunosuppressive tumor microenvironment. Studies performed on orthotopic PDAC mice showed Gal-9 siRNA exosomes could actively target the PDAC tumor site and knock down the galectin-9 dectin-1 which thus prevented immunosuppression caused by M2-polarized tumor-associated macrophages (M2-TAMs). Combination treatment with this exosome and Oxaliplatin showed a synergistic effect of anti-PDAC immunity and enhanced cytotoxicity compared to Oxaliplatin alone (Figure 7c).[194] Liu et al. developed an exosome derived from Immature Dendritic Cells expressing the membrane protein Lamp2b, which was then fused to an av integrin specific iRGD peptide (iRGD) for the targeting of avβ3 integrin receptor, a receptor commonly overexpressed in diffuse large B-cell lymphoma. These exosomes were loaded with antiBCL6 siRNA using electroporation and administered intravenously into BALB/c nude mice implanted with the cytoskeletal system of the OCI-Ly8 cells. Administration of this siRNA-iRGD-modified exosome showed reduced expression of BCL6 and a significant reduction in tumor cell proliferation without any significant toxicity to organs.

As indicated by a current Phase I Clinical Trial, the translation potential of siRNA therapy using exosomes may be possible. For example, one trial is evaluating an siRNA treatment targeting the KrasG12 D mutation in pancreatic cancer using a stromal-cell-derived exosome as a carrier (NCT03608631). This specific exosome (termed iExosome) has shown efficacy in preclinical mice with patient-derived xenograft KrasG12D tumors. It was demonstrated that iExosomes can actively target the KrasG12D mutation, leading to gene knockdown and tumor volume reduction compared to the control groups (Figure 7d).[195] The current aims of the trial include identifying the maximum tolerated dose and dose-limiting toxicities and evaluating the pharmacokinetic profile of the mesenchymal stem cell-derived exosomes carrying KrasG12D siRNA in PDAC patients with the KrasG12D mutation. One significant challenge of exosome siRNA delivery is the scale-up and manufacturing costs of the exosomes. Exosomes require intensive cell culture methods for generation and are challenging to achieve batch-to-batch consistency.[196] Additionally, downstream purification methods are needed to be further developed to yield higher purity exosomes, as current purification processes for exosomes are slow and difficult to scale up to industrial quantities.[197] However, methods are being developed to scale and manufacture bone marrow mesenchymal stem/stromal cells (MSCs) derived exosomes in cGMP conditions.[195] As this technology develops, additional clinical trials using exosomes to deliver siRNA may emerge to treat diseases.

4. CONCLUSION

Many diseases are characterized by detrimental upregulation or expression of mutated versions of proteins. siRNA therapy provides a platform whereby these unfavorable protein expression profiles can be targeted for silencing even if the proteins themselves are deemed "undruggable" by traditional drugs. The approval of five siRNA-based therapies has highlighted the potential of this technology for human use, and many more siRNA therapies are in development. Table 1 summarizes the ongoing clinical trials utilizing siRNA therapies.

Looking forward, siRNA has many different possible applications in various diseases. This is because its sequence can be engineered to target virtually any mRNA transcript. This opens the door for therapy against genes that have been historically difficult to target with small molecule and biologic therapies. To date, siRNA therapies have been approved for genetic neurological (Patisiran, Vutisiran, and Givosiran), renal (Lumasiran), and cardiovascular (Inclisiran) disorders. siRNA therapies in development broaden the indications of that can be treated with RNAi. For example, cancer is a growing area of interest in siRNA therapy, especially cancers that have proven to be difficult to treat with traditional chemotherapies and small molecule inhibitors. Several siRNA treatments have demonstrated success in the clinic. However, because cancer exhibits a multitude of oncogenic mutations to reach malignancy, a singular siRNA construct may prove insufficient to treat many cancers in clinical settings. However, using siRNA to silence singular aberrant genes may significantly decrease the risk of tumor resistance, as already supported by in *vitro* experiments exhibiting a 70% reversal of resistance after treatment with an MDR1 siRNA in a breast cancer cell line treated with doxorubicin.[198,199] Success of siRNA as cotreatment with chemotherapies has been echoed in numerous other studies.[200−202]

Although 5 siRNA drugs have to date been approved by the FDA, there are still a number of barriers and challenges that remain toward translating additional siRNA therapies from the benchtop to the clinic. For example, developing siRNA therapies that can access nonhepatic targets will be essential for realizing the full clinical potential of siRNA therapies. Specifically, developing siRNA therapies that can access the lungs, brain, reproductive organs, and gastrointestinal tract, among other targets in the body, would benefit diseases originating in these organs that are associated with the overexpression of specific genes. Accomplishing this goal may be realized through the development of novel strategies that hybridize advances in drug formulation and chemistry, particularly leveraging next-generation targeting chemistries to overcome the innate biodistribution of siRNA therapies by targeting specific receptors on nonhepatic targets.[203] Further, the development of novel injection styles and routes including priming strategies to transiently occupy the liver may prove essential for enabling nonviral delivery technologies to access nonhepatic organs.[204] Developing these advancements will likely benefit from an interdisciplinary approach that synergizes advances in biology, chemistry, engineering, and medicine,

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

each of which has to date proved to be essential in advancing siRNA therapies from the benchtop to the clinic.

Moreover, the development of the next generation of siRNA drugs may be accelerated by taking lessons from challenges and failures that have been encountered throughout their development in clinical trials. Within clinical trials, specific end points must be met at each phase to move drug candidates forward throughout the development process. For example, meeting end points including identifying drug-induced side-effects, maximal tolerated doses, and dose-limiting toxicities are central to advance drugs through Phase I clinical trials.[205] Building on this, meeting efficacy end points is essential to advance drug candidates through Phases II and III. Given their innate physiochemical properties and delivery-associated challenges, a number of potential siRNA drug candidates have—like many small molecule and biologic drugs—failed clinical trials due to reasons including toxicity, efficacy, or other concerns. Going forward, it will therefore be important to consider these failures to develop next-generation siRNA therapies that are safe and effective for the treatment of human disease, particularly taking into account strategies to balance these considerations with approaches to maximize the stability, endosomal escape, immunological toleration, and RISC complex recruitment for siRNA drugs, among other areas. Further, it will be interesting to see how advances in other gene therapies, including mRNA, may help to advance this field. For example, siRNA drugs may leverage the same LNP delivery platform used in the COVID-19 vaccine. It will be interesting to see which similarities and differences exist and how this will impact the design of future drugs. Regardless, siRNA therapy offers hope for patients suffering from traditionally undruggable diseases, and the future is bright for developing next-generation siRNA therapies for treating human disease.

AUTHOR INFORMATION

Corresponding Author

Owen S. Fenton − *Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States; Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States;* orcid.org/0000-0002-5585-9280; Email: osfenton@unc.edu

Authors

Eshan A. Narasipura − *Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States;* orcid.org/0009-0008-2821-2482

Rachel VanKeulen-Miller − *Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States*

Yutian Ma − *Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States;* orcid.org/0000-0002-0110-0244

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.bioconjchem.3c00205

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Figures [1]−[5] were created using biorender.com.

REFERENCES

(1) Pathmanathan, S.; Grozavu, I.; Lyakisheva, A.; Stagljar, I. Drugging the Undruggable Proteins in Cancer: A Systems Biology Approach. *Curr. Opin. Chem. Biol.* **2022**, *66*, 102079.

(2) Llombart, V.; Mansour, M. R. Therapeutic Targeting of "Undruggable" MYC. *EBioMedicine* **2022**, *75*, 103756.

(3) Zhang, J.-T.; Liu, J.-Y. Drugging the "Undruggable" DNA-Binding Domain of STAT3. *Oncotarget* **2016**, *7* (41), 66324−66325.

(4) Moore, A. R.; Rosenberg, S. C.; McCormick, F.; Malek, S. RAS-Targeted Therapies: Is the Undruggable Drugged? *Nat. Rev. Drug Discov* **2020**, *19* (8), 533−552.

(5) Padda, I. S.; Mahtani, A. U.; Parmar, M. Small Interfering RNA (SiRNA) Based Therapy. In *StatPearls*; StatPearls Publishing: Treasure Island (FL), 2022.

(6) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis Elegans. *Nature* **1998**, *391* (6669), 806−811.

(7) Grimm, D.; Streetz, K. L.; Jopling, C. L.; Storm, T. A.; Pandey, K.; Davis, C. R.; Marion, P.; Salazar, F.; Kay, M. A. Fatality in Mice Due to Oversaturation of Cellular MicroRNA/Short Hairpin RNA Pathways. *Nature* **2006**, *441* (7092), 537−541.

(8) Whitehead, K. A.; Langer, R.; Anderson, D. G. Knocking down Barriers: Advances in SiRNA Delivery. *Nat. Rev. Drug Discov* **2009**, *8* (2), 129−138.

(9) Lee, Y. S.; Nakahara, K.; Pham, J. W.; Kim, K.; He, Z.; Sontheimer, E. J.; Carthew, R. W. Distinct Roles for Drosophila Dicer-1 and Dicer-2 in the SiRNA/MiRNA Silencing Pathways. *Cell* **2004**, *117* (1), 69−81.

(10) Mocellin, S.; Provenzano, M. RNA Interference: Learning Gene Knock-down from Cell Physiology. *J. Transl Med.* **2004**, *2* (1), 39.

(11) Santhekadur, P. K.; Kumar, D. P. RISC Assembly and Post-Transcriptional Gene Regulation in Hepatocellular Carcinoma. *Genes & Diseases* **2020**, *7* (2), 199−204.

(12) Hannon, G. J. RNA Interference. *Nature* **2002**, *418* (6894), 244−251.

(13) Micura, R. Small Interfering RNAs and Their Chemical Synthesis. *Angew. Chem., Int. Ed.* **2002**, *41* (13), 2265−2269.

(14) Dong, Y.; Siegwart, D. J.; Anderson, D. G. Strategies, Design, and Chemistry in SiRNA Delivery Systems. *Adv. Drug Deliv Rev.* **2019**, *144*, 133−147.

(15) Beaucage, S.; Reese, C. Recent Advances in the Chemical Synthesis of RNA. In *Current Protocols in Nucleic Acid Chemistry*; 2009; Chapter 2, Unit 2.16, pp 1−31. .

(16) Marshall, W. S.; Kaiser, R. J. Recent Advances in the High-Speed Solid Phase Synthesis of RNA. *Curr. Opin. Chem. Biol.* **2004**, *8* (3), 222−229.

(17) Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C. Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles. *Mol. Pharmaceutics* **2008**, *5* (4), 505−515.

(18) Bartlett, D. W.; Davis, M. E. Effect of SiRNA Nuclease Stability on the in Vitro and in Vivo Kinetics of SiRNA-Mediated Gene Silencing. *Biotechnol. Bioeng.* **2007**, *97* (4), 909−921.

(19) Judge, A. D.; Sood, V.; Shaw, J. R.; Fang, D.; McClintock, K.; MacLachlan, I. Sequence-Dependent Stimulation of the Mammalian Innate Immune Response by Synthetic SiRNA. *Nat. Biotechnol.* **2005**, *23* (4), 457−462.

(20) *Molecular Biology of the Cell*, 4th ed., Alberts, B., Ed.; Garland Science: New York, 2002.

(21) Kumar, H.; Kawai, T.; Akira, S. Toll-like Receptors and Innate Immunity. *Biochem. Biophys. Res. Commun.* **2009**, *388* (4), 621−625.

(22) Sajid, M. I.; Moazzam, M.; Kato, S.; Yeseom Cho, K.; Tiwari, R. K. Overcoming Barriers for SiRNA Therapeutics: From Bench to Bedside. *Pharmaceuticals* **2020**, *13* (10), 294.

(23) Robbins, M.; Judge, A.; MacLachlan, I. SiRNA and Innate Immunity. *Oligonucleotides* **2009**, *19* (2), 89−102.
(24) Koshkin, A. A.; Nielsen, P.; Meldgaard, M.; Rajwanshi, V. K.; Singh, S. K.; Wengel, J. LNA (Locked Nucleic Acid): An RNA Mimic Forming Exceedingly Stable LNA:LNA Duplexes. *J. Am. Chem. Soc.* **1998**, *120* (50), 13252−13253.
(25) Veedu, R. N.; Wengel, J. Locked Nucleic Acid as a Novel Class of Therapeutic Agents. *RNA Biology* **2009**, *6* (3), 321−323.
(26) Pallan, P. S.; Allerson, C. R.; Berdeja, A.; Seth, P. P.; Swayze, E. E.; Prakash, T. P.; Egli, M. Structure and Nuclease Resistance of 2′,4′-Constrained 2′-O-Methoxyethyl (CMOE) and 2′-O-Ethyl (CEt) Modified DNAs. *Chem. Commun.* **2012**, *48* (66), 8195.
(27) Seth, P. P.; Vasquez, G.; Allerson, C. A.; Berdeja, A.; Gaus, H.; Kinberger, G. A.; Prakash, T. P.; Migawa, M. T.; Bhat, B.; Swayze, E. E. Synthesis and Biophysical Evaluation of 2′,4′-Constrained 2′ *O* -Methoxyethyl and 2′,4′-Constrained 2′ *O* -Ethyl Nucleic Acid Analogues. *J. Org. Chem.* **2010**, *75* (5), 1569−1581.
(28) Morita, K.; Hasegawa, C.; Kaneko, M.; Tsutsumi, S.; Sone, J.; Ishikawa, T.; Imanishi, T.; Koizumi, M. 2′-O,4′-C-Ethylene-Bridged Nucleic Acids (ENA): Highly Nuclease-Resistant and Thermodynamically Stable Oligonucleotides for Antisense Drug. *Bioorg. Med. Chem. Lett.* **2002**, *12* (1), 73−76.
(29) Langkjær, N.; Pasternak, A.; Wengel, J. UNA (Unlocked Nucleic Acid): A Flexible RNA Mimic That Allows Engineering of Nucleic Acid Duplex Stability. *Bioorg. Med. Chem.* **2009**, *17* (15), 5420−5425.
(30) Hoshika, S.; Minakawa, N.; Kamiya, H.; Harashima, H.; Matsuda, A. RNA Interference Induced by SiRNAs Modified with 4′-Thioribonucleosides in Cultured Mammalian Cells. *FEBS Lett.* **2005**, *579* (14), 3115−3118.
(31) Dande, P.; Prakash, T. P.; Sioufi, N.; Gaus, H.; Jarres, R.; Berdeja, A.; Swayze, E. E.; Griffey, R. H.; Bhat, B. Improving RNA Interference in Mammalian Cells by 4′-Thio-Modified Small Interfering RNA (SiRNA): Effect on SiRNA Activity and Nuclease Stability When Used in Combination with 2′- *O* -Alkyl Modifications. *J. Med. Chem.* **2006**, *49* (5), 1624−1634.
(32) Braasch, D. A.; Jensen, S.; Liu, Y.; Kaur, K.; Arar, K.; White, M. A.; Corey, D. R. RNA Interference in Mammalian Cells by Chemically-Modified RNA. *Biochemistry* **2003**, *42* (26), 7967−7975.
(33) Selvam, C.; Mutisya, D.; Prakash, S.; Ranganna, K.; Thilagavathi, R. Therapeutic Potential of Chemically Modified SiRNA: Recent Trends. *Chem. Biol. Drug Des* **2017**, *90* (5), 665−678.
(34) Wu, S. Y.; Yang, X.; Gharpure, K. M.; Sood, A. K.; et al. 2′-OMe-Phosphorodithioate-Modified SiRNAs Show Increased Loading into the RISC Complex and Enhanced Anti-Tumour Activity. *Nat. Commun.* **2014**, *5* (1), 3459.
(35) Shaw, B. R.; Moussa, L.; Sharaf, M.; Cheek, M.; Dobrikov, M. Boranophosphate SiRNA-Aptamer Chimeras for Tumor-Specific Downregulation of Cancer Receptors and Modulators. *Nucleic Acids Symp. Ser.* **2008**, *52* (1), 655−656.
(36) Eckstein, F. Phosphorothioates, Essential Components of Therapeutic Oligonucleotides. *Nucleic Acid Therapeutics* **2014**, *24* (6), 374−387.
(37) Lebreton, J.; Waldner, A.; Fritsch, V.; Wolf, R. M.; De Mesmaeker, A. Comparison of Two Amides as Backbone Replacement of the Phosphodiester Linkage in Oligodeoxynucleotides. *Tetrahedron Lett.* **1994**, *35* (29), 5225−5228.
(38) Mutisya, D.; Hardcastle, T.; Cheruiyot, S. K.; Pallan, P. S.; Kennedy, S. D.; Egli, M.; Kelley, M. L.; Smith, A. v. B.; Rozners, E. Amide Linkages Mimic Phosphates in RNA Interactions with Proteins and Are Well Tolerated in the Guide Strand of Short Interfering RNAs. *Nucleic Acids Res.* **2017**, *45* (14), 8142−8155.
(39) Hall, A. H. S. RNA Interference Using Boranophosphate SiRNAs: Structure-Activity Relationships. *Nucleic Acids Res.* **2004**, *32* (20), 5991−6000.
(40) Habibian, M.; Harikrishna, S.; Fakhoury, J.; Damha, M. J.; et al. Effect of 2′-5′/3′-5′ Phosphodiester Linkage Heterogeneity on RNA Interference. *Nucleic Acids Res.* **2020**, *48* (9), 4643−4657.
(41) Karikó, K.; Muramatsu, H.; Welsh, F. A.; Ludwig, J.; Kato, H.; Akira, S.; Weissman, D. Incorporation of Pseudouridine Into MRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability. *Molecular Therapy* **2008**, *16* (11), 1833−1840.
(42) Karikó, K.; Buckstein, M.; Ni, H.; Weissman, D. Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA. *Immunity* **2005**, *23* (2), 165−175.
(43) Anderson, B. R.; Muramatsu, H.; Jha, B. K.; Silverman, R. H.; Weissman, D.; Kariko, K. Nucleoside Modifications in RNA Limit Activation of 2′-5′-Oligoadenylate Synthetase and Increase Resistance to Cleavage by RNase L. *Nucleic Acids Res.* **2011**, *39* (21), 9329−9338.
(44) Kormann, M. S. D.; Hasenpusch, G.; Aneja, M. K.; Rudolph, C.; et al. Expression of Therapeutic Proteins after Delivery of Chemically Modified MRNA in Mice. *Nat. Biotechnol.* **2011**, *29* (2), 154−157.
(45) Lee, H.-S.; Seok, H.; Lee, D. H.; Ham, J.; Lee, W.; Youm, E. M.; Yoo, J. S.; Lee, Y.-S.; Jang, E.-S.; Chi, S. W. Abasic Pivot Substitution Harnesses Target Specificity of RNA Interference. *Nat. Commun.* **2015**, *6* (1), 10154.
(46) Liu, J.; Pendergraff, H.; Narayanannair, K. J.; Corey, D. R.; et al. RNA Duplexes with Abasic Substitutions Are Potent and Allele-Selective Inhibitors of Huntingtin and Ataxin-3 Expression. *Nucleic Acids Res.* **2013**, *41* (18), 8788−8801.
(47) Ku, S. H.; Jo, S. D.; Lee, Y. K.; Kim, K.; Kim, S. H. Chemical and Structural Modifications of RNAi Therapeutics. *Adv. Drug Delivery Rev.* **2016**, *104*, 16−28.
(48) D'Souza, A. A.; Devarajan, P. V. Asialoglycoprotein Receptor Mediated Hepatocyte Targeting — Strategies and Applications. *J. Controlled Release* **2015**, *203*, 126−139.
(49) Nair, J. K.; Willoughby, J. L. S.; Chan, A.; Manoharan, M.; et al. Multivalent *N* -Acetylgalactosamine-Conjugated SiRNA Localizes in Hepatocytes and Elicits Robust RNAi-Mediated Gene Silencing. *J. Am. Chem. Soc.* **2014**, *136* (49), 16958−16961.
(50) Foster, D. J.; Brown, C. R.; Shaikh, S.; Milstein, S.; et al. Advanced SiRNA Designs Further Improve In Vivo Performance of GalNAc-SiRNA Conjugates. *Molecular Therapy* **2018**, *26* (3), 708−717.
(51) Geuze, H. J.; Slot, J. W.; Strous, G. J. A. M.; Lodish, H. F.; Schwartz, A. L. Intracellular Site of Asialoglycoprotein Receptor-Ligand Uncoupling: Double-Label Immunoelectron Microscopy during Receptor-Mediated Endocytosis. *Cell* **1983**, *32* (1), 277−287.
(52) Springer, A. D.; Dowdy, S. F. GalNAc-SiRNA Conjugates: Leading the Way for Delivery of RNAi Therapeutics. *Nucleic Acid Therapeutics* **2018**, *28* (3), 109−118.
(53) Schwartz, A. L.; Fridovich, S. E.; Knowles, B. B.; Lodish, H. F. Characterization of the Asialoglycoprotein Receptor in a Continuous Hepatoma Line. *J. Biol. Chem.* **1981**, *256* (17), 8878−8881.
(54) Lu, J.; Swearingen, E.; Hardy, M.; Collins, P.; Wu, B.; Yuan, E.; Lu, D.; Li, C.-M.; Wang, S.; Ollmann, M. RAB18 Is a Key Regulator of GalNAc-Conjugated SiRNA-Induced Silencing in Hep3B Cells. *Molecular Therapy - Nucleic Acids* **2022**, *28*, 423−434.
(55) Schmidt, K.; Prakash, T. P.; Donner, A. J.; Kinberger, G. A.; Gaus, H. J.; Low, A.; Østergaard, M. E.; Bell, M.; Swayze, E. E.; Seth, P. P. Characterizing the Effect of GalNAc and Phosphorothioate Backbone on Binding of Antisense Oligonucleotides to the Asialoglycoprotein Receptor. *Nucleic Acids Res.* **2017**, *45* (5), 2294−2306.
(56) Matsuda, S.; Keiser, K.; Nair, J. K.; Manoharan, M.; et al. SiRNA Conjugates Carrying Sequentially Assembled Trivalent *N*-Acetylgalactosamine Linked Through Nucleosides Elicit Robust Gene Silencing *In Vivo* in Hepatocytes. *ACS Chem. Biol.* **2015**, *10* (5), 1181−1187.
(57) Burel, S. A.; Hart, C. E.; Cauntay, P.; Henry, S. P.; et al. Hepatotoxicity of High Affinity Gapmer Antisense Oligonucleotides Is Mediated by RNase H1 Dependent Promiscuous Reduction of

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

Very Long Pre-MRNA Transcripts. *Nucleic Acids Res.* **2016**, *44* (5), 2093−2109.
(58) Jackson, A. L.; Bartz, S. R.; Schelter, J.; Kobayashi, S. V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P. S. Expression Profiling Reveals Off-Target Gene Regulation by RNAi. *Nat. Biotechnol.* **2003**, *21* (6), 635−637.
(59) Janas, M. M.; Schlegel, M. K.; Harbison, C. E.; Jadhav, V.; et al. Selection of GalNAc-Conjugated SiRNAs with Limited off-Target-Driven Rat Hepatotoxicity. *Nat. Commun.* **2018**, *9* (1), 723.
(60) Zhang, L.; Liang, Y.; Liang, G.; Tian, Z.; Zhang, Y.; Liu, Z.; Ji, X. The Therapeutic Prospects of N-Acetylgalactosamine-SiRNA Conjugates. *Front Pharmacol* **2022**, *13*, 1090237.
(61) Biscans, A.; Caiazzi, J.; McHugh, N.; Hariharan, V.; Muhuri, M.; Khvorova, A. Docosanoic Acid Conjugation to SiRNA Enables Functional and Safe Delivery to Skeletal and Cardiac Muscles. *Molecular Therapy* **2021**, *29* (4), 1382−1394.
(62) Biscans, A.; Coles, A.; Haraszti, R.; Echeverria, D.; Hassler, M.; Osborn, M.; Khvorova, A. Diverse Lipid Conjugates for Functional Extra-Hepatic SiRNA Delivery in Vivo. *Nucleic Acids Res.* **2019**, *47* (3), 1082−1096.
(63) Brown, K. M.; Nair, J. K.; Janas, M. M.; Jadhav, V.; et al. Expanding RNAi Therapeutics to Extrahepatic Tissues with Lipophilic Conjugates. *Nat. Biotechnol.* **2022**, *40* (10), 1500−1508.
(64) Petrova, N. S.; Chernikov, I. V.; Meschaninova, M. I.; Dovydenko, Ii. S.; Venyaminova, A. G.; Zenkova, M. A.; Vlassov, V. V.; Chernolovskaya, E. L. Carrier-Free Cellular Uptake and the Gene-Silencing Activity of the Lipophilic SiRNAs Is Strongly Affected by the Length of the Linker between SiRNA and Lipophilic Group. *Nucleic Acids Res.* **2012**, *40* (5), 2330−2344.
(65) Wolfrum, C.; Shi, S.; Jayaprakash, K. N.; Stoffel, M.; et al. Mechanisms and Optimization of in Vivo Delivery of Lipophilic SiRNAs. *Nat. Biotechnol.* **2007**, *25* (10), 1149−1157.
(66) Osborn, M. F.; Coles, A. H.; Biscans, A.; Khvorova, A.; et al. Hydrophobicity Drives the Systemic Distribution of Lipid-Conjugated SiRNAs via Lipid Transport Pathways. *Nucleic Acids Res.* **2019**, *47* (3), 1070−1081.
(67) Chernikov, I. V.; Gladkikh, D. V.; Meschaninova, M. I.; Ven'yaminova, A. G.; Zenkova, M. A.; Vlassov, V. V.; Chernolovskaya, E. L. Cholesterol-Containing Nuclease-Resistant SiRNA Accumulates in Tumors in a Carrier-Free Mode and Silences MDR1 Gene. *Molecular Therapy - Nucleic Acids* **2017**, *6*, 209−220.
(68) Khan, T.; Weber, H.; DiMuzio, J.; Matter, A.; Dogdas, B.; Shah, T.; Thankappan, A.; Disa, J.; Jadhav, V.; Lubbers, L.; Sepp-Lorenzino, L.; Strapps, W. R.; Tadin-Strapps, M. Silencing Myostatin Using Cholesterol-Conjugated SiRNAs Induces Muscle Growth. *Molecular Therapy - Nucleic Acids* **2016**, *5*, No. e342.
(69) Murad, Y. M.; Clay, T. M. CpG Oligodeoxyneucleotides as TLR9 Agonists: Therapeutic Applications in Cancer. *BioDrugs* **2009**, *23* (6), 361−375.
(70) Vollmer, J.; Krieg, A. M. Immunotherapeutic Applications of CpG Oligodeoxynucleotide TLR9 Agonists. *Adv. Drug Delivery Rev.* **2009**, *61* (3), 195−204.
(71) Wagner, H. Interactions between Bacterial CpG-DNA and TLR9 Bridge Innate and Adaptive Immunity. *Curr. Opin. Microbiol.* **2002**, *5* (1), 62−69.
(72) Klinman, D. M. Immunotherapeutic Uses of CpG Oligodeoxynucleotides. *Nat. Rev. Immunol* **2004**, *4* (4), 249−259.
(73) Krieg, A. M.; Yi, A.-K.; Matson, S.; Waldschmidt, T. J.; Bishop, G. A.; Teasdale, R.; Koretzky, G. A.; Klinman, D. M. CpG Motifs in Bacterial DNA Trigger Direct B-Cell Activation. *Nature* **1995**, *374* (6522), 546−549.
(74) Adamus, T.; Kortylewski, M. The Revival of CpG Oligonucleotide-Based Cancer Immunotherapies. *wo* **2018**, *2018* (1), 56−60.
(75) Urban-Wojciuk, Z.; Khan, M. M.; Oyler, B. L.; Fåhraeus, R.; Marek-Trzonkowska, N.; Nita-Lazar, A.; Hupp, T. R.; Goodlett, D. R. The Role of TLRs in Anti-Cancer Immunity and Tumor Rejection. *Front Immunol* **2019**, *10*, 2388.
(76) Yu, H.; Kortylewski, M.; Pardoll, D. Crosstalk between Cancer and Immune Cells: Role of STAT3 in the Tumour Microenvironment. *Nat. Rev. Immunol* **2007**, *7* (1), 41−51.
(77) Huang, B.; He, A.; Zhang, P.; Ma, X.; Yang, Y.; Wang, J.; Wang, J.; Zhang, W. Targeted Silencing of Genes Related to Acute Monocytic Leukaemia by CpG(B)-MLAA-34 SiRNA Conjugates. *J. Drug Targeting* **2020**, *28* (5), 516−524.
(78) Kortylewski, M.; Swiderski, P.; Herrmann, A.; Yu, H.; et al. In Vivo Delivery of SiRNA to Immune Cells by Conjugation to a TLR9 Agonist Enhances Antitumor Immune Responses. *Nat. Biotechnol.* **2009**, *27* (10), 925−932.
(79) Nechaev, S.; Gao, C.; Moreira, D.; Swiderski, P.; Jozwiak, A.; Kowolik, C. M.; Zhou, J.; Armstrong, B.; Raubitschek, A.; Rossi, J. J.; Kortylewski, M. Intracellular Processing of Immunostimulatory CpG-SiRNA: Toll-like Receptor 9 Facilitates SiRNA Dicing and Endosomal Escape. *J. Controlled Release* **2013**, *170* (3), 307−315.
(80) Zhang, Q.; Hossain, D. M. S.; Nechaev, S.; Kortylewski, M.; et al. TLR9-Mediated SiRNA Delivery for Targeting of Normal and Malignant Human Hematopoietic Cells in Vivo. *Blood* **2013**, *121* (8), 1304−1315.
(81) Hossain, D. M. S.; Pal, S. K.; Moreira, D.; Duttagupta, P.; Zhang, Q.; Won, H.; Jones, J.; D'Apuzzo, M.; Forman, S.; Kortylewski, M. TLR9-Targeted STAT3 Silencing Abrogates Immunosuppressive Activity of Myeloid-Derived Suppressor Cells from Prostate Cancer Patients. *Clin. Cancer Res.* **2015**, *21* (16), 3771−3782.
(82) de Jong. Drug Delivery and Nanoparticles: Applications and Hazards. *IJN* **2008**, 133.
(83) Zhang, L.; Gu, F.; Chan, J.; Wang, A.; Langer, R.; Farokhzad, O. Nanoparticles in Medicine: Therapeutic Applications and Developments. *Clin Pharmacol Ther* **2008**, *83* (5), 761−769.
(84) Wilson, B.; Geetha, K. M. Lipid Nanoparticles in the Development of MRNA Vaccines for COVID-19. *J. Drug Deliv Sci. Technol.* **2022**, *74*, 103553.
(85) Sun, D.; Lu, Z.-R. Structure and Function of Cationic and Ionizable Lipids for Nucleic Acid Delivery. *Pharm. Res.* **2023**, *40* (1), 27−46.
(86) Hajj, K. A.; Whitehead, K. A. Tools for Translation: Non-Viral Materials for Therapeutic MRNA Delivery. *Nat. Rev. Mater.* **2017**, *2* (10), 17056.
(87) Carrasco, M. J.; Alishetty, S.; Alameh, M.-G.; Said, H.; Wright, L.; Paige, M.; Soliman, O.; Weissman, D.; Cleveland, T. E.; Grishaev, A.; Buschmann, M. D. Ionization and Structural Properties of MRNA Lipid Nanoparticles Influence Expression in Intramuscular and Intravascular Administration. *Commun. Biol.* **2021**, *4* (1), 956.
(88) Hafez, I.; Maurer, N.; Cullis, P. On the Mechanism Whereby Cationic Lipids Promote Intracellular Delivery of Polynucleic Acids. *Gene Ther.* **2001**, *8* (15), 1188−1196.
(89) Tseng, Y.-C.; Mozumdar, S.; Huang, L. Lipid-Based Systemic Delivery of SiRNA. *Adv. Drug Delivery Rev.* **2009**, *61* (9), 721−731.
(90) Fenton, O. S.; Kauffman, K. J.; McClellan, R. L.; Appel, E. A.; Dorkin, J. R.; Tibbitt, M. W.; Heartlein, M. W.; DeRosa, F.; Langer, R.; Anderson, D. G. Bioinspired Alkenyl Amino Alcohol Ionizable Lipid Materials for Highly Potent In Vivo MRNA Delivery. *Adv. Mater.* **2016**, *28* (15), 2939−2943.
(91) Hashiba, K.; Sato, Y.; Taguchi, M.; Sakamoto, S.; Otsu, A.; Maeda, Y.; Shishido, T.; Murakawa, M.; Okazaki, A.; Harashima, H. Branching Ionizable Lipids Can Enhance the Stability, Fusogenicity, and Functional Delivery of MRNA. *Small Science* **2023**, *3* (1), 2200071.
(92) Han, X.; Zhang, H.; Butowska, K.; Swingle, K. L.; Alameh, M.-G.; Weissman, D.; Mitchell, M. J. An Ionizable Lipid Toolbox for RNA Delivery. *Nat. Commun.* **2021**, *12* (1), 7233.
(93) Maier, M. A.; Jayaraman, M.; Matsuda, S.; Akinc, A.; et al. Biodegradable Lipids Enabling Rapidly Eliminated Lipid Nanoparticles for Systemic Delivery of RNAi Therapeutics. *Mol. Ther* **2013**, *21* (8), 1570−1578.

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

(94) Kulkarni, J. A.; Witzigmann, D.; Leung, J.; Tam, Y. Y. C.; Cullis, P. R. On the Role of Helper Lipids in Lipid Nanoparticle Formulations of SiRNA. *Nanoscale* **2019**, *11* (45), 21733−21739.
(95) Eygeris, Y.; Gupta, M.; Kim, J.; Sahay, G. Chemistry of Lipid Nanoparticles for RNA Delivery. *Acc. Chem. Res.* **2022**, *55* (1), 2−12.
(96) Hald Albertsen, C.; Kulkarni, J. A.; Witzigmann, D.; Lind, M.; Petersson, K.; Simonsen, J. B. The Role of Lipid Components in Lipid Nanoparticles for Vaccines and Gene Therapy. *Adv. Drug Deliv Rev.* **2022**, *188*, 114416.
(97) Krause, M. R.; Regen, S. L. The Structural Role of Cholesterol in Cell Membranes: From Condensed Bilayers to Lipid Rafts. *Acc. Chem. Res.* **2014**, *47* (12), 3512−3521.
(98) Sebastiani, F.; Yanez Arteta, M.; Lerche, M.; Cárdenas, M.; et al. Apolipoprotein E Binding Drives Structural and Compositional Rearrangement of MRNA-Containing Lipid Nanoparticles. *ACS Nano* **2021**, *15* (4), 6709−6722.
(99) Jokerst, J. V.; Lobovkina, T.; Zare, R. N.; Gambhir, S. S. Nanoparticle PEGylation for Imaging and Therapy. *Nanomedicine* **2011**, *6* (4), 715−728.
(100) Butowska, K.; Han, X.; Gong, N.; El-Mayta, R.; Haley, R. M.; Xue, L.; Zhong, W.; Guo, W.; Wang, K.; Mitchell, M. J. Doxorubicin-Conjugated SiRNA Lipid Nanoparticles for Combination Cancer Therapy. *Acta Pharmaceutica Sinica B* **2023**, *13*, 1429.
(101) Anthiya, S.; Öztürk, S. C.; Yanik, H.; Alonso, M. J.; et al. Targeted SiRNA Lipid Nanoparticles for the Treatment of KRAS-Mutant Tumors. *J. Controlled Release* **2023**, *357*, 67−83.
(102) Zimmermann, C. M.; Baldassi, D.; Chan, K.; Merkel, O. M.; et al. Spray Drying SiRNA-Lipid Nanoparticles for Dry Powder Pulmonary Delivery. *J. Controlled Release* **2022**, *351*, 137−150.
(103) Zhou, J.-E.; Sun, L.; Liu, L.; Jia, Y.; Han, Y.; Shao, J.; Wang, J.; Wang, Y.; Yu, L.; Yan, Z. Hepatic Macrophage Targeted SiRNA Lipid Nanoparticles Treat Non-Alcoholic Steatohepatitis. *J. Controlled Release* **2022**, *343*, 175−186.
(104) Adams, D.; Gonzalez-Duarte, A.; O'Riordan, W. D.; Suhr, O. B.; et al. Patisiran, an RNAi Therapeutic, for Hereditary Transthyretin Amyloidosis. *N Engl J. Med.* **2018**, *379* (1), 11−21.
(105) Kanasty, R.; Dorkin, J. R.; Vegas, A.; Anderson, D. Delivery Materials for SiRNA Therapeutics. *Nat. Mater.* **2013**, *12* (11), 967−977.
(106) Xiao, T.; Xiao, Y.; Wang, W.; Tang, Y. Y.; Xiao, Z.; Su, M. Targeting EphA2 in Cancer. *J. Hematol Oncol* **2020**, *13* (1), 114.
(107) De Luca, A.; Federici, L.; De Canio, M.; Stella, L.; Caccuri, A. M. New Insights into the Mechanism of JNK1 Inhibition by Glutathione Transferase P1−1. *Biochemistry* **2012**, *51* (37), 7304−7312.
(108) O'Brien, Z.; Wang, L.; Majeti, B.; Clamme, J.; Baclig, R.; Chu, J.; Fong, S.; Harborth, J.; Ibarra, J.; Yin, H.; Yu, J.; Zhang, C.; Adami, R.; Zabludoff, S.; Ying, W. Abstract 5917: A Novel Lipid Nanoparticle (NBF-006) Encapsulating Glutathione S-Transferase P (GSTP) SiRNA for the Treatment of KRAS-Driven Non-Small Cell Lung Cancer. *Cancer Res.* **2018**, *78*, 5917−5917.
(109) Webb, C.; Ip, S.; Bathula, N. V.; Blakney, A. K.; et al. Current Status and Future Perspectives on MRNA Drug Manufacturing. *Mol. Pharmaceutics* **2022**, *19* (4), 1047−1058.
(110) Roces, C. B.; Lou, G.; Jain, N.; Abraham, S.; Thomas, A.; Halbert, G. W.; Perrie, Y. Manufacturing Considerations for the Development of Lipid Nanoparticles Using Microfluidics. *Pharmaceutics* **2020**, *12* (11), 1095.
(111) Akinc, A.; Maier, M. A.; Manoharan, M.; Cullis, P. R.; et al. The Onpattro Story and the Clinical Translation of Nanomedicines Containing Nucleic Acid-Based Drugs. *Nat. Nanotechnol.* **2019**, *14* (12), 1084−1087.
(112) Oliver, S. E.; Gargano, J. W.; Marin, M.; Dooling, K.; et al. The Advisory Committee on Immunization Practices' Interim Recommendation for Use of Moderna COVID-19 Vaccine — United States, December 2020. *MMWR Morb Mortal Wkly Rep* **2021**, *69* (51−52), 1653−1656.
(113) Polack, F. P.; Thomas, S. J.; Kitchin, N.; Zerbini, C.; et al. Safety and Efficacy of the BNT162b2MRNA Covid-19 Vaccine. *New England journal of medicine* **2020**, *383* (27), 2603−2615.
(114) Kumar, R.; Santa Chalarca, C. F.; Bockman, M. R.; Bruggen, C. V.; Grimme, C. J.; Dalal, R. J.; Hanson, M. G.; Hexum, J. K.; Reineke, T. M. Polymeric Delivery of Therapeutic Nucleic Acids. *Chem. Rev.* **2021**, *121* (18), 11527−11652.
(115) Tzeng, S. Y.; Green, J. J. Polymeric Nucleic Acid Delivery for Immunoengineering. *Current Opinion in Biomedical Engineering* **2018**, *7*, 42−50.
(116) Mitchell, M. J.; Billingsley, M. M.; Haley, R. M.; Wechsler, M. E.; Peppas, N. A.; Langer, R. Engineering Precision Nanoparticles for Drug Delivery. *Nat. Rev. Drug Discov* **2021**, *20* (2), 101−124.
(117) Peeler, D. J.; Sellers, D. L.; Pun, S. H. PH-Sensitive Polymers as Dynamic Mediators of Barriers to Nucleic Acid Delivery. *Bioconjugate Chem.* **2019**, *30* (2), 350−365.
(118) Valcourt, D. M.; Dang, M. N.; Scully, M. A.; Day, E. S. Nanoparticle-Mediated Co-Delivery of Notch-1 Antibodies and ABT-737 as a Potent Treatment Strategy for Triple-Negative Breast Cancer. *ACS Nano* **2020**, *14* (3), 3378−3388.
(119) Gomes, M. J.; Fernandes, C.; Martins, S.; Borges, F.; Sarmento, B. Tailoring Lipid and Polymeric Nanoparticles as SiRNA Carriers towards the Blood-Brain Barrier - from Targeting to Safe Administration. *J. Neuroimmune Pharmacol* **2017**, *12* (1), 107−119.
(120) Sur, S.; Rathore, A.; Dave, V.; Reddy, K. R.; Chouhan, R. S.; Sadhu, V. Recent Developments in Functionalized Polymer Nanoparticles for Efficient Drug Delivery System. *Nano-Structures & Nano-Objects* **2019**, *20*, 100397.
(121) Dahlman, J. E.; Barnes, C.; Khan, O. F.; Anderson, D. G.; et al. In Vivo Endothelial SiRNA Delivery Using Polymeric Nanoparticles with Low Molecular Weight. *Nat. Nanotechnol.* **2014**, *9* (8), 648−655.
(122) Hussein, W. M.; Cheong, Y. S.; Liu, C.; Liu, G.; Begum, A. A.; Attallah, M. A.; Moyle, P. M.; Torchilin, V. P.; Smith, R.; Toth, I. Peptide-Based Targeted Polymeric Nanoparticles for SiRNA Delivery. *Nanotechnology* **2019**, *30* (41), 415604.
(123) Jin, M.; Jin, G.; Kang, L.; Chen, L.; Gao, Z.; Huang, W. Smart Polymeric Nanoparticles with PH-Responsive and PEG-Detachable Properties for Co-Delivering Paclitaxel and Survivin SiRNA to Enhance Antitumor Outcomes. *IJN* **2018**, *13*, 2405−2426.
(124) Patil, Y.; Panyam, J. Polymeric Nanoparticles for SiRNA Delivery and Gene Silencing. *Int. J. Pharm.* **2009**, *367* (1), 195−203.
(125) Xue, L.; Yan, Y.; Kos, P.; Chen, X.; Siegwart, D. J. PEI Fluorination Reduces Toxicity and Promotes Liver-Targeted SiRNA Delivery. *Drug Delivery and Transl. Res.* **2021**, *11* (1), 255−260.
(126) Lynn, D. M.; Langer, R. Degradable Poly(β-Amino Esters): Synthesis, Characterization, and Self-Assembly with Plasmid DNA. *J. Am. Chem. Soc.* **2000**, *122* (44), 10761−10768.
(127) Hong, C. A.; Eltoukhy, A. A.; Lee, H.; Langer, R.; Anderson, D. G.; Nam, Y. S. Dendrimeric SiRNA for Efficient Gene Silencing. *Angew. Chem., Int. Ed.* **2015**, *54* (23), 6740−6744.
(128) Inchanalkar, S.; Deshpande, N. U.; Kasherwal, V.; Jayakannan, M.; Balasubramanian, N. Polymer Nanovesicle-Mediated Delivery of MLN8237 Preferentially Inhibits Aurora Kinase A To Target RalA and Anchorage-Independent Growth in Breast Cancer Cells. *Mol. Pharmaceutics* **2018**, *15* (8), 3046−3059.
(129) Dosta, P.; Demos, C.; Ramos, V.; Kang, D. W.; Kumar, S.; Jo, H.; Borrós, S. Delivery of SiRNA to Endothelial Cells In Vivo Using Lysine/Histidine Oligopeptide-Modified Poly(β-Amino Ester) Nanoparticles. *Cardiovasc Eng. Tech* **2021**, *12* (1), 114−125.
(130) Dosta, P.; Segovia, N.; Cascante, A.; Ramos, V.; Borrós, S. Surface Charge Tunability as a Powerful Strategy to Control Electrostatic Interaction for High Efficiency Silencing, Using Tailored Oligopeptide-Modified Poly(Beta-Amino Ester)s (PBAEs). *Acta Biomaterialia* **2015**, *20*, 82−93.
(131) Tzeng, S. Y.; Hung, B. P.; Grayson, W. L.; Green, J. J. Cystamine-Terminated Poly(Beta-Amino Ester)s for SiRNA Delivery to Human Mesenchymal Stem Cells and Enhancement of Osteogenic Differentiation. *Biomaterials* **2012**, *33* (32), 8142−8151.

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197

(132) Tang, Y.; Liu, Y.; Xie, Y.; Chen, J.; Dou, Y. Apoptosis of A549 Cells by Small Interfering RNA Targeting Survivin Delivery Using Poly-β-Amino Ester/Guanidinylated O-Carboxymethyl Chitosan Nanoparticles. *Asian Journal of Pharmaceutical Sciences* **2020**, *15* (1), 121−128.
(133) Vandenbroucke, R. E.; De Geest, B. G.; Bonné, S.; Sanders, N. N.; et al. Prolonged Gene Silencing in Hepatoma Cells and Primary Hepatocytes after Small Interfering RNA Delivery with Biodegradable Poly(B-amino Esters). *J. Gene Med.* **2008**, *10* (7), 783−794.
(134) Lee, J. S.; Deng, X.; Han, P.; Cheng, J. Dual Stimuli-Responsive Poly(β-Amino Ester) Nanoparticles for On-Demand Burst Release: Dual Stimuli-Responsive Poly(β-Amino Esters) Nanoparticles. *Macromol. Biosci.* **2015**, *15* (9), 1314−1322.
(135) Segovia, N.; Pont, M.; Oliva, N.; Ramos, V.; Borrós, S.; Artzi, N. Hydrogel Doped with Nanoparticles for Local Sustained Release of SiRNA in Breast Cancer. *Adv. Healthcare Mater.* **2015**, *4* (2), 271−280.
(136) Smith, A. E.; Sizovs, A.; Grandinetti, G.; Xue, L.; Reineke, T. M. Diblock Glycopolymers Promote Colloidal Stability of Polyplexes and Effective PDNA and SiRNA Delivery under Physiological Salt and Serum Conditions. *Biomacromolecules* **2011**, *12* (8), 3015−3022.
(137) Van Bruggen, C.; Hexum, J. K.; Tan, Z.; Dalal, R. J.; Reineke, T. M. Nonviral Gene Delivery with Cationic Glycopolymers. *Acc. Chem. Res.* **2019**, *52* (5), 1347−1358.
(138) Sizovs, A.; Xue, L.; Tolstyka, Z. P.; Ingle, N. P.; Wu, Y.; Cortez, M.; Reineke, T. M. Poly(Trehalose): Sugar-Coated Nanocomplexes Promote Stabilization and Effective Polyplex-Mediated SiRNA Delivery. *J. Am. Chem. Soc.* **2013**, *135* (41), 15417−15424.
(139) Kwak, E.; Kim, T.; Yang, K.; Kim, Y. M.; Han, H. S.; Park, K. H.; Choi, K. Y.; Roh, Y. H. Surface-Functionalized Polymeric SiRNA Nanoparticles for Tunable Targeting and Intracellular Delivery to Hematologic Cancer Cells. *Biomacromolecules* **2022**, *23* (6), 2255−2263.
(140) Li, H.; Yu, S. S.; Miteva, M.; Nelson, C. E.; Werfel, T.; Giorgio, T. D.; Duvall, C. L. Matrix Metalloproteinase Responsive, Proximity-Activated Polymeric Nanoparticles for SiRNA Delivery. *Adv. Funct. Mater.* **2013**, *23* (24), 3040−3052.
(141) He, C.; Yin, L.; Tang, C.; Yin, C. Trimethyl Chitosan-Cysteine Nanoparticles for Systemic Delivery of TNF-α SiRNA via Oral and Intraperitoneal Routes. *Pharm. Res.* **2013**, *30* (10), 2596−2606.
(142) Song, Y.; Wu, Y.; Xu, L.; Jiang, T.; Tang, C.; Yin, C. Caveolae-Mediated Endocytosis Drives Robust SiRNA Delivery of Polymeric Nanoparticles to Macrophages. *ACS Nano* **2021**, *15* (5), 8267−8282.
(143) Guţoaia, A.; Schuster, L.; Margutti, S.; Laufer, S.; Schlosshauer, B.; Krastev, R.; Stoll, D.; Hartmann, H. Fine-Tuned PEGylation of Chitosan to Maintain Optimal SiRNA-Nanoplex Bioactivity. *Carbohydr. Polym.* **2016**, *143*, 25−34.
(144) Nguyen, J.; Steele, T. W. J.; Merkel, O.; Reul, R.; Kissel, T. Fast Degrading Polyesters as SiRNA Nano-Carriers for Pulmonary Gene Therapy. *J. Controlled Release* **2008**, *132* (3), 243−251.
(145) Steinbach, J. M.; Weller, C. E.; Booth, C. J.; Saltzman, W. M. Polymer Nanoparticles Encapsulating SiRNA for Treatment of HSV-2 Genital Infection. *J. Controlled Release* **2012**, *162* (1), 102−110.
(146) Zuckerman, J. E.; Gritli, I.; Tolcher, A.; Heidel, J. D.; Lim, D.; Morgan, R.; Chmielowski, B.; Ribas, A.; Davis, M. E.; Yen, Y. Correlating Animal and Human Phase Ia/Ib Clinical Data with CALAA-01, a Targeted, Polymer-Based Nanoparticle Containing SiRNA. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111* (31), 11449−11454.
(147) Golan, T.; Khvalevsky, E. Z.; Hubert, A.; Galun, E.; et al. RNAi Therapy Targeting KRAS in Combination with Chemotherapy for Locally Advanced Pancreatic Cancer Patients. *Oncotarget* **2015**, *6* (27), 24560−24570.
(148) Belda-Iniesta, C.; Ibáñez de Cáceres, I.; Barriuso, J.; de Castro Carpeño, J.; González Barón, M.; Feliú, J. Molecular Biology of Pancreatic Cancer. *Clin Transl Oncol* **2008**, *10* (9), 530−537.
(149) Stawikowski, M.; Fields, G. B. Introduction to Peptide Synthesis. In *Current Protocols in Protein Science*; 2012; Vol *69*, Unit-18.1. DOI: 10.1002/0471140864.ps1801s69.
(150) Temsamani, J.; Vidal, P. The Use of Cell-Penetrating Peptides for Drug Delivery. *Drug Discovery Today* **2004**, *9* (23), 1012−1019.
(151) Silva, S.; Almeida, A. J.; Vale, N. Combination of Cell-Penetrating Peptides with Nanoparticles for Therapeutic Application: A Review. *Biomolecules* **2019**, *9* (1), 22.
(152) Gazit, E.; Lee, W.-J.; Brey, P. T.; Shai, Y. Mode of Action of the Antibacterial Cecropin B2: A Spectrofluorometric Study. *Biochemistry* **1994**, *33* (35), 10681−10692.
(153) Lehto, T.; Ezzat, K.; Wood, M. J. A.; EL Andaloussi, S. Peptides for Nucleic Acid Delivery. *Adv. Drug Delivery Rev.* **2016**, *106*, 172−182.
(154) Boisguérin, P.; Konate, K.; Josse, E.; Vivès, E.; Deshayes, S. Peptide-Based Nanoparticles for Therapeutic Nucleic Acid Delivery. *Biomedicines* **2021**, *9* (5), 583.
(155) Alhakamy, N. A.; Dhar, P.; Berkland, C. J. Charge Type, Charge Spacing, and Hydrophobicity of Arginine-Rich Cell-Penetrating Peptides Dictate Gene Transfection. *Mol. Pharmaceutics* **2016**, *13* (3), 1047−1057.
(156) EL Andaloussi, S.; Lehto, T.; Mäger, I.; Langel, D. C.; et al. Design of a Peptide-Based Vector, PepFect6, for Efficient Delivery of SiRNA in Cell Culture and Systemically in Vivo. *Nucleic Acids Res.* **2011**, *39* (9), 3972−3987.
(157) Rajeswari, M. R.; Montenay-Garestier, T.; Helene, C. Does Tryptophan Intercalate in DNA? A Comparative Study of Peptide Binding to Alternating and Non-Alternating A.Cntdot.T Sequences. *Biochemistry* **1987**, *26* (21), 6825−6831.
(158) Jobin, M.-L.; Blanchet, M.; Henry, S.; Chaignepain, S.; Manigand, C.; Castano, S.; Lecomte, S.; Burlina, F.; Sagan, S.; Alves, I. D. The Role of Tryptophans on the Cellular Uptake and Membrane Interaction of Arginine-Rich Cell Penetrating Peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2015**, *1848* (2), 593−602.
(159) Li, Y.; Li, Y.; Wang, X.; Lee, R. J.; Teng, L. Fatty Acid Modified Octa-Arginine for Delivery of SiRNA. *Int. J. Pharm.* **2015**, *495* (1), 527−535.
(160) McCarthy, H. O.; McCaffrey, J.; McCrudden, C. M.; Robson, T.; et al. Development and Characterization of Self-Assembling Nanoparticles Using a Bio-Inspired Amphipathic Peptide for Gene Delivery. *J. Controlled Release* **2014**, *189*, 141−149.
(161) Bennett, R.; Yakkundi, A.; McKeen, H. D.; McClements, L.; McKeogh, T. J.; McCrudden, C. M.; Arthur, K.; Robson, T.; McCarthy, H. O. RALA-Mediated Delivery of FKBPL Nucleic Acid Therapeutics. *Nanomedicine* **2015**, *10* (19), 2989−3001.
(162) Mulholland, E. J.; Ali, A.; Robson, T.; Dunne, N. J.; McCarthy, H. O. Delivery of RALA/SiFKBPL Nanoparticles via Electrospun Bilayer Nanofibres: An Innovative Angiogenic Therapy for Wound Repair. *J. Controlled Release* **2019**, *316*, 53−65.
(163) Jafari, M.; Xu, W.; Naahidi, S.; Chen, B.; Chen, P. A New Amphipathic, Amino-Acid-Pairing (AAP) Peptide as SiRNA Delivery Carrier: Physicochemical Characterization and in Vitro Uptake. *J. Phys. Chem. B* **2012**, *116* (44), 13183−13191.
(164) Xu, W.; Pan, R.; Zhao, D.; Chen, P.; et al. Design and Evaluation of Endosomolytic Biocompatible Peptides as Carriers for SiRNA Delivery. *Mol. Pharmaceutics* **2015**, *12* (1), 56−65.
(165) Xu, W.; Jafari, M.; Yuan, F.; Chen, P.; et al. In Vitro and in Vivo Therapeutic SiRNA Delivery Induced by a Tryptophan-Rich Endosomolytic Peptide. *J. Mater. Chem. B* **2014**, *2* (36), 6010.
(166) Konate, K.; Crombez, L.; Deshayes, S.; Decaffmeyer, M.; Thomas, A.; Brasseur, R.; Aldrian, G.; Heitz, F.; Divita, G. Insight into the Cellular Uptake Mechanism of a Secondary Amphipathic Cell-Penetrating Peptide for SiRNA Delivery. *Biochemistry* **2010**, *49* (16), 3393−3402.
(167) Konate, K.; Dussot, M.; Aldrian, G.; Vaissière, A.; Viguier, V.; Neira, I. F.; Couillaud, F.; Vivès, E.; Boisguerin, P.; Deshayes, S. Peptide-Based Nanoparticles to Rapidly and Efficiently "Wrap 'n Roll" SiRNA into Cells. *Bioconjugate Chem.* **2019**, *30* (3), 592−603.
(168) Konate, K.; Josse, E.; Tasic, M.; Redjatti, K.; Aldrian, G.; Deshayes, S.; Boisguérin, P.; Vivès, E. WRAP-Based Nanoparticles for SiRNA Delivery: A SAR Study and a Comparison with Lipid-Based Transfection Reagents. *J. Nanobiotechnol* **2021**, *19* (1), 236.

(169) Ren, Y.; Cheung, H. W.; von Maltzhan, G.; Bhatia, S. N. Targeted Tumor-Penetrating SiRNA Nanocomplexes for Credentialing the Ovarian Cancer Oncogene *ID4*. *Sci. Transl. Med.* **2012**, *4* (147), 1 DOI: 10.1126/scitranslmed.3003778.
(170) Asai, T.; Tsuzuku, T.; Takahashi, S.; Okamoto, A.; Dewa, T.; Nango, M.; Hyodo, K.; Ishihara, H.; Kikuchi, H.; Oku, N. Cell-Penetrating Peptide-Conjugated Lipid Nanoparticles for SiRNA Delivery. *Biochem. Biophys. Res. Commun.* **2014**, *444* (4), 599−604.
(171) Kwon, E. J.; Bergen, J. M.; Pun, S. H. Application of an HIV Gp41-Derived Peptide for Enhanced Intracellular Trafficking of Synthetic Gene and SiRNA Delivery Vehicles. *Bioconjugate Chem.* **2008**, *19* (4), 920−927.
(172) Xu, X.; Wu, J.; Liu, Y.; Shi, J.; et al. Multifunctional Envelope-Type SiRNA Delivery Nanoparticle Platform for Prostate Cancer Therapy. *ACS Nano* **2017**, *11* (3), 2618−2627.
(173) Kim, G. C.; Cheon, D. H.; Lee, Y. Challenge to Overcome Current Limitations of Cell-Penetrating Peptides. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **2021**, *1869* (4), 140604.
(174) Freimann, K.; Arukuusk, P.; Kurrikoff, K. M.; Langel, D. C.; et al. Optimization of in Vivo DNA Delivery with NickFect Peptide Vectors. *J. Controlled Release* **2016**, *241*, 135−143.
(175) Aldrian, G.; Vaissière, A.; Konate, K.; Boisguerin, P.; et al. PEGylation Rate Influences Peptide-Based Nanoparticles Mediated SiRNA Delivery in Vitro and in Vivo. *J. Controlled Release* **2017**, *256*, 79−91.
(176) Kirkpatrick, K.; Ogunkolade, W.; Elkak, A.; Bustin, S.; Jenkins, P.; Ghilchik, M.; Mokbel, K. The MRNA Expression of Cyclo-Oxygenase-2 (COX-2) and Vascular Endothelial Growth Factor (VEGF) in Human Breast Cancer. *Current Medical Research and Opinion* **2002**, *18* (4), 237−241.
(177) Syed, V. TGF-β Signaling in Cancer. *J. Cell. Biochem.* **2016**, *117* (6), 1279−1287.
(178) van Niel, G.; Porto-Carreiro, I.; Simoes, S.; Raposo, G. Exosomes: A Common Pathway for a Specialized Function. *Journal of Biochemistry* **2006**, *140* (1), 13−21.
(179) EL Andaloussi, S.; Mäger, I.; Breakefield, X. O.; Wood, M. J. A. Extracellular Vesicles: Biology and Emerging Therapeutic Opportunities. *Nat. Rev. Drug Discov* **2013**, *12* (5), 347−357.
(180) El-Andaloussi, S.; Lee, Y.; Lakhal-Littleton, S.; Li, J.; Seow, Y.; Gardiner, C.; Alvarez-Erviti, L.; Sargent, I. L.; Wood, M. J. A. Exosome-Mediated Delivery of SiRNA in Vitro and in Vivo. *Nat. Protoc* **2012**, *7* (12), 2112−2126.
(181) Amiri, A.; Bagherifar, R.; Ansari Dezfouli, E.; Kiaie, S. H.; Jafari, R.; Ramezani, R. Exosomes as Bio-Inspired Nanocarriers for RNA Delivery: Preparation and Applications. *Journal of Translational Medicine* **2022**, *20* (1), 125.
(182) Faruqu, F. N.; Xu, L.; Al-Jamal, K. T. Preparation of Exosomes for SiRNA Delivery to Cancer Cells. *J. Vis Exp* **2018**, 142.
(183) Wood, M. J.; O'Loughlin, A. J; Lakhal, S. Exosomes and the blood-brain barrier: implications for neurological diseases. *Therapeutic Delivery* **2011**, *2*, 1095.
(184) Perets, N.; Betzer, O.; Shapira, R.; Brenstein, S.; Angel, A.; Sadan, T.; Ashery, U.; Popovtzer, R.; Offen, D. Golden Exosomes Selectively Target Brain Pathologies in Neurodegenerative and Neurodevelopmental Disorders. *Nano Lett.* **2019**, *19* (6), 3422−3431.
(185) Wahlgren, J.; Karlson, T. D. L.; Brisslert, M.; Vaziri Sani, F.; Telemo, E.; Sunnerhagen, P.; Valadi, H. Plasma Exosomes Can Deliver Exogenous Short Interfering RNA to Monocytes and Lymphocytes. *Nucleic Acids Res.* **2012**, *40* (17), No. e130.
(186) Lunavat, T. R.; Jang, S. C.; Nilsson, L.; Park, H. T.; Repiska, G.; Lässer, C.; Nilsson, J. A.; Gho, Y. S.; Lötvall, J. RNAi Delivery by Exosome-Mimetic Nanovesicles - Implications for Targeting c-Myc in Cancer. *Biomaterials* **2016**, *102*, 231−238.
(187) Shtam, T. A.; Kovalev, R. A.; Varfolomeeva, E. Y.; Makarov, E. M.; Kil, Y. V.; Filatov, M. V. Exosomes Are Natural Carriers of Exogenous SiRNA to Human Cells in Vitro. *Cell Communication and Signaling* **2013**, *11* (1), 88.
(188) Evers, M. J. W.; van de Wakker, S. I.; de Groot, E. M.; de Jong, O. G.; Gitz-François, J. J. J.; Seinen, C. S.; Sluijter, J. P. G.; Schiffelers, R. M.; Vader, P. Functional SiRNA Delivery by Extracellular Vesicle-Liposome Hybrid Nanoparticles. *Adv. Healthcare Mater.* **2022**, *11* (5), 2101202.
(189) Didiot, M.-C.; Hall, L. M.; Coles, A. H.; Khvorova, A.; et al. Exosome-Mediated Delivery of Hydrophobically Modified SiRNA for Huntingtin MRNA Silencing. *Molecular Therapy* **2016**, *24* (10), 1836−1847.
(190) Huang, W.; Qu, M.; Li, L.; Liu, T.; Lin, M.; Yu, X. SiRNA in MSC-Derived Exosomes Silences CTGF Gene for Locomotor Recovery in Spinal Cord Injury Rats. *Stem Cell Res. Ther* **2021**, *12* (1), 334.
(191) Lentz, T. L. Rabies Virus Binding to an Acetylcholine Receptor α-Subunit Peptide. *J. Mol. Recognit.* **1990**, *3* (2), 82−88.
(192) Alvarez-Erviti, L.; Seow, Y.; Yin, H.; Betts, C.; Lakhal, S.; Wood, M. J A Delivery of SiRNA to the Mouse Brain by Systemic Injection of Targeted Exosomes. Nat Biotechnol 29:341−345. *Nature biotechnology* **2011**, *29*, 341−345.
(193) Liu, Y.; Li, D.; Liu, Z.; Zhang, C.-Y.; et al. Targeted Exosome-Mediated Delivery of Opioid Receptor Mu SiRNA for the Treatment of Morphine Relapse. *Sci. Rep* **2015**, *5* (1), 17543.
(194) Zhou, W.; Zhou, Y.; Chen, X.; Ning, T.; Chen, H.; Guo, Q.; Zhang, Y.; Liu, P.; Zhang, Y.; Li, C.; Chu, Y.; Sun, T.; Jiang, C. Pancreatic Cancer-Targeting Exosomes for Enhancing Immunotherapy and Reprogramming Tumor Microenvironment. *Biomaterials* **2021**, *268*, 120546.
(195) Mendt, M.; Kamerkar, S.; Sugimoto, H.; Kalluri, R. Generation and Testing of Clinical-Grade Exosomes for Pancreatic Cancer. *JCI Insight* **2018**, *3* (8), No. e99263.
(196) Colao, I. L.; Corteling, R.; Bracewell, D.; Wall, I. Manufacturing Exosomes: A Promising Therapeutic Platform. *Trends in Molecular Medicine* **2018**, *24* (3), 242−256.
(197) Ahn, S.-H.; Ryu, S.-W.; Choi, H.; You, S.; Park, J.; Choi, C. Manufacturing Therapeutic Exosomes: From Bench to Industry. *Mol. Cells* **2022**, *45* (5), 284−290.
(198) Zhang, Y.-K.; Wang, Y.-J.; Gupta, P.; Chen, Z.-S. Multidrug Resistance Proteins (MRPs) and Cancer Therapy. *AAPS J.* **2015**, *17* (4), 802−812.
(199) Dönmez, Y.; Gündüz, U. Reversal of Multidrug Resistance by Small Interfering RNA (SiRNA) in Doxorubicin-Resistant MCF-7 Breast Cancer Cells. *Biomedicine & Pharmacotherapy* **2011**, *65* (2), 85−89.
(200) Luo, K.; Gao, Y.; Yin, S.; Yao, Y.; Yu, H.; Wang, G.; Li, J. Co-Delivery of Paclitaxel and STAT3 SiRNA by a Multifunctional Nanocomplex for Targeted Treatment of Metastatic Breast Cancer. *Acta Biomaterialia* **2021**, *134*, 649−663.
(201) Sun, W.; Chen, X.; Xie, C.; Wang, Y.; Lin, L.; Zhu, K.; Shuai, X. Co-Delivery of Doxorubicin and Anti-BCL-2 SiRNA by PH-Responsive Polymeric Vector to Overcome Drug Resistance in In Vitro and In Vivo HepG2 Hepatoma Model. *Biomacromolecules* **2018**, *19* (6), 2248−2256.
(202) Meel, R.; Chen, S.; Zaifman, J.; Kulkarni, J. A.; Zhang, X. R. S.; Tam, Y. K.; Bally, M. B.; Schiffelers, R. M.; Ciufolini, M. A.; Cullis, P. R.; Tam, Y. Y. C. Modular Lipid Nanoparticle Platform Technology for SiRNA and Lipophilic Prodrug Delivery. *Small* **2021**, *17* (37), 2103025.
(203) Tibbitt, M. W.; Dahlman, J. E.; Langer, R. Emerging Frontiers in Drug Delivery. *J. Am. Chem. Soc.* **2016**, *138* (3), 704−717.
(204) Saunders, N. R. M.; Paolini, M. S.; Fenton, O. S.; Langer, R.; et al. A Nanoprimer To Improve the Systemic Delivery of SiRNA and MRNA. *Nano Lett.* **2020**, *20* (6), 4264−4269.
(205) McEvoy, J. P.; Freudenreich, O. Issues in the Design and Conductance of Clinical Trials. In *Animal and Translational Models for CNS Drug Discovery*; Elsevier, 2008; pp 75−95. DOI: 10.1016/B978-0-12-373861-5.00003-5.
(206) *Alnylam Submits CTA Application for ALN-APP, an Investigational RNAi Therapeutic for the Treatment of Alzheime*. Investor

Relations | Alnylam Pharmaceuticals, Inc. https://investors.alnylam.com/press-release?id=26296 (accessed 2023-04-27).
(207) *SLN124, a GalNAc Conjugated 19-Mer Double-Stranded SiRNA Reduces Iron and Increases Hepcidin Levels of Healthy Volunteers in a Phase 1 Clinical Study | Blood | American Society of Hematology*. https://ashpublications.org/blood/article/138/Supplement%201/2009/482523/SLN124-a-GalNAc-Conjugated-19-Mer-Double-Stranded (accessed 2023-04-27).
(208) Rider, D. A.; Eisermann, M.; Löffler, K.; Campion, G.; et al. Pre-Clinical Assessment of SLN360, a Novel SiRNA Targeting LPA, Developed to Address Elevated Lipoprotein (a) in Cardiovascular Disease. *Atherosclerosis* **2022**, *349*, 240−247.
(209) Montali, I.; Vecchi, A.; Rossi, M.; Tiezzi, C.; Penna, A.; Reverberi, V.; Laccabue, D.; Missale, G.; Boni, C.; Fisicaro, P. Antigen Load and T Cell Function: A Challenging Interaction in HBV Infection. *Biomedicines* **2022**, *10* (6), 1224.
(210) Ranasinghe, P.; Addison, M. L.; Webb, D. J. Small Interfering RNA Therapeutics in Hypertension: A Viewpoint on Vasopressor and Vasopressor-Sparing Strategies for Counteracting Blood Pressure Lowering by Angiotensinogen-Targeting Small Interfering RNA. *Journal of the American Heart Association* **2022**, *11* (20), No. e027694.
(211) *Alnylam Development Pipeline of Investigational RNAi Therapeutics*. https://www.alnylam.com/alnylam-rnai-pipeline (accessed 2023-04-27).
(212) *Information About Dicerna's Lead Development Candidate, Nedosiran*. Dicerna Pharmaceuticals. https://dicerna.com/pipeline/nedosiran/ (accessed 2023-04-27).
(213) Badri, P.; Jiang, X.; Borodovsky, A.; Najafian, N.; Kim, J.; Clausen, V. A.; Goel, V.; Habtemariam, B.; Robbie, G. J. Pharmacokinetic and Pharmacodynamic Properties of Cemdisiran, an RNAi Therapeutic Targeting Complement Component 5, in Healthy Subjects and Patients with Paroxysmal Nocturnal Hemoglobinuria. *Clin Pharmacokinet* **2021**, *60* (3), 365−378.
(214) *Pipeline*. Arrowhead Pharmaceuticals, Inc. https://arrowheadpharma.com/pipeline/ (accessed 2023-04-27).
(215) Cui, H.; Zhu, X.; Li, S.; Wang, P.; Fang, J. Liver-Targeted Delivery of Oligonucleotides with N-Acetylgalactosamine Conjugation. *ACS Omega* **2021**, *6* (25), 16259−16265.
(216) *Products-Suzhou Ribo Life Science Co., Ltd*. https://www.ribolia.com/En/major-procucts (accessed 2023-04-27).
(217) *EphA2 Gene Targeting using Neutral Liposomal Small Interfering RNA Delivery (IND# 72924): A Phase I Clinical Trial*. MD Anderson Cancer Center. https://www.mdanderson.org/patients-family/diagnosis-treatment/clinical-trials/clinical-trials-index/clinical-trials-detail.html (accessed 2023-04−27).
(218) Sirnaomics: A Unique, Clinical-Phase RNA Therapeutic Innovator on the Global Stage. Press Release | Sirnaomics. https://sirnaomics.com/en/news-room/press-release/20161107/ (accessed 2023-07-06)
(219) Hu, B.; Zhong, L.; Weng, Y.; Peng, L.; Huang, Y.; Zhao, Y.; Liang, X.-J. Therapeutic SiRNA: State of the Art. *Sig Transduct Target Ther* **2020**, *5* (1), 101.
(220) *Oneness Biotech Co., Ltd*. https://www.onenessbio.com/en/ (accessed 2023-04-27).
(221) *News | ADARx Pharmaceuticals*. ADARX. https://www.adarx.com/news/ (accessed 2023-04-27).

https://doi.org/10.1021/acs.bioconjchem.3c00205
Bioconjugate Chem. 2023, 34, 1177−1197