NUCLEIC ACID THERAPEUTICS
Volume 28, Number 3, 2018

DOI: 10.1089/nat.2018.0725

Improving siRNA Delivery *In Vivo* Through Lipid Conjugation

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RNA interference (RNAi)-based therapeutics are approaching clinical approval for genetically defined diseases. Current clinical success is a result of significant innovations in the development of chemical architectures that support sustained, multi-month efficacy *in vivo* following a single administration. Conjugate-mediated delivery has established itself as the most promising platform for safe and targeted small interfering RNA (siRNA) delivery. Lipophilic conjugates represent a major class of modifications that improve siRNA pharmacokinetics and enable efficacy in a broad range of tissues. Here, we review current literature and define key features and limitations of this approach for *in vivo* modulation of gene expression.

Keywords: siRNA, lipid conjugation, delivery

Introduction

Oligonucleotide drugs hold tremendous promise to treat genetically defined diseases inaccessible to conventional small molecule and antibody-based therapies. The primary challenge facing the clinical development of small interfering RNAs (siRNA) has been overcoming barriers that impede *in vivo* delivery. siRNAs are large, polyanionic macromolecules with intrinsically poor pharmacological properties. Unmodified siRNAs have a half-life of less than 5 min in circulation, and they do not permeate intact cellular membranes. Both nanoscale and molecular-scale delivery strategies have been employed to surmount these limitations.

Lipid- and polymer-based nanoparticles prolong circulation time, stability, and bioavailability of siRNAs *in vivo* (reviewed in [1]). A lipid-formulated siRNA will likely be the first drug in this class to receive FDA approval (www.alnylam.com). However, nanoparticle-based delivery is typically limited to clearance organs with fenestrated or discontinuous endothelium (e.g., liver, spleen, and certain tumors) and may necessitate intravenous (IV) administration [2]. Molecular-scale delivery of siRNAs conjugated to small ligands, carbohydrates, cell-penetrating peptides, aptamers, or lipids offers a simple and effective alternative to carrier-based methods. The most clinically advanced siRNA conjugate is a trivalent N-acetylgalactosamine (GalNAc)-siRNA, which binds with high selectivity to the asialoglycoprotein receptor on hepatocytes. GalNAc-siRNAs trigger potent and durable (6–9 month) gene silencing in patients ([3], reviewed in [4]). The second widely used class of molecular siRNA conjugates are lipids, which greatly enhance circulation time and promote local and systemic delivery and efficacy. The goal of this review is to identify the key characteristics, factors, and limitations of lipophilic siRNAs as an approach for *in vivo* delivery.

Cholesterol conjugation promotes siRNA delivery and distribution in vivo

One of the most well-studied lipid moieties enabling efficient cellular and tissue delivery following direct oligonucleotide conjugation is cholesterol. Cholesterol constitutes 15%–30% of cellular membranes [5] and spontaneously intercalates into lipid membranes upon co-incubation with cells, fulfilling its biological role of supporting membrane structure and fluidity [6]. There are two primary mechanisms by which cholesterol conjugation promotes siRNA uptake. In the first, the cholesterol conjugate intercalates into the plasma membrane and oligonucleotide is internalized by endocytosis. In the second, the cholesterol conjugate binds circulating plasma lipoproteins and siRNA uptake is driven by interactions with lipoprotein receptors [7]. *In vitro*, cholesterol-conjugated siRNA is rapidly internalized—within seconds of exposure—by any cell type via EEA1-associated endocytosis [8]. Because internalization is rapid and only partially inhibited by serum, it is likely dominated by direct membrane incorporation. These features positioned cholesterol-conjugated siRNAs as ideal candidates for local *in vivo* delivery, particularly to the skin [9–11], eye [12,13], and brain parenchyma [14–16]. Rapid membrane association and uptake upon local administration *in vivo* typically leads to restricted distribution from the site of injection (e.g., >1 cm in mouse striatum) [16].

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Cholesterol was the first reported conjugate to be used for systemic delivery of siRNA [17]. This study showed that cholesterol conjugation dramatically improved the pharmacokinetic parameters of siRNA, including half-life and bioavailability in serum. Cholesterol-conjugated siRNAs were hypothesized to target the liver due to its discontinuous, fenestrated endothelium, and to the high capacity for hepatocytes to internalize cholesterol in native lipid trafficking pathways [17]. However, cholesterol and other lipid-conjugated oligonucleotides were subsequently discovered to bind circulating lipoproteins, for example, low density lipoprotein (LDL), and their uptake by hepatocytes *in vivo* was found to depend on LDL receptor expression [18].

Several cholesterol-conjugated siRNAs have advanced to clinical evaluation. RXi Pharmaceuticals' cholesterol-conjugated siRNA RXI-109 was evaluated in several Phase II clinical studies (www.rxipharma.com/technology/rxi-109) for its ability to reduce hypertrophic scarring. RXI-109 targets connective tissue growth factor, a key regulator of cellular pathways related to fibrosis. RXI-109 was also evaluated in a Phase I/II trial in the eyes of age-related macular degeneration patients at risk for subretinal fibrosis. Arrowhead Pharmaceuticals used cholesterol-conjugated oligonucleotides to target the liver to treat hepatitis B. In this case, cholesterol-conjugated siRNAs were co-administrated with a polymeric carrier that enhanced endosomal release and *in vivo* potency [19]. This trial was later discontinued when the cationic formulation alone was found to cause toxicity in non-human primates. We have summarized the main published *in vivo* work on the local and systemic delivery of cholesterol-conjugate siRNAs in Table 1.

Impact of siRNA chemical architecture on lipid conjugate-mediated delivery

Although cholesterol conjugation broadly promotes cellular internalization, the nucleic acid modifications also significantly affect the overall efficacy of siRNA *in vivo*. The relative potency, safety, and duration of conjugated siRNAs are predominantly dictated by the chemical architecture and siRNA stabilization strategy (Table 1). Because most early studies were performed using minimally modified siRNAs, high doses or repetitive/continuous administration was necessary to achieve silencing with a relatively limited duration of effect. Recently, fully chemically stabilized siRNA scaffolds that are compatible with Ago2 loading (and assembly of the RNA-induced silencing complex) have allowed durable silencing by a single, bolus injection of lipid-conjugated siRNAs.

Completely unmodified siRNAs are rapidly degraded in serum by endo- and exonucleases. A wide variety of chemical modifications to the phosphodiester backbone and ribose have been explored to protect siRNAs from nucleases (reviewed in [20]; Fig. 1). Early studies of lipid conjugate-mediated delivery *in vivo* used siRNA scaffolds that were minimally modified with terminal phosphorothioate (PS) and 2′-*O*-methyl modifications to shield the 3′ single-stranded overhang. In these studies, repetitive, high doses (cumulatively 150 mg/kg delivered over 3 days) were required to silence ApoB in liver and jejunum of mice [17], but limited potency blocked progression toward clinical development. Nevertheless, this work provided a critical proof-of-concept for the use of lipophilic conjugates for *in vivo* delivery. Subsequent research on lipid conjugation utilized siRNAs where all pyrimidine nucleotides were modified with 2′-*O*-methyl or 2′-fluoro [21]. In one case, a single high-dose injection (50 mg/kg) was sufficient to induce ~50% gene silencing in the liver using either cholesterol or the saturated fatty acid docosanoic acid (DCA) as the conjugate [18].

It is now clear that the defining factor that limits siRNA potency—regardless of the nature of the conjugate—is siRNA stability. Indeed, a direct comparison of fully modified (all ribose 2′-OH substituted) versus partially modified (~60% of ribose 2′-OH substituted) siRNA revealed that total 2′-OH substitution improved oligonucleotide stability, tissue accumulation, and efficacy by several orders of magnitude following a single 10 mg/kg dose [22]. Furthermore, a fully stabilized siRNA scaffold permitted extrahepatic siRNA delivery, resulting in moderate levels of accumulation in the kidney, spleen, and heart, among other tissues [23]. These levels of accumulation reach the threshold required for silencing activity. For instance, administration of 50 mg/kg of a cholesterol-conjugated, metabolically stabilized siRNA resulted in >90% myostatin silencing in the muscle that persisted for 3 weeks [24]. Similar durations of effect have been noted for liver and kidney [25]. In contrast, the efficacy of partially modified siRNAs *in vivo* is typically limited to several days postinjection (Table 1).

Iterative improvements continue to be made to the siRNA architecture to enhance siRNA efficacy and reduce immunogenicity. Based on structural analysis of siRNA-loaded Ago2 and the requirement for a 5′-phosphate to anchor the siRNA guide strand in Ago2 [26], the 5′-phosphate of siRNA guide has been replaced with the stable analog, 5′-vinylphosphonate. This modification enhances siRNA accumulation, silencing activity, and duration of effect in multiple organs, including heart, where systemically administered siRNAs had not been detected [25, 27–30]. Combining modification strategies—for example, complete elimination of the ribose 2′-OH, stabilization of the phosphodiester backbone, and incorporation of 5′ guide strand phosphate analogs—will likely become a prerequisite for future clinical development of lipophilic siRNA drugs [25,29,30].

Another interesting consideration is oligonucleotide structure. To date, lipophilic conjugates have been used for delivery of both single- and double-stranded RNAs, including siRNAs (reviewed in Table 1), Dicer substrates [31,32], single-stranded siRNAs [33], antisense oligonucleotides (ASOs) [34], and asymmetric siRNAs [16,25,35,36]. Single-stranded RNAs are inherently less stable than duplex RNAs. Substituting the phosphodiester linkage with PS is the simplest modification to resist hydrolysis and increase oligonucleotide half-life both *in vitro* and *in vivo* [37]. PS modifications have therefore become an essential chemistry in oligonucleotide delivery [38]. Indeed, they are perhaps most responsible for *in vivo* efficacy of ASOs [39]. For unconjugated ASOs, a minimum of ~14 PS linkages are believed necessary to promote plasma protein binding and liver deposition [33]. In contrast, uniformly PS-modified siRNAs show reduced activity compared to unmodified siRNAs, presumably due to interference with Ago2 loading [40,41]. However, as few as 6–8 terminal PS linkages are sufficient for increased siRNA stability, uptake, and efficacy in tissues such as the kidney (Osborn, Khvorova, unpublished data). To date, most published

Table 1. *In Vivo* Activity of Cholesterol-Conjugated Small Interfering RNAs

Oligonucleotide chemistry	*Target gene*	*Target tissue*	*Dose*	*mRNA silencing*	*Year*	*DOI*
Cholesterol-conjugated siRNAs: systemic administration						
Terminal 2′-*O*-methyl and PS modifications	Apolipoprotein B	Liver	Three doses of 50 mpk	40%–60% (4 days)	2004	10.1038/nature03121
		Jejunum	Three doses of 50 mpk	50%–70% (4 days)		
2′-fluoro pyrimidines, terminal dT, PS modifications	Apolipoprotein B	Liver	Single 50 mpk injection	50% (7 days)	2007	10.1038/nbt1339
Terminal 2′-*O*-methyl and PS modifications	12/15-lipoxygenase	Kidney	Biweekly 400 μg injection for 7 weeks	50%–70%	2008	10.1152/ajprenal.90268.2008
Accell siRNA	Krüppel-like factor 6	Tumor xenograft	Six doses of 3 mpk	80% (30 days)	2010	10.1158/0008-5472.CAN-08-4282
Unmodified RNA nucleotides, terminal dT	Pokemon	Tumor xenograft	25 μg	0% silencing (14 days)	2012	10.1016/j.biomaterials.2012.08.057
2′-fluoro pyrimidines, terminal dT, PS modifications	Plasma factor 7	Liver	5 mpk	0% silencing (2 days)	2012	10.1089/nat.2012.0389
Accell siRNA	Mannan-binding lectin associated serine proteases 1,3	Liver	Three doses of 8 μg	58%–62% (7 days)	2016	10.4049/jimmunol.1600719
	Complement factor D	Adipose tissue	Three doses of 8 μg	53% (7 days)		
Alternating 2′-fluoro and 2′-*O*-methyl, vP, PS modifications	Myostatin	Muscle	50 mpk	85%–95% (21 days)	2016	10.1038/mtna.2016.55
Alternating 2′-fluoro and 2′-*O*-methyl, vP, PS modifications, Cy3	Cyclophilin B, Huntingtin	Liver	10 mpk	50%–70% (7 days)	2017	10.1093/nar/gkx507
		Kidney	20 mpk	40%–60% (7 days)		
		Heart	20 mpk	50%–55% (7 days)		
2′-*O*-methyl modified at nuclease cleavage site	P-glycoprotein	Tumor xenograft	10 mpk	65% (6 days)	2017	10.1016/j.omtn.2016.12.011
Alternating 2′-fluoro and 2′-*O*-methyl, vP, PS modifications	Cyclophilin B	Liver	20 mpk	60% (7 days)	2018	*
		Kidney	20 mpk	22% (7 days)		
		Adrenal Gland	20 mpk	37% (7 days)		
		Ovary	20 mpk	20% (7 days)		
Cholesterol-conjugated siRNAs: Local administration						
Terminal 2′-*O*-methyl and PS modifications	Huntingtin	CNS	Single 20 nmol injection	50%–60% (3 days; protein)	2007	10.1073 pnas.0708285104
Accell siRNA	Luciferase	Skin	Six doses of 120 ng	25%–50% silencing (1 days)	2010	10.1038/mt.2010.126
Accell siRNA	MAP kinase-activated protein kinase 2	Oral	4 nmol/injection for 3–5 injections	30%–50% silencing (9–16 days)	2010	10.1124/jpet.110.172395
Partially 2′-*O*-methyl, terminal dT	2′,3′-Cyclic-nucleotide 3′-phosphodiesterase	CNS	Infusion of 0.72 mg/day for 3–7 days	60%	2010	10.1016/j.jconrel.2010.02.011

(*continued*)

Table 1. (Continued)

Oligonucleotide chemistry	*Target gene*	*Target tissue*	*Dose*	*mRNA silencing*	*Year*	*DOI*
2′-fluoro pyrimidines in guide strand, 2′-*O*-methyl pyrimidines in sense strand, PS modifications	Cyclophilin B, Mitogen-activated protein kinase 4	Eye	10 μg injection	50% (2 days)	2013	10.1089/jop.2013.0148
Accell siRNA	Green fluorescent protein	Skin	Microinjection of 0.5 mg every other day 80% for 10 days	80%	2013	10.1038/mtna.2013.56
Accell siRNA	Microtubule-associated protein tau	CNS	Bilateral intraparenchymal (0.4 nmol)	∼50% (protein) 14 days	2014	10.2174/156652321405140926160602
Accell siRNA	DNMT1, 3a, and 3b	CNS (rat)	Intraparenchymal (0.1 nmol)	∼30%–70% (2 days)	2014	10.1111/ejn.12819
Accell siRNA	Phosphoinositide 3-Kinase Gamma	CNS (rat)	ICV 500 nmol	∼30% (1 days)	2015	10.1523/JNEUROSCI.0546-15.2015
Accell siRNA	Glyceradehyde-3-phosphate dehydrogenase (GAPDH)	CNS (rat)	ICV 5 μg	-	2015	10.1074/jbc.M114.635607
Alternating 2′-fluoro and 2′-*O*-methyl, PS modifications	Huntingtin	CNS	2 nmol	70% (7 days)	2015	10.1038/mtna.2015.38
-	Glyceraldehyde 3-phosphate dehydrogenase	Skin	75 μg	66% (1 days)	2016	10.1038/srep21422
-	Glyceraldehyde 3-phosphate dehydrogenase	Ear	15 μg	∼70% (1 days)	2016	10.1038/srep21422
Alternating 2′-fluoro and 2′-*O*-methyl, PS modifications	Luciferase	CNS (Tumor xenograft)	5 nmol	90% (7 days; protein)	2018	*

'-' Data unavailable.
*Osborn, Khvorova, unpublished data.
CNS, central nervous system; dT, deoxythymidine; ICV, intracerebroventricular; mpk, mg/kg; PS, phosphorothioate; siRNA, small interfering RNA; vP, vinylphosphonate.

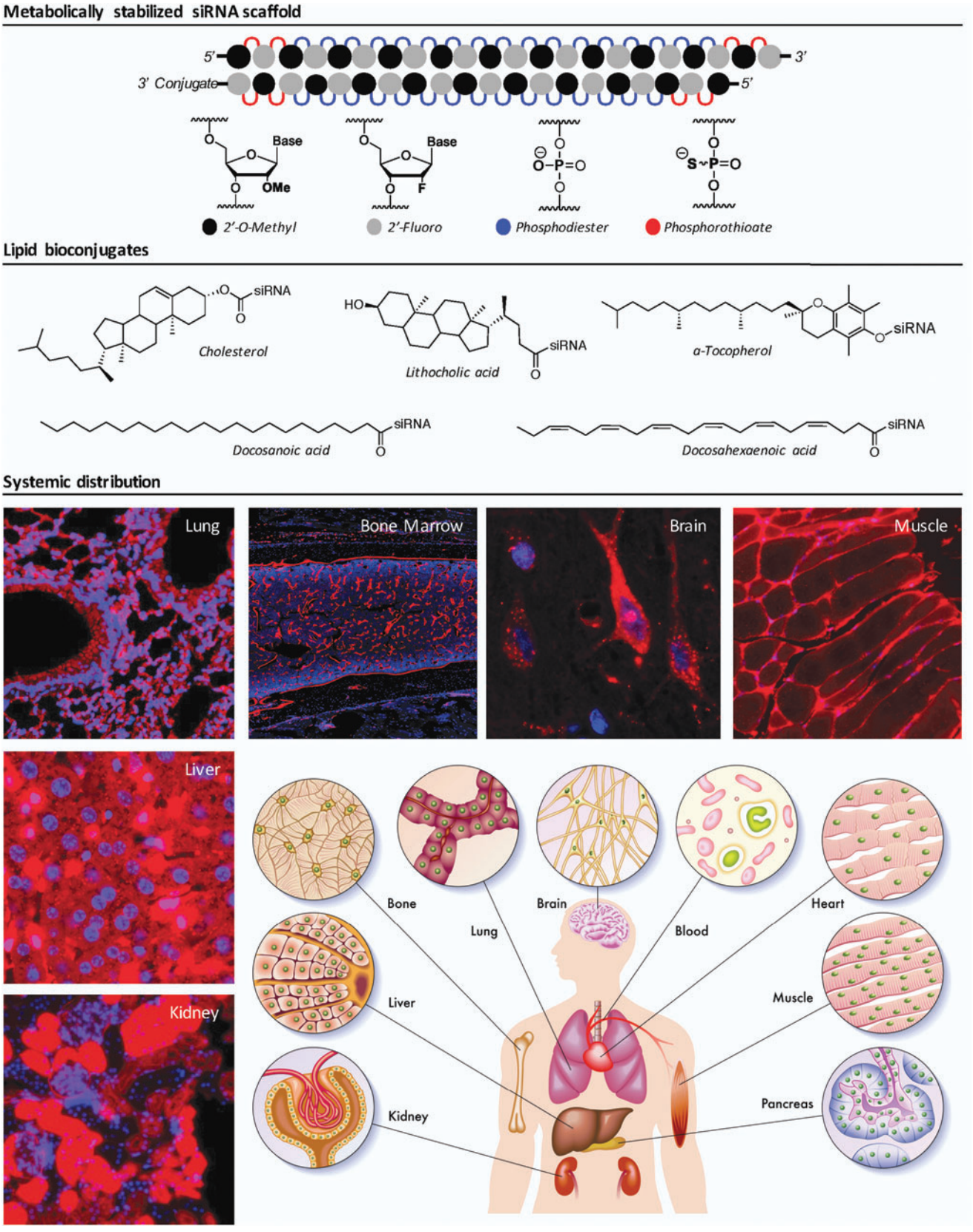

FIG. 1. Architecture, chemical modifications, and systemic distribution of metabolically stabilized lipid-siRNA conjugates. *Upper panel:* a fully chemically modified siRNA scaffold, depicting a 2′ overhang at the 3′ end of the antisense (guide) strand and the lipid conjugate at the 3′ end of the sense (passenger) strand. *Middle panel:* chemical structures of biologically occurring lipids commonly used as siRNA conjugates *in vivo*. *Lower panel:* representative fluorescence microscopy of Cy3-labeled, lipid-conjugated siRNA accumulation in a variety of tissues following a single SC (for kidney, liver, lung, bone marrow, muscle; 20 mg/kg) or intracerebroventricular (for brain; 40 nmol) injection in wild-type female mice (Osborn, Khvorova, unpublished data). SC, subcutaneous; siRNA, small interfering RNA.

studies reporting the distribution and efficacy of lipophilic siRNAs use scaffolds that contain multiple PS bonds, either in the context of single-stranded or base-paired regions (Table 1).

In addition to enhancing siRNA stability, PS modifications likely contribute to oligonucleotide delivery by providing an additional anchor for membrane binding and cellular internalization (ref). How PS linkages promote uptake are unknown, but binding to serum proteins or cell surface receptors (e.g., ASGPR) presumably contribute to cellular internalization [42]. Stabilizing the backbone and 5′ phosphate of GalNAc-siRNAs (via PS bonds and 5′-vinylphosphonate) together significantly enhances siRNA retention in the liver [28]. We recently compared the systemic distribution of PS-modified siRNAs with different structures. As a general rule, the presence of PS modifications markedly enhances lipid-conjugated siRNA accumulation in tissues. For instance, we observe that in the presence of a PS-modified, single-stranded overhang, lipid-conjugated siRNA uptake into liver hepatocytes is increased >10-fold (Osborn *et al.*, 2018, manuscript submitted). A detailed understanding of how PS linkages and RNA structure promote uptake will allow optimization of conjugate-mediated delivery.

Impact of lipid conjugation on siRNA distribution, efficacy, and safety

Although cholesterol has been extensively explored as a ligand for promoting oligonucleotide delivery, recent studies have shown that a wide variety of lipids affect siRNA pharmacokinetics, many with improved safety profiles (Biscans *et al.*, 2018, manuscript submitted). The chemical compositions of lipophilic conjugates dramatically influence siRNA clearance kinetics [43]. Compared to unconjugated siRNA, for example, a diacyl lipid conjugate resulted in six to ninefold increases in circulation half-life and siRNA bioavailability [44]. A direct comparison between cholesterol (more hydrophobic) and phosphatidylcholine-docosahexanoic acid (DHA; less hydrophobic) revealed that cholesterol-modified siRNAs have approximately twofold longer terminal elimination half-life following subcutaneous (SC) or IV injection. However, after SC administration, the time to maximum blood concentration was much faster for phosphatidylcholine-DHA (15 min) than cholesterol (2–3 h). This is likely due to cholesterol-siRNA accumulation in the skin around the injection site [43].

Altering the nature of the lipid conjugate also profoundly affects tissue distribution [18,33]. One rationale for this observation is that the structure and physicochemical properties of the lipid conjugate largely define protein binding capacity in circulation. For instance, in the context of lipid-conjugated asymmetric siRNAs, highly hydrophobic lipids (e.g., cholesterol, DCA) promote association with LDL, while less hydrophobic lipids (e.g., DHA) predominantly associate with high-density lipoprotein (HDL) or albumin (Osborn *et al.*, 2018, manuscript submitted). Protein or lipoprotein association drives tissue-specific internalization via recognition by cell surface receptors. It is important to note that although lipid conjugation increases siRNA-albumin binding *in vitro*, albumin association may not persist *in vivo* in the presence of higher affinity, competing chaperones, such as LDL and HDL.

While lipid conjugation enables siRNA uptake by a variety of tissues, the concentration required to induce RNA interference (RNAi)-mediated gene silencing is tissue- and conjugate-specific. As much as 50–100 ng/mg siRNA is necessary for efficacy in the kidney, while as little as 1–2 ng/mg in sufficient in muscle and fat (Biscans *et al.*, 2018, manuscript submitted). This supports the hypothesis that both ''productive'' and ''non-productive'' oligonucleotide uptake pathways exist, although the molecular players in each pathway have yet to be defined [42]. For instance, α-tocopherol-siRNA was active against ApoB in the liver at a concentration as low as 2 mg/kg, yet cholesterol-siRNA was inactive at that dose [45]. Similarly, following equimolar (10 mg/kg) administration of GalNAc- or cholesterol-conjugated siRNA, cholesterol-siRNA accumulated to higher levels in the liver, but GalNAc-siRNA was more potent (Osborn, Khvorova, unpublished data). While the underlying mechanism is still under investigation, the nature of the conjugate clearly determines siRNA bioavailability.

In addition to driving siRNA clearance kinetics and tissue tropism, lipid conjugates directly impact oligonucleotide safety profile. Acute toxicity following local administration of highly hydrophobic lipid-siRNA conjugates has been reported in the central nervous system [35]. Whereas intraparenchymal injection of up to 200 μg (limit of solubility) fully chemically stabilized DHA-conjugated siRNAs showed no evidence of neuronal toxicity or innate immune activation, as little as 25 μg cholesterol-conjugated siRNA induced significant neuronal death. In the brain, exceedingly high local concentrations of cholesterol-siRNA likely disturb membrane potential and activate glial cells. RNA stability also contributed to the observed toxicity of the fully stabilized cholesterol-conjugated siRNA: when cholesterol is conjugated to a partially modified siRNA, a 25 μg dose is safe and well tolerated [16].

The maximum tolerated dose and therapeutic index for DHA-conjugated siRNAs is also much higher than that of cholesterol-conjugated siRNA when administered systemically (Turanov *et al.*, 2018, manuscript in preparation). Systemic administration of 100–200 mg/kg DHA-conjugated siRNA were well tolerated, but at the same dose, cholesterol-conjugated siRNA triggered detectable cytokine activation. How and why do cholesterol-siRNA conjugates stimulate an innate immune response? One theory may relate to the deposition of cholesterol-conjugated siRNAs in monocytes that reside in spleen and bone marrow (Coles, Khvorova, unpublished data). Indeed, lipid conjugates that show poor uptake by monocytes *in vivo* exhibit lower levels of cytokine activation (Turanov *et al.*, 2018, manuscript in preparation).

Future of lipid conjugate mediated siRNA delivery

GalNAc conjugation, when used in conjunction with a fully chemically stabilized siRNA scaffold, has solved the quandary of targeted delivery to liver hepatocytes [22]. The clinical development of GalNAc-siRNA conjugates has been reviewed extensively, and as low as 2–5 mg/kg is sufficient to silence liver-expressed genes for up to 6 months in the clinic [3,46]. There are over 15 clinical trials relying on this delivery concept for a variety of nucleic acid drugs (ASOs, siRNAs, and microRNAs). Though highly potent and clinically convenient (defined by ease of manufacturing, SC administration, long duration of effect, saline formulation, and long shelf life), the GalNAc-siRNA platform is limited to one cell type—hepatocytes. Lipid conjugation supports a much broader

therapeutic distribution, and co-optimization of the siRNA scaffold and lipid conjugate has enabled functional delivery to liver, kidneys, lung, heart, muscle, spleen, and adipose tissue after a single injection.

Though lipid conjugation is a potentially transformative strategy for functional genomics and therapeutic intervention, the approach has several major caveats. Delivery is not tissue- or cell type-specific, and a significant fraction of the injected dose will be delivered to primary clearance tissues, including liver, kidney, and spleen. Thus, for clinical utility, target selection is a critical parameter. Ideally, lipid-conjugated siRNAs should be generated against disease-causing genes whose expression is limited to a targetable disease tissue or whose silencing in normal tissue is well tolerated. This is not an insurmountable task. For example, lipid-conjugated siRNAs efficiently reach the placenta (>8% of injected dose) to silence soluble fms-like tyrosine kinase 1 (sFlt1), the primary cause of preeclampsia. As the placenta is responsible for sFlt1 production in pregnant animals, sFlt1 silencing in the liver and kidney is well tolerated and does not contribute to disease progression (Turanov *et al.*, 2018, under review). There are other targets that are only expressed in the affected tissues, including double homeobox 4 (*DUX4*) in muscular dystrophy [47]. Thus, lipophilic conjugates can be broadly used to modulate gene expression, provided that the target and clinical indication are carefully considered and matched to the lipid conjugate pharmacological and safety profiles.

Another interesting area of application for lipophilic conjugates is tumor delivery following local or systemic injection. Despite being a major limitation for use in neurodegenerative disorders, the restricted diffusion and resultant toxicity of cholesterol-siRNAs could be an advantage for local tumor treatment. Cholesterol-conjugated siRNAs broadly penetrate and induce >90% gene silencing in orthotopic glioma brain tumors (Osborn, Coles *et al.*, 2018, in press). Lipid-modified siRNAs and ASOs have also been successfully used to reach implanted tumors after systemic administration [36,44]. Cancer immunotherapy is a burgeoning field that may benefit greatly from advances in lipid-siRNA conjugation and tumor delivery.

For lipid nanoparticle-based siRNA delivery, a considerable and concerted effort over several years yielded chemical formulations that improved efficacy by several orders of magnitude [2]. We predict that a similar degree of combinatorial chemistry will be required to achieve the same results for lipid-conjugated oligonucleotides. Naturally, overall distribution will be driven by protein binding properties and restricted to currently targetable tissues. However, further chemical engineering will likely improve the efficiency of endosomal escape, the primary factor limiting siRNA bioavailability [48].

Lipid conjugate-mediated delivery of other classes of oligonucleotides

We have focused this review on the use of lipophilic modification (i.e., ''hydrophobization'') to improve siRNA delivery *in vivo*. Although we have provided extensive evidence that the chemical architecture of the nucleic acid in question contributes to pharmacokinetic behavior, general trends remain consistent. Therefore, the concept of lipid conjugation for nucleic acid delivery represents an area worth further exploration. Indeed, ligand-conjugated antisense (LICA) technology comprises the next frontier of ASO development, with conjugates ranging from sugars (e.g., GalNAc, [42,49]) to peptides [50–52], and lipids [33,53]. While our current understanding of the impact of lipid conjugation on nucleic acid delivery is limited to siRNAs and ASOs, these fundamental discoveries will likely be applicable during future clinical development of other oligonucleotide classes, including aptamers, messenger RNAs, and CRISPR components.

Acknowledgments

The authors thank Annabelle Biscans and Julia Alterman for help preparing the figures and NIH F32 NS095508-03 and GM108803 for funding.

Author Disclosure Statement

M.F.O. has no competing financial interests to disclose. A.K. owns stock of RXi Pharmaceuticals and Advirna.

References

1. Schroeder A, CG Levins, C Cortez, R Langer and DG Anderson. (2010). Lipid-based nanotherapeutics for siRNA delivery. J Intern Med 267:9–21.
2. Zatsepin TS, YV Kotelevtsev and V Koteliansky. (2016). Lipid nanoparticles for targeted siRNA delivery—going from bench to bedside. Int J Nanomedicine 11:3077–3086.
3. Fitzgerald K, S White, A Borodovsky, BR Bettencourt, A Strahs, V Clausen, P Wijngaard, JD Horton, J Taubel, *et al.* (2017). A highly durable RNAi therapeutic inhibitor of PCSK9. N Engl J Med 376:41–51.
4. Huang Y. (2017). Preclinical and clinical advances of GalNAc-decorated nucleic acid therapeutics. Mol Ther Nucleic Acids 6:116–132.
5. van Meer G and AI de Kroon. (2011). Lipid map of the mammalian cell. J Cell Sci 124:5.
6. Razin S. (1975). Cholesterol incorporation into bacterial membranes. J Bacteriol 124:570–572.
7. Gaibelet G, S Allart, F Tercé, V Azalbert, J Bertrand-Michel, S Hamdi, X Collet and S Orlowski. (2015). Specific cellular incorporation of a pyrene-labelled cholesterol: lipoprotein-mediated delivery toward ordered intracellular membranes. PLoS One 10:e0121563.
8. Ly S, DM Navaroli, MC Didiot, J Cardia, L Pandarinathan, JF Alterman, K Fogarty, C Standley, LM Lifshitz, *et al.* (2017). Visualization of self-delivering hydrophobically modified siRNA cellular internalization. Nucleic Acids Res 45:15–25.
9. Hickerson RP, WC Wey, DL Rimm, T Speaker, S Suh, MA Flores, E Gonzalez-Gonzalez, D Leake, CH Contag and RL Kaspar. (2013). Gene silencing in skin after deposition of self-delivery siRNA with a motorized microneedle array device. Mol Ther Nucleic Acids 2:e129.
10. Chong RH, E Gonzalez-Gonzalez, MF Lara, TJ Speaker, CH Contag, RL Kaspar, SA Coulman, R Hargest and JC Birchall. (2013). Gene silencing following siRNA delivery to skin via coated steel microneedles: in vitro and in vivo proof-of-concept. J Control Release 166:211–219.
11. Deng Y, J Chen, Y Zhao, X Yan, L Zhang, K Choy, J Hu, HJ Sant, BK Gale and T Tang. (2016). Transdermal

delivery of siRNA through microneedle array. Sci Rep 6: 21422.
12. Byrne M, R Tzekov, Y Wang, A Rodgers, J Cardia, G Ford, K Holton, L Pandarinathan, J Lapierre, *et al.* (2013). Novel hydrophobically modified asymmetric RNAi compounds (sd-rxRNA) demonstrate robust efficacy in the eye. J Ocul Pharmacol Ther 29:855–864.
13. Cheng C, R Haasdijk, D Tempel, EHM van de Kamp, R Herpers, F Bos, WK Den Dekker, LAJ Blonden, R de Jong, *et al.* (2012). Endothelial cell-specific FGD5 involvement in vascular pruning defines neovessel fate in mice. Circulation 125:3142–3158.
14. DiFiglia M, M Sena-Esteves, K Chase, E Sapp, E Pfister, M Sass, J Yoder, P Reeves, RK Pandey, *et al.* (2007). Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. Proc Natl Acad Sci U S A 104:17204–17209.
15. Chen Q, D Butler, W Querbes, RK Pandey, P Ge, MA Maier, L Zhang, KG Rajeev, L Nechev, *et al.* (2010). Lipophilic siRNAs mediate efficient gene silencing in oligodendrocytes with direct CNS delivery. J Control Release 144:227–232.
16. Alterman JF, LM Hall, AH Coles, MR Hassler, MC Didiot, K Chase, J Abraham, E Sottosanti, E Johnson, *et al.* (2015). Hydrophobically modified siRNAs silence huntingtin mRNA in primary neurons and mouse brain. Mol Ther Nucleic Acids 4:e266.
17. Soutschek J, A Akinc, B Bramlage, K Charisse, R Constien, M Donoghue, S Elbashir, A Geick, P Hadwiger, *et al.* (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432: 173–178.
18. Wolfrum C, S Shi, KN Jayaprakash, M Jayaraman, G Wang, RK Pandey, KG Rajeev, T Nakayama, K Charrise, *et al.* (2007). Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nat Biotech 25:1149–1157.
19. Wong SC, JJ Klein, HL Hamilton, Q Chu, CL Frey, VS Trubetskoy, J Hegge, D Wakefield, DB Rozema and DL Lewis. (2012). Co-injection of a targeted, reversibly masked endosomolytic polymer dramatically improves the efficacy of cholesterol-conjugated small interfering RNAs in vivo. Nucleic Acid Ther 22:380–390.
20. Khvorova A and JK Watts. (2017). The chemical evolution of oligonucleotide therapies of clinical utility. Nat Biotechnol 35:238–248.
21. Allerson CR, N Sioufi, R Jarres, TP Prakash, N Naik, A Berdeja, L Wanders, RH Griffey, EE Swayze and B Bhat. (2005). Fully 2′-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. J Med Chem 48:901–904.
22. Nair JK, JLS Willoughby, A Chan, K Charisse, MR Alam, Q Wang, M Hoekstra, P Kandasamy, AV Kel'in, *et al.* (2014). Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. J Am Chem Soc 136:16958–16961.
23. Hassler MR, AA Turanov, JF Alterman, RA Haraszti, AH Coles, MF Osborn, D Echeverria, M Nikan, WE Salomon, *et al.* (2018). Comparison of partially and fully chemically-modified siRNA in conjugate-mediated delivery in vivo. Nucleic Acids Res 46:2185–2196.
24. Khan T, H Weber, J DiMuzio, A Matter, B Dogdas, T Shah, A Thankappan, J Disa, V Jadhav, *et al.* (2016). Silencing myostatin using cholesterol-conjugated siRNAs induces muscle growth. Mol Ther Nucleic Acids 5:e342.
25. Haraszti RA, L Roux, AH Coles, AA Turanov, JF Alterman, D Echeverria, B Godinho, N Aronin and A Khvorova. (2017). 5-Vinylphosphonate improves tissue accumulation and efficacy of conjugated siRNAs in vivo. Nucleic Acids Res 45:7581–7592.
26. Schirle NT, GA Kinberger, HF Murray, WF Lima, TP Prakash and IJ MacRae. (2016). Structural analysis of human Argonaute-2 bound to a modified siRNA guide. J Am Chem Soc 138:8694–8697.
27. Parmar RG, CR Brown, S Matsuda, JLS Willoughby, CS Theile, K Charisse, DJ Foster, I Zlatev, V Jadhav, *et al.* (2018). Facile synthesis, geometry, and 2′-substituent-dependent in vivo activity of 5′-(E)- and 5′-(Z)-vinylphosphonate-modified siRNA conjugates. J Med Chem 61:734–744.
28. Prakash TP, GA Kinberger, HM Murray, A Chappell, S Riney, MJ Graham, WF Lima, EE Swayze and PP Seth. (2016). Synergistic effect of phosphorothioate, 5′-vinylphosphonate and GalNAc modifications for enhancing activity of synthetic siRNA. Bioorg Med Chem Lett 26: 2817–2820.
29. Parmar R, JL Willoughby, J Liu, DJ Foster, B Brigham, CS Theile, K Charisse, A Akinc, E Guidry, *et al.* (2016). 5′-(E)-vinylphosphonate: a stable phosphate mimic can improve the RNAi activity of siRNA-GalNAc conjugates. Chembiochem 17:985–989.
30. Elkayam E, R Parmar, CR Brown, JL Willoughby, CS Theile, M Manoharan and L Joshua-Tor. (2017). siRNA carrying an (E)-vinylphosphonate moiety at the 5 end of the guide strand augments gene silencing by enhanced binding to human Argonaute-2. Nucleic Acids Res 45: 3528–3536.
31. Kubo T, Y Takei, K Mihara, K Yanagihara and T Seyama. (2012). Amino-modified and lipid-conjugated dicer-substrate siRNA enhances RNAi efficacy. Bioconjug Chem 23:164–173.
32. Kubo T, K Yanagihara, Y Takei, K Mihara, Y Sato and T Seyama. (2012). Lipid-conjugated 27-nucleotide double-stranded RNAs with dicer-substrate potency enhance RNAi-mediated gene silencing. Mol Pharm 9:1374–1383.
33. Lima WF, TP Prakash, HM Murray, GA Kinberger, W Li, AE Chappell, CS Li, SF Murray, H Gaus, *et al.* (2012). Single-stranded siRNAs activate RNAi in animals. Cell 150:883–894.
34. Karaki S, S Benizri, R Mejias, V Baylot, N Branger, T Nguyen, B Vialet, K Oumzil, P Barthelemy and P Rocchi. (2017). Lipid-oligonucleotide conjugates improve cellular uptake and efficiency of TCTP-antisense in castration-resistant prostate cancer. J Control Release 258:1–9.
35. Nikan M, MF Osborn, AH Coles, BM Godinho, LM Hall, RA Haraszti, MR Hassler, D Echeverria, N Aronin and A Khvorova. (2016). Docosahexaenoic acid conjugation enhances distribution and safety of siRNA upon local administration in mouse brain. Mol Ther Nucleic Acids 5:e344.
36. Nikan M, MF Osborn, AH Coles, A Biscans, B Godinho, RA Haraszti, E Sapp, D Echeverria, M DiFiglia, *et al.* (2017). Synthesis and evaluation of parenchymal retention and efficacy of a metabolically stable O-phosphocholine-N-docosahexaenoyl-l-serine siRNA conjugate in mouse brain. Bioconjug Chem 28:1758–1766.
37. Eckstein F and G Gish. (1989). Phosphorothioates in molecular biology. Trends Biochem Sci 14:97–100.

38. Eckstein F. (2014). Phosphorothioates, essential components of therapeutic oligonucleotides. Nucleic Acid Ther 24:374–387.
39. Geary RS, D Norris, R Yu and CF Bennett. (2015). Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. Adv Drug Deliv Rev 87:46–51.
40. Jahns H, M Roos, J Imig, F Baumann, Y Wang, R Gilmour and J Hall. (2015). Stereochemical bias introduced during RNA synthesis modulates the activity of phosphorothioate siRNAs. Nat Commun 6:6317.
41. Braasch DA, Z Paroo, A Constantinescu, G Ren, OK Oz, RP Mason and DR Corey. (2004). Biodistribution of phosphodiester and phosphorothioate siRNA. Bioorg Med Chem Lett 14:1139–1143.
42. Tanowitz M, L Hettrick, A Revenko, GA Kinberger, TP Prakash and PP Seth. (2017). Asialoglycoprotein receptor 1 mediates productive uptake of N-acetylgalactosamine-conjugated and unconjugated phosphorothioate antisense oligonucleotides into liver hepatocytes. Nucleic Acids Res 45:12388–12400.
43. Godinho B, JW Gilbert, RA Haraszti, AH Coles, A Biscans, L Roux, M Nikan, D Echeverria, M Hassler and A Khvorova. (2017). Pharmacokinetic profiling of conjugated therapeutic oligonucleotides: a high-throughput method based upon serial blood microsampling coupled to peptide nucleic acid hybridization assay. Nucleic Acid Ther 27:323–334.
44. Sarett SM, TA Werfel, L Lee, MA Jackson, KV Kilchrist, D Brantley-Sieders and CL Duvall. (2017). Lipophilic siRNA targets albumin in situ and promotes bioavailability, tumor penetration, and carrier-free gene silencing. Proc Natl Acad Sci U S A 114:E6490–E6497.
45. Nishina K, T Unno, Y Uno, T Kubodera, T Kanouchi, H Mizusawa and T Yokota. (2008). Efficient in vivo delivery of siRNA to the liver by conjugation of [alpha]-tocopherol. Mol Ther 16:734–740.
46. Khvorova A. (2017). Oligonucleotide therapeutics—a new class of cholesterol-lowering drugs. N Engl J Med 376: 4–7.
47. Ansseau E, C Vanderplanck, A Wauters, SQ Harper, F Coppee and A Belayew. (2017). Antisense oligonucleotides used to target the DUX4 mRNA as therapeutic approaches in faciosscapulohumeral muscular dystrophy (FSHD). Genes (Basel) 8:E93.
48. Dowdy SF. (2017). Overcoming cellular barriers for RNA therapeutics. Nat Biotechnol 35:222–229.
49. Schmidt K, TP Prakash, AJ Donner, GA Kinberger, HJ Gaus, A Low, ME Ostergaard, M Bell, EE Swayze and PP Seth. (2017). Characterizing the effect of GalNAc and phosphorothioate backbone on binding of antisense oligonucleotides to the asialoglycoprotein receptor. Nucleic Acids Res 45:2294–2306.
50. Henke E, J Perk, J Vider, P de Candia, Y Chin, DB Solit, V Ponomarev, L Cartegni, K Manova, *et al.* (2008). Peptide-conjugated antisense oligonucleotides for targeted inhibition of a transcriptional regulator in vivo. Nat Biotechnol 26:91–100.
51. Jarver P, T Coursindel, SE Andaloussi, C Godfrey, MJ Wood and MJ Gait. (2012). Peptide-mediated cell and in vivo delivery of antisense oligonucleotides and siRNA. Mol Ther Nucleic Acids 1:e27.
52. Yin H, HM Moulton, Y Seow, C Boyd, J Boutilier, P Iverson and MJ Wood. (2008). Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function. Hum Mol Genet 17:3909–3918.
53. Nishina T, J Numata, K Nishina, K Yoshida-Tanaka, K Nitta, W Piao, R Iwata, S Ito, H Kuwahara, *et al.* (2015). Chimeric antisense oligonucleotide conjugated to alpha-tocopherol. Mol Ther Nucleic Acids 4:e220.

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Received for publication February 16, 2018; accepted after revision March 22, 2018.