Unlocking the therapeutic potential of locked nucleic acids through lipid nanoparticle delivery

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Locked nucleic acids (LNAs) are a subtype of antisense oligonucleotides (ASOs) that are characterized by a bridge within the sugar moiety. LNAs owe their robustness to this chemical modification, which as the name suggests, locks it in one conformation. This perspective includes two components: a general overview on ASOs from one side and on delivery issues focusing on lipid nanoparticles (LNPs) on the other side. Throughout, a screening of the ongoing clinical trials involving ASOs is given, as well as a take on the versatility and challenges of using LNAs. Finally, we highlight the potential of LNPs as carriers for the successful delivery of LNAs.

INTRODUCTION

Ever since the discovery of DNA as the heredity vector in 1940, our understating of nucleic acids has been growing, allowing us to harness them as therapeutic agents. Although most therapies in the market today are small molecules, the top-down approach in their development makes their discovery a tedious task with a very low chance of success. A staggering number of 10,000 molecules need to be tested for one of them to make it to the market.[1] In contrast, oligonucleotides are normally developed in a bottom-up approach, where once the mechanism of a disease or the agents causing it is known, a specific sequence can be tailored to execute the needed effect. Furthermore, the development of several oligonucleotide drugs, e.g., Onpattro (Patisiran, for familial amyloid polyneuropathy) and Defibrotide (for veno-occlusive disease), paved the way for the regulatory rules and conduct of this area.[2]

As part of developing several oligonucleotide therapeutic approaches, in 1978, Paul Zamecnik et al. published papers in which ODN (oligodeoxynucleotide; synthetic DNA molecules) were used to inhibit the translation of the E1 start region of human papillomavirus by stimulation of RNAse H, demonstrating antiviral activity *in vivo*. These sequences would be later called antisense oligonucleotides (ASOs).[3] Though there was enthusiasm around their discovery, the field was forsaken for a while due to the challenges it posed. However, with the development of chemical modifications, the elucidation of the mechanism of action, and general advancement in gene therapy, we are now witnessing the renaissance of ASOs.

In this perspective, we will concentrate on locked nucleic acids (LNA), a specific subtype of ASOs, which were introduced in the late 1990s.[4]

The chemical modification that makes an LNA monomer is a methylene bridge between the second oxygen and the 4th carbon in the sugar moiety. We will also highlight the potential future of this field, adding possible improvements.[5]

ASO GENERATIONS

Nucleic acid-based therapy agents are analogs of RNA and DNA (Figure 1) that were created to solve the challenge of instability among these agents. ASOs were designed to bind their RNA target via a Watson-Crick interaction.[6]

The stability, specificity, and potency of ASOs have been developed gradually over time so that the new chemistry tackles the problems of its predecessor. According to these chemical modifications, ASOs are classified into three generations.

First-generation ASOs

In the first generation of ASOs, a chemical modification was added in the phosphate backbone. Namely, they are obtained by the replacement of one of the non-bridging oxygen atoms in the phosphodiester bond by a sulfur (phosphorothioates), a methyl (methyl-phosphonates), or an amine group (phosphoramidates) (Figure 2). These modifications made a major leap in the field of nucleic acid-based therapy, by increasing their stability and extending their half-life from minutes to days. Furthermore, this generation has the unique ability to recruit RNAse H once meeting its target mRNA.[7–9] RNAse H will then degrade the mRNA, eventually leading to the downregulation of the corresponding protein. This chemical modification remains the most clinically used.[10]

Second-generation ASOs

In the case of the second-generation ASOs, the chemical modification is done in the 2° position of the sugar moiety, yielding RNA analogs. The most common of these modifications are 2-O-methoxyethyl (2°-MOE), and 2-O-methyl (2°-OME) (Figure 3).[7] These modifications grant this generation a higher binding affinity while lowering
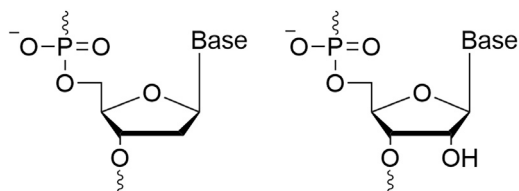
Figure 1. Chemical structures of natural oligonucleotides
A structure of a deoxyribonucleic acid (DNA) nucleotide (left) and a ribonucleic acid (RNA) nucleotide (right).

the toxicity and immunogenicity. Unlike the first generation, the agents of this group lack the ability to recruit RNAse H and thus cannot lead to RNA degradation. Nevertheless, protein downregulation may be obtained by this group through sterically blocking the mRNA-ribosome interaction. This group also has the capacity for splice modulation, leading to the inclusion of excluded exons to repair the mRNA and restore the functionality of the target protein.[10] Most of the ASOs that are approved for clinical use today use the 2-MOE technology.[8,11,12]

Third generation and the development of locked nucleic acids
While first-generation chemical modifications are in the phosphate backbone, and second-generation modifications are done in the second position in the sugar moiety, yielding RNA analogs, the third generation includes versatile chemical modifications done within the furanose ring, yielding a group of diverse nucleotide analogs (Figure 4). Among this generation are LNAs, phosphorodiamidate morpholino, and peptide nucleic acid. While all 3 are characterized by high potency, LNAs are particularly potent and robust among them.[13]

LNAs were first described by Obika et al. (1997) and Singh, Koshkin, Wengel, and Nielsen (1998) and consist of a bridging between the second and fourth carbons of the furanose ring via an oxymethylene link.[14] As the name suggests, the introduced chemical modification locks this RNA analog in one conformation. This change reduces the entropy of LNA hybridization with their target DNA/RNA, or in other words, it increases the chance for such a duplex to form.[15] Eventually, the synthesis of LNAs introduced a leap in the affinity of ASOs to their targets.

As mentioned before, the chemical modifications done in each generation grant its monomers a unique feature. Ita and Obika et al. have referred to this concept as "unit chemistry."[16] We can think of the

different monomers as LEGO bricks that synergistically complement each other when combined, creating different entities with different abilities.

Clinical trials of ASOs
Many clinical trials were conducted to evaluate the potential therapeutic benefits, as well as the safety profile of ASOs in treating various diseases, including genetic disorders, cancer, and neurological conditions. Although the development of ASOs has faced challenges, so far 10 drugs of this therapeutic class have been approved for marketing for many indications including Duchenne muscular dystrophy (DMD); this includes Fomivirsen, an agent withdrawn due to a reduction in the number of CMV cases following the development of highly active antiretroviral therapy.[17,18] The field has continued to show steady progress over the years, with oligonucleotides being under clinical development for approximately three decades, demonstrating the enduring interest and potential of this therapeutic approach (Table 1).[19] Most of the ASOs that are approved for clinical use today are second-generation 2-MOE chemical modifications.

In the case of ASOs, the drug development is disease target centered: i.e., once a biological target is linked to the pathophysiology of a disease, a sequence is tailored in a specific manner to that target, considering the features that can be achieved using different chemical modifications. In the case of small molecules, however, the development is molecule centered, as it starts from the discovery of a compound, after which the chemical properties, features, and biological effects are elucidated. After these tests, the molecule can be matched to a specific disease target and enter preclinical and clinical development. This methodical difference significantly shortens the discovery stage of gene therapy compared to the classic small molecule development. It is estimated that out of 10,000 molecules, only 250 make it to preclinical development, and only 1 is eventually marketed (0.4% from preclinical to marketing).[20] According to available information, thus far, 446 ASOs agents have been assessed pre-clinically, out of which 8 are marketed (~1.8%), with some of the studies summarized in Table 1.[17] Despite the higher rate in comparison to small molecules, we believe that ASOs are not reaching their full potential, partially since smart delivery systems are not being harnessed. As an example, clinical studies of ASOs revealed an association with increases of hepatic enzymes, thrombocytopenia, and proteinuria, which are believed to be a consequence of systemic exposure, as well as off-target-sequence-related effects.[21] Both problems may be
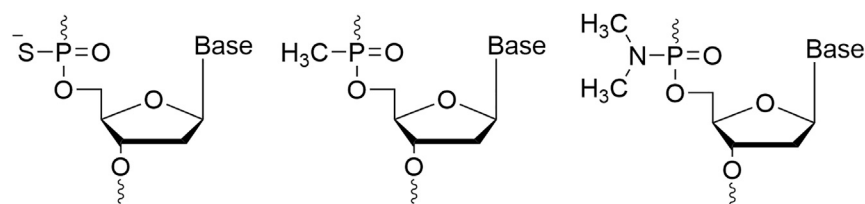

Figure 2. Chemical structures of first-generation ASOs
A structure of a phosphorothioate, methyl-phosphonate, and phosphoramidate nucleotide, respectively.
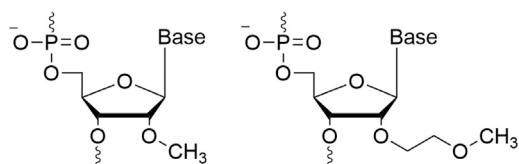
Figure 3. Chemical structures of second-generation ASOs
A structure of a 2-O-methyl (2°-OME) nucleotide (left) and a 2-O-methoxyethyl (2°-MOE) nucleotide (right).

mitigated via a targeted delivery in a cell-specific manner. One example is a drug developed for acute myeloid leukemia (AML) and tested on other types of cancers: Prexigebersen. The chemically modified DNA is incorporated inside a neutrally charged lipid bilayer, creating lipid nanoparticles that reach the target cells without toxic effects.

In conclusion, ASOs represent a therapeutic strategy with significant potential in treating a wide range of diseases. While progress has been made in clinical trials and in understanding their mechanisms of action, further research is needed to address existing challenges and optimize the efficacy and safety of ASO-based therapies, with an emphasis and encouragement to employ delivery systems to achieve those goals.

INCORPORATING LNAs FOR VERSATILE USES

LNAs brought a set of remarkable features into the ASOs field. Their most prominent and important feature is their stable conformation, making them very potent compounds with high affinity. In line with the LEGO bricks approach, the incorporation of LNAs in existing oligonucleotide technologies yielded novel biotechnological and therapeutic applications.

The incorporation of LNAs with DNA nucleotides has yielded what is termed antisense LNA. This can be divided into mixmers and gapmers; in the former, LNA units are dispersed across the sequence, while in the latter, there is a central gap of DNA between two over-hinged LNAs.[10] The LNA involvement gives the sequence a long half-life, with high potency, while the native DNA allows for RNAse H recruitment once hybridized with complementary RNA.[22]

The conformational lock within the furanose ring facilitates and favors the binding of LNAs to their complementary sequence. Accord-

ingly, when incorporated with siRNAs, they ensure the loading of the sequence into the RNA-induced silencing complex.[23]

One of the most interesting applications in which LNAs are employed is pre-mRNA splice switching. Pre-mRNA maturation is a process that takes place in the nucleus and involves a range of ribonucleoproteins comprising a "spliceosome."[24] The creation of different mRNA variants is obtained by sequences around introns and exons, which either enhance or silence spliceosomes. Accordingly, in cases where faulty splicing occurs, splice-switching oligonucleotides (SSOs) may be utilized to tilt the balance back to normal. This process, however, requires a high level of precision; well-defined sequences need to be excluded/included, without degrading the pre-mRNA, which makes LNAs a prominent candidate for the mission, since they lack the ability to recruit RNAse H on one hand and are very potent and precise on the other.[23] Roberts et al. used an LNA SSO (LNA SSO-654) to demonstrate the prevention of aberrant splicing of EGFP in transgenic mice. SSOs including LNAs rather than 2′OMe fully modified ASOs were more efficient.[25]

Indeed, this versatility has thus far proven to be effective in various preclinical and clinical studies; for example, Tassone et al. demonstrated a good safety profile and a promising anti-tumoral activity in a first-in-human, phase 1, open-label, dose-escalation study. In the study, 17 patients received a 13-mer LNA with a phosphorothioate (PS)-modified backbone that inhibits microRNA-221 (LNA-i-miR-221).[26] Another target that has been tested in the context of LNAs is miR-21, an oncomiR that plays a pivotal role in many cancers. Javanmard et al. have demonstrated preclinical anticancer activity using an LNA sequence to treat melanoma-bearing male C57BL/6 mice.[27] Further highlighting their versatility, it should be noted that LNAs' efficacy is not limited to cancer. For example, a work by Bockstahler et al. has demonstrated anti-inflammatory activity in A/J mice suffering from (induced) autoimmune myocarditis, ultimately reducing cardiac inflammation and fibrosis.[28] The various successes in the works mentioned above, as well as the various clinical trials found in Table 1, show the versatile nature of LNAs in multiple indications, from cancer and inflammatory autoimmunity to genetic diseases such as DMD.

TOXICITY

The driving force for chemical modification throughout the generations was to improve the therapeutic window of ASOs; namely, the
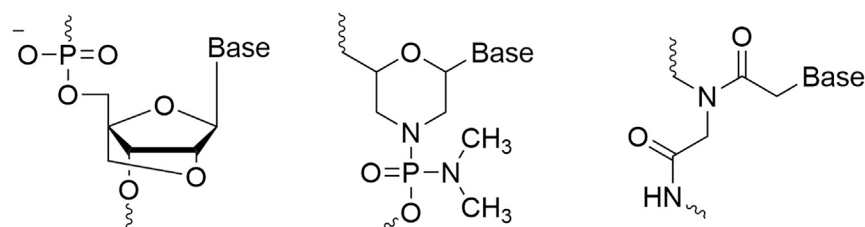

Figure 4. Chemical structures of third-generation ASOs
Structures of a locked nucleic acid (LNA), phosphorodiamidate morpholino (PMO), and peptide nucleic acid (PNA) nucleotide, left to right, respectively.

Table 1. Clinical trials of ASOs

Company	Candidate	Route	Biological target	Condition or disease	Clinical stage	Latest update
Genzyme Corporation	ISIS-301012/Kynamro/Mipomersen	subcutaneous injection	Apolipoprotein B-100	hyperlipoproteinemia type IIa	marketed	preclinical for atherosclerosis was discontinued
Isis Pharmaceuticals	Fomivirsen/ISIS 2922/Vitravene	intravitreal injection	CMV protein IE2 (blocks translation of UL123 gene)	cytomegalovirus infections	market withdrawal	market withdrawal
Sarepta Therapeutics	Eteplirsen/AVI-4658 PMO/Exondys 51	intravenous infusion	Exon 51 of Dystrophin	Duchenne muscular dystrophy	marketed	marketed
Genzyme Corporation	Nusinersen/ISIS-396443/Spinraza	intrathecal injection by lumbar puncture	SMN2 gene	spinal muscular atrophy	marketed	marketed
Sarepta Therapeutics	Golodirsen/SRP 4053	intravenous infusion	Exon 53 of dystrophin	Duchenne muscular dystrophy	marketed	marketed
National Center of Neurology and Psychiatry	Viltolarsen/Biltrarsen/NCNP-01	intravenous infusion	Exon 53 of dystrophin	Duchenne muscular dystrophy	marketed	marketed
Santaris Pharma	SPC 4955	subcutaneous injection	Apolipoprotein B-100 (ApoB)	hypercholesterolaemia	phase I	completed
eFFECTOR Therapeutics	Zotatifin/eFT 226	intravenous/subcutaneous	Eukaryotic Initiation Factor-4A (EIF4A1)	COVID 2019 infections	phase I	favorable safety results and positive trends in multiple antiviral activity measures
Acasti Pharma	GTX-102	oral spray	Paternal UBE3A	ataxia telangiectasia	phase I	pharmacokinetic (PK) study for GTX-102 met all outcome measures
University of Texas Southwestern Medical Center/Viridian Therapeutics	MRG-110 (Anti-Mir92a; S95010)	local intradermal injection	Microrna-92	heart failure, ischemia, wounds	phase I	well-tolerated safety profile; efficacy will be evaluated for heart failure and ischemia
University of Texas M. D. Anderson Cancer Center/Bio-Path Holdings	BP1002	intravenous infusion (liposomal)	Proto-oncogene protein c-Bcl-2	acute myeloid leukemia, advanced lymphoid malignancies	phase I	ongoing
Texas A&M University/Ultragenyx Pharmaceutical	GTX-102	intrathecal (it) injection	Paternal UBE3A	Angelman syndrome	phase I/II	recruiting
University of Texas M. D. Anderson Cancer Center/Bio-Path Holdings	Prexigebersen (BP1001)	intravenous infusion (liposomal)	Grb2	cancer	phase I/II	updated efficacy and safety data from the phase II trial in acute myeloid leukemia (AML) was released
Ionis Pharmaceuticals	ISIS-GCGRRx (ISIS 449884)	subcutaneous injection	Glucagon Receptor (GCGR),	type 2 diabetes	phase II	positive data from the phase II
GSK	GSK3389404 (IONIS HBV LRx)	subcutaneous injection	Hbv RNA	hepatitis B	phase II	acceptable safety, but no efficacious dosing regimen was identified

Table 1. Continued

Company	Candidate	Route	Biological target	Condition or disease	Clinical stage	Latest update
University of Marburg/ Sterna biologicals	SB-010	inhalation	GATA3 Transcription Factor	allergic asthma; chronic obstructive pulmonary disease; ulcerative colitis	phase II	completed
University of Marburg/ Sterna biologicals	SB-011	topical (water/oil/water emulsion) emulsion	GATA3 Transcription Factor	mild to moderate atopic dermatitis	phase II	completed
University of Marburg/ Sterna biologicals	SB-012	intrarectally	GATA3 Transcription Factor	ulcerative colitis	phase II	completed
Ionis Pharmaceuticals	Sapablursen (ISIS 702843, IONIS-TMPRSS6-LRx)	subcutaneous injection	Transmembrane Protease Serine 6 (TMPRSS6)	beta thalassemia, polycythemia vera	phase II	development of sapablursen in beta thalassemia was discontinued, while that of polycythemia vera is on going
Sarepta Therapeutics	Vesleteplirsen/SRP 5051	intravenous infusion	Exon 51 of Dystrophin	Duchenne muscular dystrophy	phase II	ongoing
AstraZeneca/Ionis Pharmaceuticals	Danvatirsen (AZD9150, IONIS-STAT3-2.5Rx)	intravenous infusion	Stat3	cancer	phase II	promising data in combination with durvalumab
eFFECTOR Therapeutics	Zotatifin/eFT 226	intravenous	Eukaryotic Initiation Factor-4A (EIF4A1)	breast cancer; non-small cell lung cancer	phase II	safe and well-tolerated, with encouraging signals of efficacy
Ionis Pharmaceuticals	QR 1123 (IONIS-RHO-2.5Rx; ISIS-GSK5-2.5Rx)	intravitreal (ivt) injection	Rhodopsin	retinitis pigmentosa	phase II	suspended
Geron Corporation/ Janssen Biotech	Imetelstat (GRN 140719; GRN 163; GRN 163-L)	intravenous infusion	RNA Template of Telomerase Reverse Transcriptase.	cancer	phase II/III	commercial launch announced May 2023
BioMarin Nederland	Drisapersen (GSK2402968)	subcutaneous injection	Exon 51 of Dystrophin mRNA.	muscular dystrophies	phase II/III	discontinued (FDA disapproval)
Ionis Pharmaceuticals	Tominersen/ IONIS-HTTRx; ISIS-443139; RG 6042	intrathecal injection	Huntingtin (Htt) Protein	Huntington's disease	phase II/III	ongoing
Ionis Pharmaceuticals/ Roche	IONIS-FB-LRX	subcutaneous injection	Complement Factor B (CFB)	primary Iga nephropathy/ geographic atrophy	phase II/III	phase II showed promising clinical data
Ionis Pharmaceuticals	Olezarsen (AKCEA-APOCIII-LRx; ISIS 678354)	subcutaneous injection	ApoC-III	hypertriglyceridemia/ cardiovascular diseases	phase III	granted a fast-track designation
Ludwig Institute for Cancer Research/ Biogen/Ionis Pharmaceuticals	Tofersen (BIIB067)	intrathecal injection	Superoxide Dismutase 1 (SOD1)	amyotrophic lateral sclerosis (ALS)	phase III	approved

Table 1. Continued

Company	Candidate	Route	Biological target	Condition or disease	Clinical stage	Latest update
Atlantic Healthcare	Alicaforsen (ISIS 2302)	enema/intravenous infusion	Intercellular Adhesion Molecule-1 (ICAM-1)	ulcerative colitis/Crohn's disease/pouchitis	phase III	approved
Sarepta Therapeutics	Casimersen/ AMONDYS 45; SRP-4045	intravenous infusion	Exon 45 of Dystrophin	Duchenne muscular dystrophy	phase III	interim results show casimersen safe, efficacious
Akcea Therapeutics/ Ionis Pharmaceuticals	Inotersen (GSK 2998728)	subcutaneous injection	Transthyretin (TTR)	amyloidosis	phase III	marketed
Ionis Pharmaceuticals	Donidalorsen (IONIS-PKK-LRX)	subcutaneous injection	Prekallikrein (PKK)	hereditary angioedema	phase III	was found to reduce the number of monthly swelling attacks
Ionis Pharmaceuticals	Eplontersen (AKCEA-TTR-LRx)	subcutaneous injection	Transthyretin (TTR)	hereditary transthyretin-mediated amyloid polyneuropathy (hattr-pn)	phase III/pre-registration	completed
Korea Institute of Science and Technology/ ISIS Pharmaceuticals	ISIS 353512 (ISIS CRPRx)	subcutaneous injection	C-Reactive Proteins (Crp)	inflammatory diseases	phase I	discontinued
Enzon Pharmaceuticals	RG 6061/EZN-2968/RO-7070179	intravenous infusion	Hypoxia-Inducible Factor-1 Alpha	liver cancer; lymphoma; solid tumors	phase I	discontinued
Santaris Pharma	SPC 5001	subcutaneous injection	Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)	hypercholesterolemia	phase I	discontinued
Ionis Pharmaceuticals	Tadnersen (BIIB078)	intrathecal injection	Chromosome 9 Open Reading Frame 72 (C9orf72)	amyotrophic lateral sclerosis	phase I	discontinued due to unmet secondary efficacy endpoints and no clinical benefit
National Institute of Allergy and Infectious Diseases/Sarepta Therapeutics	radavirsen (AVI-7100)	intravenous infusion	Matrix 1 & 2 Segment Of Influenza A	influenza a virus infections	phase I	discontinued
Santaris Pharma	RG 6061 (EZN-2968; RO-7070179; SPC-2968)	intravenous infusion	Hypoxia-Inducible Factor-1 Alpha (HIF-1α)	liver cancer; lymphoma; solid tumors	phase I	discontinued
Santaris Pharma	EZN-4176	intravenous infusion	Androgen Receptor (Ar)	bone cancer; prostate cancer	phase I	discontinued
Georgetown University	LErafAON-ETU	intravitreal injection (liposome entrapped)	C-Raf	solid tumors	phase I	discontinued
Santaris Pharma	SPC 2996	intravenous infusion	B cell Lymphoma 2 (Bcl-2)	chronic lymphocytic leukemia	phase I/II	discontinued
Santaris Pharma	Miravirsen/SPC-3649	subcutaneous injection	Mirn122	chronic hepatitis C	phase I/II	discontinued
AstraZenec/Regulus Therapeutics	RG 125 (AZD 4076)	subcutaneous injection	Microrna-103/107	type 2 diabetic with nonalcoholic fatty liver disease	phase I/II	discontinued
Daiichi Sankyo Inc	Renadirsen (DS-5141b)	subcutaneous injection	Dystrophin Exon 45	Duchenne muscular dystrophy	phase I/II	discontinued
Temple University	G 4460 (c-myb ASO G4460; INX 3001; LR3001)		Proto Oncogene Proteins C Myb	chronic myeloid leukemia; leukemia	phase I/II	discontinued

Table 1. Continued

Company	Candidate	Route	Biological target	Condition or disease	Clinical stage	Latest update
		intravenous infusion/parenteral: aso treated bone marrow				
WaVe life Sciences	Rovanersen/BMTR5150; WV-1092; WVE-120101	intrathecal injection	Huntington (HTT)	Huntington's disease	phase I/II	terminated
Idera Pharmaceuticals	AEG 35156	intravenous infusion	X-Linked Inhibitor Of Apoptosis (XIAP)	cancer	phase I/II	terminated; failed to reach endpoints; neurotoxicity
WaVe life Sciences	Lexanersen/WV-2603; WVE-120102	intrathecal injection	Huntington (HTT)	Huntington's cisease	phase I/II	termination due to lack of efficacy
ProQR Therapeutics	Sepofarsen (EW9I3PJC3R; QR-110)	intravitreal (ivt) injection	Cep290 Protein	Leber congenital amaurosis	phase I/II	termination due to sponsor's decision
Ionis Pharmaceuticals	Vupanorsen (IONIS ANGPTL3-LRx/AKCEA-ANGPTL3-LRX/ISIS 703802)	subcutaneous injection	Angiopoietin-Like 3 (Angptl3)	dyslipidemia/nonalcoholic fatty liver disease/diabetes mellitus, type 2	phase II	discontinuation for hypertriglyceridaemia
University of Nebraska Medical Center	Cenersen (Aezea; EL625; EL831; OL(1)p53)	intravenous infusion	Exon 10 In P53	myelodysplastic syndromes; acute myeloid leukemia; chronic lymphocytic leukemia	phase II	Discontinued.
Regulus Therapeutics; Sanofi	Lademirsen (RG 012; RG 456070; SAR 339375)	subcutaneous injection	Microrna-21	Alport syndrome	phase II	discontinued
Isis Pharmaceuticals	AZD 4785 (IONIS-KRAS-2.5Rx)	intravenous infusion	Kras	non-small cell lung cancer, advanced solid tumors	phase II	discontinued
Isis Pharmaceuticals	IONIS AR 2.5Rx (AZD 5312; ISIS ARRX; ISIS AZ1Rx; ISIS-560131)	intravenous infusion	Androgen Receptor (Ar)	prostate cancer; solid tumors	phase II	discontinued
Isis Pharmaceuticals	ISIS-FXIRx (ISIS 416858)	subcutaneous injection	Factor XI	end-stage renal disease (ESRD)/venous thromboembolism	phase II	discontinued
Ionis Pharmaceuticals	Cimdelirsen (IONIS-GHR-LRx; ISIS 766720)	subcutaneous injection	Growth Hormone Receptor (Ghr)	acromegaly	phase II	discontinued
Isis Pharmaceuticals	ISIS 5132 (CGP 69846A)	intravenous infusion	C-Raf	breast cancer/ovarian cancer	phase II	discontinued
Ionis Pharmaceuticals/University of British Columbia	Apatorsen (ISIS-306053; OGX-427)	intravenous infusion	Heat Shock Protein Hsp27	cancer	phase II	discontinued
Isis Pharmaceuticals	Aprinocarsen (Affinitac; Aprenocarsen sodium; CGP 64128A; ISIS 3521)	intravenous infusion	Protein Kinase C (PKC)	cancer	phase II	discontinued, N/A

Table 1. Continued

Company	Candidate	Route	Biological target	Condition or disease	Clinical stage	Latest update
University of Pennsylvania	Oblimersen (Augmerosen; BCL-2 ASO - Genta; Genasense)	intravenous infusion	B cell Lymphoma 2 (Bcl-2)	cancer	phase II/III	discontinued
Prosensa	Drisapersen (GSK2402968)	subcutaneous injection	Exon 51 Of Dystrophin Mrna	muscular dystrophies	phase II/III	discontinued (FDA disapproval)
ProQR Therapeutics	Ultevursen (QR-421)	intravitreal (ivt) injection	Exon 13 Of The USH2A Gene	Usher syndromes/ retinitis pigmentosa	phase II/III	termination of phase II in Usher syndromes due to the sponsor's decision; termination of phase II/III trial in retinitis pigmentosa
University of British Columbia	Custirsen (ISIS 112989; OGX-011; TV-1011)	intravenous infusion	Clusterin	cancer	phase III	discontinued; did not meet endpoint of significantly extending overall survival
WaVe life Sciences	Suvodirsen/ WV-3473; WVE-210201	intravenous	Exon 51 Of Distrophin	Duchenne muscular dystrophy	phase II/III	terminated

effective dose should be well below the dose that causes significant adverse events. In the context of adverse events of ASOs, adverse events are divided into two categories: hybridization-dependent off-target effects and non-hybridization-dependent off-target effects.[16]

If we take the phosphorothioate backbones of the first-generation ASOs as an example, while efficient, they seem to be immunostimulatory, exerting a proinflammatory effect through nucleic acid-sensing TLR receptors among other suggested mechanisms. This adverse effect falls under the definition of non-hybridization-dependent off-target effects, as it is not a result of an ASO-target Watson/Crick base-pairing but rather linked to the high dose in which phosphorothioates should be administered.[16,29]

Using LNAs with their conformational lock, as well as their short length, makes them very potent compared to other ASOs, which allow them to be administered in lower doses. Unfortunately, most research shows that this comes at the expense of increased hybridization-dependent off-target effects, in which a sequence can bind sequences other than its intended target. This seems to correlate with the short length of the LNAs. Swayze et al. tested different corresponding sequences of ASOs, using MOE versus LNA chemical modification as the sole variation between each correspondent pair. Their results demonstrate a superior efficacy of LNAs *in vivo* compared to MOEs in the downregulation of TNFRSF1A-associated via death domain mRNA in the livers of Balb/c mice. However, all LNA treatment arms showed a significant increase in transaminases, to an extent where the high-dose group of the most potent sequence had to be sacrificed following a 25% loss of body weight because of hepatotoxicity. Hepatotoxicity in MOE arms was lower according to all the parameters.[30]

DELIVERING THE ASOs VIA LIPID NANOPARTICLES

As shown in Table 1, the majority of ASO clinical trials have utilized systemic intravenous administration, which means that they will be evenly dispersed throughout tissues and cells, necessitating a high dosage to achieve adequate concentration at the disease site. Consequently, as higher doses of ASOs are more likely to induce adverse effects, this method of general, unguided administration increases the chances for undesired side effects.[31,32]

Getting to the site of action with adequate quantity is just one of the challenges for ASOs. Effective treatment relies also on sufficient cellular uptake as well as successful exit from subcellular compartments and accurate hybridization to the target sequence. The cellular uptake of unmodified ASOs is typically poor, with only a fraction of ASO molecules managing to enter the cell, often showing less than 1% of the molecules arriving to the cytoplasm.[33,34]

Even when tested in controlled conditions (*in vitro*), the specific mechanisms of cellular uptake depend on various factors including chemistry, molecule length, conformation, and concentration, as well as cell type and cell culture conditions or environment among others. For instance, the PS group generally enhances cell uptake but diminishes RNA target affinity, a drawback that can be mitigated by $2'$-sugar substituents.[33]

Despite the chemical modifications that are meant to solve issues of delivery and targeting, challenges such as limited efficacy, off-target effects, and toxicity persist as barriers to broader adoption in clinical practice.[33,34]

Accordingly, drug delivery systems were created as a method to control the release and the behavior of a therapeutic agent in the body. To date, there is a versatile arsenal of nano-scale delivery systems available. The ADME of these systems may be tailored to the purpose, via modifying the system's physicochemical properties, with the most important factors being the nanoparticle size, charge, hydrophobicity, and morphology.[35] In addition to the intrinsic factors, additional features may be added and fine-tuned to reach a certain goal; for instance, PEGylation of nanoparticles increases their circulation time by lowering their uptake by the mononuclear cells of the immune system.[36]

Vehicles have been recently implemented in the context of ASO delivery. Among the prominent examples of this are Prexigebersen/BP1001 and BP1002 (Bio-Path Holding). Prexigebersen, a neutral liposomal formulation of an ASO against Grb2 mRNA, is currently being evaluated for its efficacy in combination with chemotherapy in untreated, elderly AML patients as well as in relapsed or refractory AML patients, while BP1002, a neutral liposomal formulation of ASO against Bcl-2, is currently in phase 1 clinical trials in patients with advanced lymphoid malignancies (NCT04072458) and patients with refractory/relapsed AML (NCT05190471), and these are ongoing.[37]

In addition to the above, many delivery techniques were and are tested, showing potential results such as bio-reducible LNPs that can both achieve a good performance in encapsulating the ASO and in delivering it to intracellular sites of action, as well as to target organs.[38]

Despite those efforts, we believe that drug delivery systems are not sufficiently employed to benefit the delivery and therapeutic efficacy of ASOs. One category of potentially suitable carriers that has been rising to fame in recent years are lipid nanoparticles (LNPs), which as of today are the most advanced non-viral nucleic acid delivery vehicles. The building blocks of LNPs include 3 helper lipids in the form of PEG-lipids, phospholipids, and cholesterol, and an amino ionizable cationic lipid.[39,40] The ionizable cationic lipid was a game changer in the field of nanoparticles due its ability to change its charge in different environments. While in acidic pH the ionizable lipid is positively charged, which increases both payload encapsulation efficiency and intracellular delivery, it is neutrally charged in physiological pH, greatly lowering its immunogenicity in the blood.[41,42] Vast libraries of ionizable lipids have been and are still being developed, opening different avenues of targeting, with some being cell specific and others nucleic acid specific.[39,40]

To achieve efficient treatment, LNPs should first reach the cell population of interest, after which they should be internalized to finally
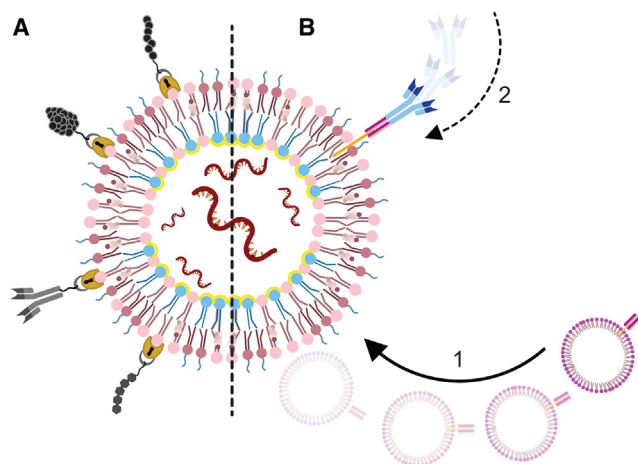
Figure 5. Targeting options and techniques for LNPs

(A) Peptides, proteins, antibodies, and carbohydrates (top to bottom respectively) may be incorporated through a covalent bound to a surface lipid. Alternatively, (B) a system developed in our lab (ASSET): (1) membrane-anchored-scFv vesicles are fused with nucleic acid-containing LNPs, yielding LNPs that can (2) non-covalently bind the Fc constant region of any rat IgG2a.

release their cargo into the cell cytosol. Over the years, many targeting options and techniques have been developed (e.g., antibodies and ligands) to direct the particles to unload their payload at the disease site or to a specific cell subset. One example of such a system is the ASSET, a self-assembled modular platform developed at our lab, which is a ready-to-use LNP system on which an unlimited repertoire of targeting moieties can be linked, eliminating the need for extensive calibration (Figure 5).[43]

After arriving at the target, LNPs can be internalized through dynamin-dependent pathway, clathrin-dependent pathway, macropinocytosis, or direct fusion with the cell membrane.[44] Eventually the internalization mechanism depends on many factors, including size, ionizable lipid of choice, and helper lipids.[45] Finally, once inside the endosome, the ionizable cationic lipid becomes positively charged due to the low pH in the endosome, and this initiates the destabilization of the endosome with the LNP-endosomal membrane adhesion, fusion, and final disruption facilitated by cholesterol and helper lipids through phase transition of the LNPs (Figure 6).[46]

Just 3 years ago, the world witnessed the unprecedented approval of LNPs as the carrier system for the delivery of mRNA vaccines against the COVID-19 virus. Even though ASOs are far smaller than the mRNA used in the approved COVID-19 vaccines, LNPs have also been proven in the past to be efficient carriers for far smaller molecules such as siRNA, as LNPs delivering siRNA were clinically approved even before the COVID-19 vaccines in the form of Patisiran, a transthyretin-targeting siRNA encapsulated in an LNP. Therefore, it is logical to assume that LNPs can also efficiently encapsulate and deliver ASOs *in vivo*, potentially reducing the required dosage for efficacy and thus the adverse side effects. The mentioned approval of the COVID-19 vaccine drove a much-needed advancement in the research and characterization in the field of LNPs, opening new avenues for their implementation, and we believe that delivering ASOs can be one new approach.

FUTURE OUTLOOK

In this perspective, we have discussed the use of ASOs with an emphasis on LNAs. What seems to be apparent is that the chemistry and sequence of ASOs dictate not only the function but also the ADME profile of the molecules. Despite the unprecedented potency of LNAs compared to other ASOs, several studies have found that this potency leads to higher incidents of hybridization-dependent off-target effects. Although the dose needed to obtain an effect is lower in the case of LNAs as noted by some studies, their lack of specificity will cause off-target effects in undesired organs, while not delivering an adequate effective dose to the intended site of action. This issue might be lowered to a degree by their incorporation into LNPs. Due to their physicochemical properties, LNPs tend to have a more specific accumulation profile, and accordingly, a lower systemic dose of LNAs will be needed to exert the needed effect at the relevant site of disease. For example, in the case of cancer or inflammatory disease, the enhanced permeability and retention may be used as passive targeting to deliver a concentrated dose of LNAs. Alternatively, LNPs may be decorated with an active targeting moiety, delivering the payload, LNAs in this case, to a further specific site.

Diving closely into cellular distribution, it was shown that when LNAs are endocytosed, they will persist in an "inactive" state as long as they are in the endosome, which acts as their trap, and will be "activated" only once shed into the cytoplasm.[23,49] We hypothesize that the usage of LNPs as a carrier of LNAs might help in increasing the percentage of "activated" LNAs out of a given dose, particularly LNPs that are fabricated using ionizable lipids. These ionizable lipids become positively charged in the acidic environment of the endosome, which facilitates electrostatic interactions, leading to their fusion with the negatively charged endosomal membrane.

Collectively, we believe that the introduction of LNPs will lower the required dose by tens of folds compared to naked LNAs and may ultimately open new therapeutic modalities.

AUTHOR CONTRIBUTIONS

S.Q. wrote the review. S.Q., D.B., and I.H.-H. contributed to the revision. G.S.N. created the figures of the chemical structures. D.P. supervised the writing and editing of the article.
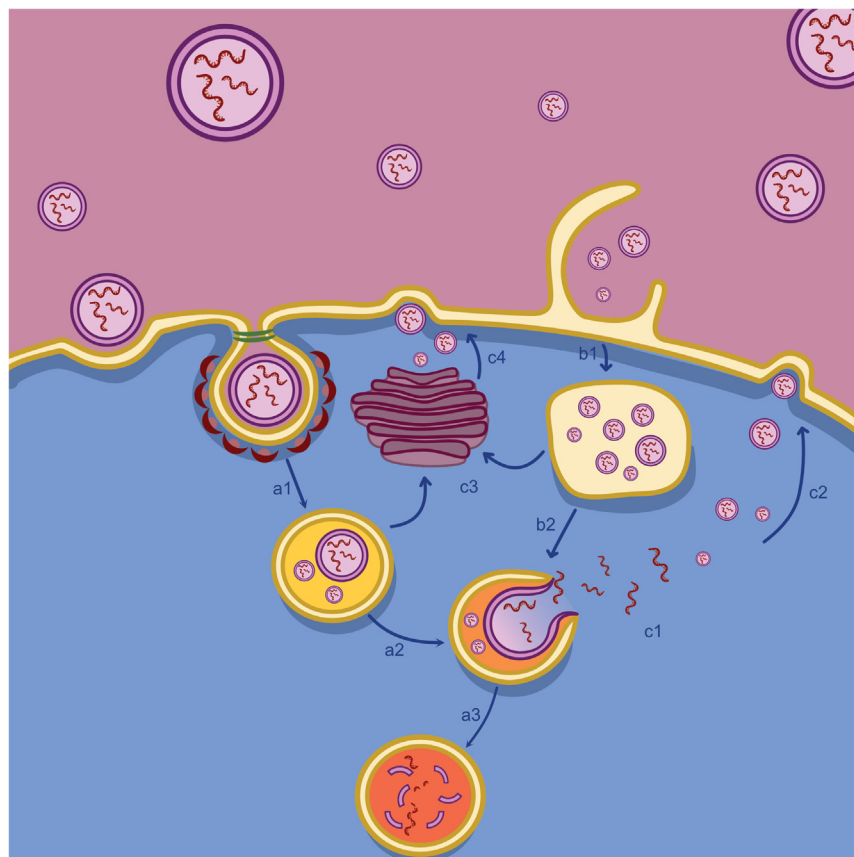
Figure 6. Possible pathways of LNP internalization

(A1) Internalization may start with clathrin-coated vesicles that mature into an early endosome; (A2) endosomal escape of the cargo (nucleic acid) may occur in the next step where an early endosome matures into a late endosome; alternatively, (A3) the late endosome might mature into a lysosome, where the cargo is likely to be destroyed. LNPs may also be internalized (B1) via macropinocytosis, where (B2) endosomal escape might occur at a later stage from the late endosome. LNPs can exit the cell through vesicle-related and non-vesicle-related routes.[47,48]

DECLARATION OF INTERESTS

D.P. receives licensing fees (to patents on which he was an inventor) from, invested in, consults (or on scientific advisory boards or boards of directors) for, lectured (and received a fee), or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNtech SE, Earli Inc., Kernal Biologics, Geneditor Biologics, Newphase Ltd., NeoVac Ltd., Roche, SirTLabs Corporation, and Teva Pharmaceuticals Inc.

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