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Criteria for developing active cellular targeting miRNA oligonucleotide therapeutics with a peptide nucleic acid backbone: Combating cardiometabolic pandemics

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Abstract

Oligonucleotide therapeutics (ONTs) represent a growing new class of therapeutic agents aimed at addressing chronic diseases that remain untreatable by small molecules and antibodies. Our goal was to establish a selection of several criteria to design and develop miRNA-based ONTs, focusing on improved chemistry, pharmacokinetics/pharmacodynamics (PK/PD) profiles, and safety characteristics to combat cardiometabolic pandemics. By leveraging our own experimental data obtained from experiments involving miR-22-3p antagomirs and a careful review of the literature, we established a set of seven criteria to optimize the design of miRNA ONTs. These criteria prioritize simplified drug synthesis, optimized PK/PD properties, and reduced potential toxicities. This proposed set of seven criteria represents a novel strategy for developing active cellular targeting miRNA ONTs for various therapeutic indications.

Keywords: MiRNAs; Antagomirs; Oligonucleotide therapeutics; Active cellular targeted delivery; Cardio-metabolic pandemics

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1. Introduction

Twenty-five years after the initial discovery of RNA interference by Fire *et al.*,[1] innovations in RNA chemistry and delivery strategies have facilitated the design and advancement of RNA-based therapies.[2-4] Oligonucleotide therapeutics (ONTs) exhibit the ability to target almost any gene, including those associated with so-called "un-druggable diseases"—conditions where genes are implicated beyond the reach of small molecules and antibodies.[5] RNA-based ONTs are distinct from small molecules and protein drugs in several aspects,[6] particularly their mechanisms of action and clinical pharmacology features. This distinction is especially notable in ONT conjugates designed for specific tissue delivery.[7,8]

The different classes and characteristics of ONTs currently being developed are shown in Table 1.

Nineteen ONTs, mainly antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), have been approved by regulatory authorities for the treatment of patients (Table 2).[9-12]

Table 1. Characteristics of the classes of oligonucleotide therapeutics

Class	Structure	Target	Site of action	Mechanism of action
Anti-sense oligonucleotides • Gapmers • Splice-switching	Single-stranded molecule	mRNA and pre-mRNA	Nucleus and cytoplasm	Gapmer: RNA degradation by RNase H and splice site switch
Small interfering RNAs	Double-stranded molecule	mRNA	Cytoplasm	mRNA degradation
miRNA antagomirs	Single-stranded molecule	miRNA	Cytoplasm	Steric inhibition
miRNA agomirs	Double-stranded molecule	mRNAs	Cytoplasm	Modulation of mRNA targets
Aptamers	Single-stranded molecule	Extracellular protein and cell surface	Extracellular site	Inhibition of protein function

Chemical modifications of ONTs have been introduced to improve the "druggability" of ONT candidates. Most of the ONT drugs currently approved for human use include (i) "first generation," which involves backbone modifications (mainly phosphorothioate [PS]) and (ii) "second generation," which involves nucleobase and sugar modifications (2'-fluoro [2'F], 2'O-methyl [2'-OMe], 2'-methoxyethyl [2'-MOE]), cytosine methylated at position 5 [m^5C], gapmers, 5'-phosphate stabilization, as well as backbone modifications (locked nucleic acid [LNA], phosphorodiamidate morpholino oligomers [PMO], and glycol nucleic acid).[9,13-15] These chemical modifications were introduced to:

(i) Improve compound stability, bioavailability, and efficacy
(ii) Avoid off-target toxicity
(iii) Elude innate immune response.

To enhance the efficacy, safety profile, and therapeutic index of ONTs, generation 2.5 ONTs (also referred to as third-generation ONTs) were developed. They include various delivery formulations (cholesterol, liposomes, lipids, nanoparticles, polymers, cell-penetrating peptides, and antibody conjugates)[16,17] and/or a cellular targeting moiety such as N-acetyl-galactosamine (GalNac) for specific delivery to the liver at a much-reduced effective dose.[18]

Our ultimate objective is to develop safe and effective therapies with reduced cost of synthesis and improved patient compliance, two key factors in prevalent and chronic diseases.

2. MiRNAs

Mature miRNAs are conserved 18 – 24 nucleotides long non-coding RNAs that post-transcriptionally regulate gene functions through direct degradation of their target messenger RNAs (mRNAs) and/or translational repression.[19] MiRNAs are stable, usually cell-type specific, easily measured in body fluids and tissues, and involved in exosomal cell-to-cell communications.[20-24]

MiRNAs serve as useful diagnostic, prognostic, and therapeutic markers for many human diseases,[25-30] including cancers.[31-34] Furthermore, miRNAs are relevant modulators of disease pathogenesis and valuable molecular therapeutic targets.[35,36] Due to their specific expression patterns and their ability to target multiple transcripts through a one-to-multiple pattern, miRNA modulators (known as agomirs and antagomirs) can simultaneously alter the expression of several genes and proteins involved in complex diseases that cannot be adequately treated by small-molecule drugs.[37]

3. MiRNA antagomirs

MiRNA antagomirs (or anti-miRs) are synthetic, single-stranded ONTs that bind by Watson–Crick base-pairing to their complementary miRNAs, preventing these miRNAs from modulating their target mRNAs (Figure 1). Antagomirs do not induce RNAse H-mediated target RNA degradation. The risk of off-target effects of antagomirs is low due to their high binding specificity and affinity.[13] Compared to other ONTs, the mechanism of action of antagomirs is straightforward (steric blockade) and localized into the cytoplasm (Figure 1).

Various agomirs and antagomirs are in pre-clinical development, especially for oncology indications (miR-10b, miR-21, miR-29, miR-34a, miR-221, and miR-710).[2,36,38-42] MiRNA-based therapies that have reached clinical trials target miR10b, miR-16, miR-17, miR-21, miR-34a, miR-92a, miR-103/107, miR-122, miR-132, miR-155, and miR-200a/c.[36,40] Several miRNA-based ONTs have entered clinical development (Table 3).[37,43-46]

4. Selection of structural and chemical criteria for new generation 2.5 antagomirs

We propose a set of seven chemical modifications to optimize the properties of miRNA antagomirs as therapeutic agents for prevalent and chronic diseases (Table 4).

Table 2. Oligonucleotide therapeutics approved by regulatory authorities

Year of approval	Compound (Trade name)	Category	Chemical changes	Coupling agent	Route of administration	Indication
1998	Fomivirsen (VITRAVENE®)	21-mer ASO	PS, DNA	-	Intravitreal	CMV retinitis
2004	Pegaptanib (MACUGEN®)	27-mer Aptamer	2'-OH, 2'-F, 2'-OMe, DNA	PEG	Intravitreal	Neovascular AMD
2013	Mipomersen (KYNAMRO®)	20-gapmer ASO	PS, 2'-MOE, m^5C	-	Subcutaneous	Hypercholesterolemia
2016	Eteplirsen (EXONDYS 51®)	30-mer SSO	2'MOE, PMO	-	Intra-venous	Duchenne muscular dystrophy
2016	Nusinersen (SPINRAZA®)	18-mer SSO	PS, 2'-MOE, m^5C	-	Intrathecal	Spinal muscular atrophy
2018	Patisiran (ONPATTRO®)	21-mer/21-mer siRNA	2'-OH, 2'-OMe	Liposomal	Intravenous	Hereditary transthyretin amyloidosis (ATTR amyloidosis)
2018	Inotersen (TEGSEDI®)	20-gapmer ASO	PS, 2'-MOE, m^5C	-	Subcutaneous	Hereditary ATTR amyloidosis
2019	Givosiran (GIVLAARI®)	21-mer/23-mer siRNA	PS, 2'-OMe, 2'-F	3 GalNAc residues	Intravenous	Acute hepatic porphyria
2019	Golodirsen (VYONDUS 53®)	25-mer SSO	2'-MOE, PMO	-	Intravenous	Duchenne muscular dystrophy
2019	Volanesorsen (WAYLIVRA®)	20-gapmer ASO	PS, 2'-MOE m^5C	-	Subcutaneous	Familial chylomicronemia syndrome
2020	Lumasiran (OXLUMO®)	21-mer/23-mer siRNA	PS, 2'-OMe, 2'-F	3 GalNAc residues	Subcutaneous	Primary hyperoxaluria
2020	Inclisiran (LEQVIO®)	21-mer/23-mer siRNA	PS, 2'-OMe, 2'-F	3 GalNAc residues	Subcutaneous	Hyper cholesterolemia
2020	Viltolarsen (VILTEPSO®)	21-mer SSO	2'-OMe, PMO	-	Intravenous	Duchenne muscular dystrophy
2021	Casimersen (AMONDYS 45®)	22-mer SSO	PMO	-	Intravenous	Duchenne muscular dystrophy
2022	Vultrisiran (AMVUTTRA®)	21-mer/23-mer siRNA	PS, 2'-F, 2'-OMe	3 GalNAc residues	Subcutaneous	Hereditary ATTR amyloidosis
2023	Eplontersen (WAINUA®)	20-gapmer ASO	PS, 2'-MOE, m^5C	1 trivalent GalNAc residue	Subcutaneous	Transthyretin-mediated amyloidosis
2023	Nedosiran (RIVFLOZA®)	22-mer/36-mer siRNA	PS, 2'-F, 2'-OMe	4 GalNAc residues	Subcutaneous	Primary hyperoxaluria
2023	Tofersen (QALSODY®)	20-gapmer ASO	PS, 2'-MOE, m^5C	-	Intrathecal	Amyotrophic lateral sclerosis with superoxide dismutase (*SOD1*) gene mutation
2023	Avacincaptad pegol (IZERVAY®)	39-mer RNA Aptamer	2'-OMe, 2'-F, inverted thymidine to cap 3' end	2 PEG chains	Intravitreal	Age-related macular degeneration

Abbreviations: ASO: Antisense oligonucleotide; AMD: Age-related macular degeneration; CMV: Cytomegalovirus; GalNAc: N-acetyl-D-galactosamine; m^5C: Cytosine methylated at position 5; PEG: Polyethylene glycol; PMO: Phosphorodiamidate morpholino oligomers; PS: Phosphorothioate; siRNA: Small interfering RNA; SSO: Single stranded oligonucleotide; 2'-F: 2'-Fluoro; 2'-MOE: 2'-methoxyethyl; 2'-OH: 2'-hydroxyl; 2'-OMe: 2'-O-methyl; 2'-MOE: 2'-Methoxyethyl.

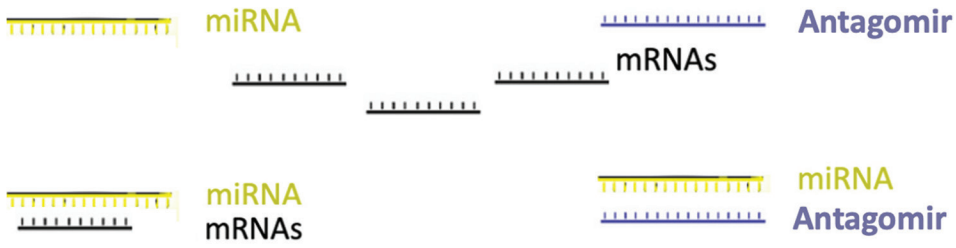


Figure 1. Mechanism of action of miRNA antagomirs. No binding of miRNA to complementary mRNAs in the presence of antagomir.

Table 3. MiRNA-based oligonucleotide therapeutics that have entered clinical development

Product	Sponsor	MiRNA	Stage of development	Indication
Miravirsen (SPC36490)	Roche/Santaris	MiR-122-5p antagomir	Phase II; NCT01200420; discontinued for strategic reasons	Hepatitis C
CDR132L	Cardior Pharmaceuticals	MiR-132-3p antagomir	Phase II; NCT05350969	MI and AHF
Lademirsen (SAR339375/ RG-012)	Sanofi/Genzyme	MiR-21 antagomir	Phase II; NCT03373786; NCT02855268	Alport nephropathy
RG-125 (AZD4076)	Astra-Zeneca	GalNAc-conjugated miR103/107 antagomir	Phase I/IIa; NCT02612662; NCT02826525; returned to Regulus	NAFLD/NASH
RGLS4326	Regulus Therapeutics	MiR-17-5p antagomir	Phase Ib; NCT04536688	PKD
RGLS8429	Regulus Therapeutics	GalNAc-conjugated miR17 Antagomir	Phase1b; NCT06621191	ADPKD
TargomiRs	Asbestos Disease Research Foundation	Nanoparticles delivered by miR-16 mimic	Phase I; NCT02369198	MPM and NSCLC
MRX34	Mirna Therapeutics	Liposomal miR-34a-5p mimic	Phase I; NCT018229971; terminated for immune-related SAE	Various cancers
Cobomarsen (MRG-106)	Miragen	MiR-155-5p antagomir (received Orphan Drug Product designation by the US FDA)	Phase I; NCT02580552; Phase II; NCT03713320	Cutaneous T-cell lymphoma
MRG-110 (S95010)	Miragen	MiR-92a-3p antagomir	Phase I; NCT03603431; NCT03494712	Wound healing
Remlarsen (MRG-201)	Miragen	Cholesterol-conjugated miR-29 mimic	Phase II; NCT026033224; NCT03601052	Keloid and scar formation

Abbreviations: ADPKD: Autosomal dominant polycystic kidney disease; AHF: Acute heart failure; US FDA: United States Food and Drug Administration; GalNAc: N-acetyl-D-galactosamine; MI: Myocardial infarction; MPM: Malignant pleural mesothelioma; NAFLD/NASH: Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis; NSCLC: Non-small cell lung cancer; PKD: Polycystic kidney disease; SAE: Serious adverse event.

4.1. Criterion 1: Select a specific miRNA target that is unique, universal, phylogenetically conserved, and significantly expressed in the tissues and organs of interest

MiRNAs are present across the animal kingdom and are usually phylogenetically conserved.[47] About 2,000 human miRNAs have been identified and experimentally validated.[48,49] Furthermore, most miRNAs exhibit physiological effects at high expression levels in tissues and organs. Some of them belong to families (e.g., let-7 family of 15 miRNAs, miR-1-133 family, miR-34 family, miR-103/107 family, miR-17-92 cluster),[50,51] while others are unique. If one were to develop a specific miRNA antagomir, it would be intuitive to choose a miRNA target that is unique instead of a member of a miRNA family to avoid the risk of so-called on/off-target effects.[52]

To design generation 2.5 pharmacologic antagonists of mature miRNA guide strands, we used miR-22-3p as a therapeutic target for treating cardio-metabolic diseases.[53,54] The miR-22-3p nucleotide sequence is highly conserved across vertebrate species, including mice, rats, dogs, pigs, and primates (relevant information can be obtained from www.targetscan.org), suggesting that experimental data could be extrapolated from one species to another. We examined the expression of hsa-miR-22-3p across human tissues and organs using the TissueAtlas2

Table 4. Criteria selected to develop active cellular targeting miRNA ONTs

Criteria	Description
1	Select a specific miRNA target that is unique, universal, phylogenetically conserved, and significantly expressed in the tissues and organs of interest to optimize the benefit/risk ratio.
2	Replace the chemical modifications (phosphorothioate and locked nucleic acid) utilized in generations 1 and 2 compounds with a gamma peptide nucleic acid backbone to avoid potential toxicities linked to specific chemical groups.
3	Optimize the backbone and nucleoside modifications to increase resistance to nucleases and proteases/peptidases, improving the ONT PK/PD profile.
4	Avoid chirality to increase the ONT purity, stability, PK/PD profile, and safety.
5	Limit binding to serum proteins to favor uptake by the tissues and organs of interest before clearance/elimination by the liver and kidneys.
6	Optimize and simplify chemical synthesis to increase drug purity and reduce the cost of goods.
7	Conjugate the ONT to a fatty acid or a short peptide for enhanced and active targeted delivery to the tissues and organs of interest, delivering a much-reduced therapeutic dose with an extended mean residence time.

Abbreviations: PK/PD: Pharmacokinetic/pharmacodynamics; ONTs: Oligonucleotide therapeutics.

program.[55] The tissue specificity index (TSI) gives each non-coding RNA a value from 0 to 1, where 1 indicates that the RNA is detected in only one specific tissue and 0 indicates it is detected in all tissues. While miRNAs show a broad range of TSI values, the TSI for hsa-miR-22-3p is 0.855, signifying an elevated tissue-specific expression pattern, specifically in the adipocytes, myocardium, and skeletal muscle.[56]

We implemented a small throughput-high yield R and D strategy[56] as an alternative to the classic high throughput screening strategy implemented by pharmaceutical companies to find "hit compounds" from libraries of millions of samples. Several *in silico*, *in vitro*, and *in vivo* tools, we reported that miR-22-3p (a unique miRNA evolutionarily conserved from flies to mammals) modulates several metabolic genes involved in lipid oxidation, mitochondrial activity, energy expenditure, fat accumulation, inflammation, and necrosis.[53,54] In animal studies, we demonstrated that miR-22-3p antagomirs with first- and second-generation chemical modifications produced several metabolic improvements: Reduction of fat mass without change of lean mass while food intake remained unchanged, improvement of glucose, cholesterol, insulin sensitivity, and a marked reduction of liver steatosis.

In addition to our work, several other investigators have independently confirmed the metabolic roles of miR-22. Yang *et al.*[57,p.322] concluded that "miR-22 inhibitors may have potential as candidate drugs for nonalcoholic fatty liver disease (NAFLD) and obesity." Kaur *et al.*[58] reported abnormally high levels of miR-22-3p in the livers of *db/db* mouse models of insulin resistance and Type 2 diabetes. They showed that silencing miR-22-3p by antagomir administration lowered random and fasting glucose in diabetic mice and improved glucose tolerance and insulin sensitivity. Hu *et al.*[59,p2] concluded that "reducing miR-22 enhances hepatic fibroblast growth factor 21 and activates AMP-activated protein kinase, which could be a novel approach to treat steatosis and insulin resistance." In a more recent study, Castano *et al.*[60] reported that elevated circulating miR-22-3p is a relevant biomarker for the stratification of patients with metabolic dysfunction-associated fatty liver disease (MAFLD). These authors suggested that elevated circulating miR-22-3p levels may be used to identify patients with a high fatty liver index. Panella *et al.*[61] used knockout and transgenic mouse models to report that miR-22 loss-of-function protects against obesity and hepatic steatosis, while its overexpression promotes both phenotypes even when mice are fed a regular chow diet. These studies included the use of first- and second-generation antagomirs. A biotechnology company (www.resalistherapeutics.com) is developing LNA/DNA "mixmer" miR-22 antagomirs for the treatment of obesity. One of their candidates is conjugated to GalNAc to treat metabolic dysfunction associated with steatohepatitis.

4.2. Criterion 2: Avoid potential toxicities by replacing PS and LNA chemical modifications utilized in generations 1 and 2 compounds with a gamma peptide nucleic acid (PNA) backbone

The first generation of ONTs, already advanced into clinical trials, incorporated several medicinal chemistry modifications, including:[62-64]

(i) PS backbone modifications, which were used to diminish nuclease degradation and augment plasma protein binding to promote tissue uptake.
(ii) Gapmer oligodeoxynucleotides, which cause RNase H cleavage of the target RNA.
(iii) Ribose modifications (mainly 2' position modifications, e.g., 2'-F, 2'-OMe, 2′-MOE, and 2' – 4' LNA), which were used to increase stability and specificity.

A panel of experts (DARTER group [https://antisenserna.eu/]) recently published considerations in the preclinical assessment of the safety of ASOs.[65] Based on animal and human data, they suggested that blood platelets and complement activation should be examined for PS-ASOs that renal and hepatic toxicities should be assessed for LNA gapmers. Therefore, alternative chemical

modifications that are not hampered by these potential toxicities are needed.

PNAs were discovered by Nielsen[66] and Nielsen *et al.*[67] in 1991. PNAs are synthetic DNA mimics with the deoxyribose phosphate backbone replaced by N-(2-aminoethyl)-glycine units (Figure 2). PNA molecules combine the properties of both peptides and nucleic acids. The neutral pseudopeptide backbone of PNAs enhances binding affinity, improves biological stability, and prolongs half-life.[68] Nucleobases (adenine, guanine, cytosine, and thymine/uridine) are attached to the backbone through a carboxy methylene linkage. The chemical properties of PNA ensure a similar distance between nucleobases, allowing tight hybridization with their complementary nucleic acid targets through Watson–Crick base-pairing. A given PNA recognizes its complementary nucleic acid strand with high affinity and excellent specificity, surpassing those of natural nucleic acids and their analogs.[69] Unlike gapmer ASOs, PNA–RNA hybrids are not targets for RNase H1 enzyme degradation.

The initial PNA structure included a neutral and achiral backbone, which is hampered by poor cellular penetration. Consequently, several chemical modifications (α-PNAs, β-PNAs, and γ-PNAs) were implemented to improve their hybridization properties and cellular penetration (Figure 3).

Gamma backbone modifications convert a randomly folded PNA into a helical structure.[70,71] The introduction of an L-serine side chain at the γ-position produces a right-handed helix PNA and enhances hybridization with its target. Regarding the risks of on/off-target effects, a PNA–RNA heteroduplex is more stable and more susceptible to mismatches than a PNA–DNA heteroduplex. A single base-pair mismatch in a PNA–RNA heteroduplex significantly diminishes the thermal melting temperature by approximately 12°C. PNAs require only 9 – 12 monomers to effectively discriminate and efficiently bind to their specific targets.[72] For reference, a 17-mer oligonucleotide sequence is expected to occur only once in the human genome.[9] Therefore, a 17 – 22-mer PNA ONT would not trigger on/off-target effects.

Mechanistically, PNA antagomirs bind to their target miRNAs by Watson–Crick base pairing, consequently sterically blocking downstream miRNA-mRNA interactions.[73] Thus, our single-stranded miR-22-3p ONTs act by Watson–Crick base-pairing complementary to the human miR-22-3p sequence in a specific manner.

The preclinical efficacy and safety of chemically modified PNAs have been established for multiple targets in several animal models. Furthermore, the capacity of PNAs to evade immune surveillance is a substantial benefit over ASOs.[68]

PNA antagomirs have been successfully tested to target miR-155,[74-76] miR-122,[77,78] miR-122-3p,[79] miR-21,[80,81] miR-101-3p, miR-145-5p and miR-335-5p,[82] miR-210,[83] miR-584-5p and miR-425-3p,[84] miR-141-3p,[85] miR-155-5p and miR-221-3p,[86] miR-10b and 21,[87] and miR-33.[88,89] As reported recently by Pradeep *et al.*, PNAs targeting mRNA, pre-mRNA, and miRNA can be used to treat various human pathologies.[68]

The therapeutic relevance of PNAs was initially hampered by their poor cellular permeability and the absence of efficient delivery methods. Recent advances in delivery methods of PNAs, using lipid or peptide conjugates or nanoparticles, have improved their *in vivo* efficacy and safety.[74,90] This topic is specifically addressed in Criterion 7 below.

4.3. Criterion 3: Maintain resistance to nucleases and proteases/peptidases

In contrast to small molecules and protein drugs, which are respectively metabolized or catabolized, ONTs can be degraded by endogenous RNases. ONTs share similar absorption, distribution, metabolism, and excretion (ADME) profiles within each chemistry category.[91,92] ONTs undergo prompt cellular uptake enabled by chemical conjugates and delivery methods, leading to a short circulating blood half-life. Such conjugated ONTs have a unique pharmacokinetic/pharmacodynamic (PK/PD) profile whereby their local PD effects in the targeted organs and tissues are distinct from their systemic PK.[6,93] After cellular/tissue uptake, the intracellular concentration of the ONT, not its systemic concentration, interacts with the targeted mRNAs, leading to an extended PD half-life and dosing frequency schedule. The PK/PD and ADME features of GalNAc-conjugated siRNAs are similar across species, predictable, and precisely scaled up to human subjects, allowing for safe and effective dosing regimens before assessing human liver PK profiles.[94]

The peculiar backbone of PNAs provides resistance against both peptidase and nuclease degradation.[68,95] No known endogenous nucleases and/or proteases recognize PNAs as substrates.[96]

4.4. Criterion 4: Avoid chirality

ONTs often include a PS backbone to decrease nuclease degradation.[97] Given that the substitution of oxygen in the ONT backbone with sulfur introduces chirality, a full PS 16-mer ONT includes 2^{15} (32,768) distinct stereoisomers, with inherent risks of inconsistent therapeutic effects and unintended off-target effects.[97-99] Other chemical

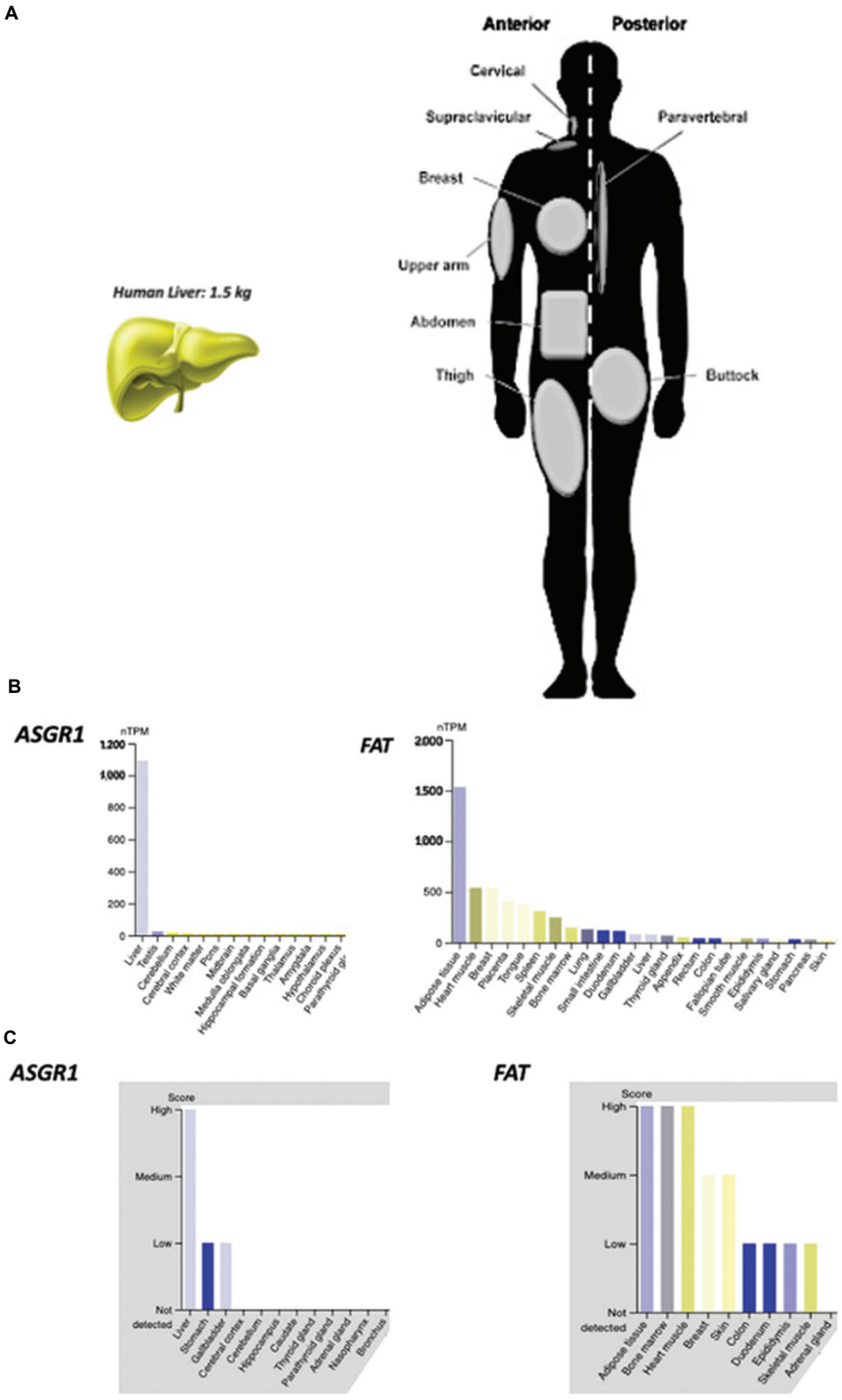


Figure 2. Comparison of targeted organs/tissues in various human tissues and organs of the asialoglycoprotein receptor 1 and the fatty acid translocase membrane transporter. (A) Size of organs/tissues: Human liver (1.5 kg), adipose tissue (40% of body weight in an obese individual), (B) mRNA expression levels, and (C) protein expression levels. Images adapted from.[56]
Abbreviation: nTPM: Normalized transcript per million.

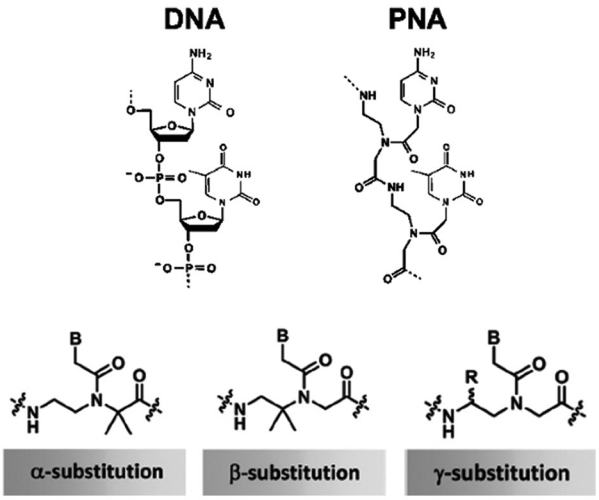


Figure 3. Various chemical modifications are applied to the structure of peptide nucleic acids (PNAs). Note: B=Nucleic acid base.

modifications, such as PMOs, also exhibit chirality. This issue of chirality is a matter of concern for regulatory authorities.[100] Since the 1980s, there has been a regulatory preference to approve single enantiomer drugs.[101] Consequently, strategies were implemented to reduce the number of PS modifications[9] or synthesize stereopure ONTs.[98,102] Scientists from Alnylam® Pharmaceuticals demonstrated that stereo-defined PS linkages at the termini of siRNAs improved their *in vivo* pharmacology.[103] Scientists from Wave Life Sciences Company reported that stereopure PS and phosphoryl guanidine backbone chemistries increase the potency and durability of mRNA silencing. They showed that a GalNAc-siRNA targeting human HSD17B13 leads to 80% silencing for at least 14 weeks after administration of a single subcutaneous dose in transgenic mice.[104]

Another option is to rely on chemical modifications that do not result in chirality, as is the case for PNA compounds, which have an achiral protein-like backbone.[105,106]

4.5. Criterion 5: Limit binding to serum proteins

The tissue bioavailability and potential toxicity of ONTs are modulated by plasma protein binding.[91,107] PS modifications increase plasma stability and plasma protein binding. In contrast, ONTs that lack charges or are less extensively bound to plasma proteins (e.g., unmodified and unformulated siRNA, PNAs, and PMOs) are rapidly cleared from the blood through either metabolism or excretion in urine. The systemic distribution of generations 1 and 2 ONTs is broad, with the highest concentrations achieved in the liver and kidneys. Gapmer PS-ASOs with constrained ethyl, LNA, and/or 2'-MOE modifications bind with high affinity to cellular proteins, modifying their functions, distribution, and half-lives.[108] Coupling an ONT to a cellular targeting agent dramatically alters the systemic PK/PD profile of the conjugate and reduces concerns about serum protein binding, as shown for the generation 2.5 N-acetyl-galactosamine conjugates.[95,109]

4.6. Criterion 6: Optimize and simplify the chemical synthesis of ONTs

Various chemical modification and conjugation strategies have been introduced to improve ONT drug characteristics, such as nuclease resistance, RNA-binding affinity, and PK profiles.[110,111] PNA compounds are synthesized using a straightforward method similar to peptide synthesis. This cost-effective solid-phase peptide synthesis method is detailed in the literature.[106,112] Furthermore, using a Fmoc/Boc protection scheme for both PNA monomers and amino acid building blocks, in association with microwave-assisted solid-phase synthesis, allows continuous assembly of PNA-peptide conjugates.[113,114]

To efficiently select PNA sequences of interest, a group of scientists recently developed machine learning algorithms and automated synthesis technology to predict PNA and peptide-PNA conjugates' synthesis efficiency and to guide rational PNA sequence design.[115,116] Another group of scientists incorporated an ultrasonication step in the solid-phase synthesis of PNAs.[106] This "United States (US)-PNAs" approach improves the purities and yields of synthesized PNAs, such as short or medium compounds (5-mer and 9-mer), complex purine-rich sequences, and longer oligomers.

4.7. Criterion 7: Conjugate the ONTs to a fatty acid or a short peptide for greater and active targeted delivery to the tissues and organs of interest at a much-reduced therapeutic dose

For therapeutic efficacy, after systemic or local administration, an ONT must be selectively delivered to the organs/tissues of interest, efficiently transported into cells, and translocated to the proper cellular compartment to interact with its molecular target. The rapid renal clearance and potential toxicity of unconjugated ONTs can be mitigated by using local targeted delivery methods. Therefore, the conjugation of ONTs is crucial to achieve safe and effective druggability.[13,41,110]

For instance, ONTs targeting the liver, the central nervous system, and muscles have been successfully developed and approved for clinical use.[9,109,117,118] The use of triantennary N-acetylgalactosamine (GalNAc3) ligand and the hepatocyte asialoglycoprotein receptor illustrates the advantages of receptor-mediated uptake of ligand-conjugated antisense drugs targeting RNAs in

hepatocytes.[119] Fifteen GalNAc3-conjugated 2'-MOE-modified ASOs are currently in clinical trials. Scientists from Ionis Pharmaceutical compared the tolerability and safety of four GalNAc3-conjugated and four identical unconjugated 2'-MOE ASOs, demonstrating an enhanced safety and tolerability of the GalNAc3-conjugated compounds versus their unconjugated controls.[119] They concluded that "the improved tolerability of the GalNAc3-conjugated 2'MOE ASO likely reflects the use of reduced doses and smaller sub-cutaneous injection volumes while increasing the ASO potency."[119,p24]

The therapeutic utility of PNAs was initially hindered by their own poor cellular permeability and the absence of effective delivery strategies. However, recent advances in PNA delivery using nanoparticles, ligands, and lipid or peptide conjugates have improved their efficacy and safety *in vivo*.[71,90,116,120-122] For instance, the South Korean drug company OliPass is developing PNA compounds for five different therapeutic indications using bases containing cationic lipid groups to increase stability and cell penetration.[123] Effective therapeutic doses in animal studies were as low as 10 ng/kg, several orders of magnitude lower than existing ASOs.

We opted to conjugate our miR-22-3p antagomir drug candidates to a fatty acid or a short peptide for enhanced targeted delivery to adipocytes and metabolic organs through the cellular membrane transporter fatty acid translocase (FAT). Our goal is to deliver a significantly reduced therapeutic dose to metabolic tissues and organs of interest with an extended intracellular duration of action (measured in mean residence time).

We chose the cellular membrane transporter FAT (FAT/CD36/SCARB3), which is the main route of uptake by adipose tissues of long-chain fatty acids as well as short peptides, such as hexarelin, prohibitin, and thrombospondin peptide-1.[124] It is worth noting that FAT is present not only on the cell surface membrane but also in intracellular compartments, notably the endosomes.[125] Thus, the reversible translocation of FAT from endosomes to the plasma membrane facilitates fatty acid cellular uptake.

The membrane transporter FAT is present in cells and tissues such as adipocytes, hepatocytes, skeletal and cardiac myocytes, pancreatic β-cells, kidney glomeruli and tubules cells, monocytes, and macrophages, which are sensitive to metabolic dysfunctions (Figure 4). In an obese male patient weighing around 200 lbs with 40% adipose tissue, there is a substantial quantity and density of FAT available to transport our new generation 2.5 of miR-22-3p antagomirs coupled to a fatty acid or a peptide into the adipocytes. To illustrate this point, we compared the

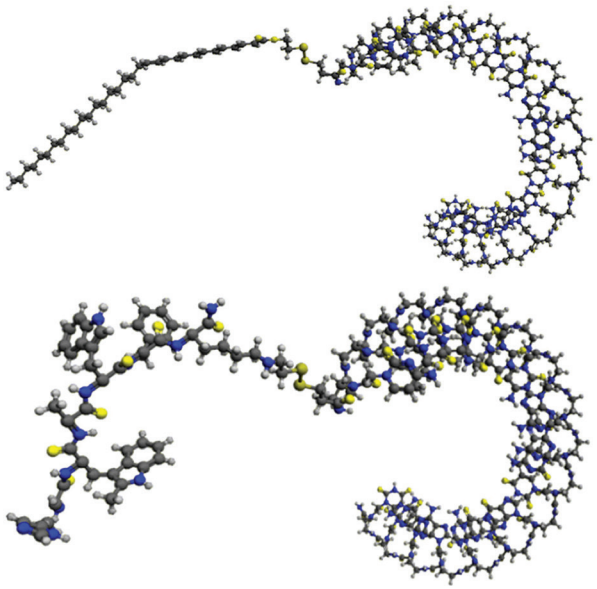


Figure 4. Graphic representations of an 18 mer miR-22-3p antagomir with a PNA backbone coupled with a long chain fatty acid (C32:6 dotriacontahexaenoic acid) (A) and a hexapeptide (hexarelin) (B). Images adapted from Thibonnier and Ghosh.[56]

mRNA and protein expression levels of FAT across human tissues to those of the asialoglycoprotein receptor 1 used for the preferred delivery of ONTs to the liver (Figure 2). This comparison underpins our rationale for targeting FAT for the preferred delivery of ONTs to metabolic organs.

Examples of the structure and composition of AptamiR's generation 2.5 active targeting miR-22-3p antagomirs are shown in Figure 4.

5. Discussion

MiRNA-based ONTs could provide an effective and convenient management of diseases, especially chronic and prevalent ones. By shifting from the classical "one drug-one target" strategy to a "one drug-many targets" paradigm, miRNA ONTs should produce significant therapeutic benefits while reducing polypharmacy, offering substantial advantages in drug cost, efficacy, safety, and patient compliance.

Our work, along with others,' has identified miR-22-3p as a promising candidate for the treatment of metabolic disorders associated with obesity and metabolic dysfunction-associated steatotic liver disease (MASLD).[53,54,56,61] We have established the efficacy of miR-22-3p antagomirs both *in vitro* in human cells in culture and *in vivo* preclinical animal models. The metabolic roles of miR-22 and the benefits of miR-22-3p antagomirs have been confirmed by several independent investigators, as summarized in a review paper by investigators at the

University of California, Davis, United States.[126] Early clinical trials for miR-22-3p antagomirs should be initiated in the near future.

Relying on an extensive and critical review of the literature and our own experience, we selected a set of seven criteria based on scientific parameters, PK/PD profile, ease, and cost of development to design generation 2.5 miRNA ONTs. Once the target miRNA of interest is selected according to criterion 1, five other criteria (criterions 2 – 6) can be simultaneously addressed by selecting a modified PNA backbone. The critical criterion 7, which involves enhanced selective delivery to the tissues and organs of interest, can be satisfied by coupling the PNA antagomir to a fatty acid or peptide actively transported by a cellular membrane transporter expressed in the tissue/organ of interest. Therefore, the modified PNA with its targeting moiety can achieve all these criteria (reduced potential toxicity, chemical stability, no chirality, superior PK/PD profile, ease, and cost of synthesis). This set of criteria could be applied to the development of miRNA ONTs for various therapeutic indications.

In the case of specific organs or tissues, such as the eyes, the brain, and the lungs, the mode and type of local administration represent another targeted delivery option. Several ONTs approved for ocular diseases (cytomegalovirus retinitis and age-related macular degeneration) are delivered through intravitreal administration (Table 1). Various ONT formulations directly delivered to the lungs using aerosolized inhalers for the treatment of fibrotic lung diseases, as well as asthma and lung cancer, are currently in clinical trials and display few systemic side effects.[127]

The clinical relevance of ONTs in the context of cardiometabolic pandemics can now be addressed by the example of inclisiran, the first-in-class siRNA targeting PCSK9 approved by both the US Food and Drug Administration (FDA) and European Medicines Evaluation Agency. Inclisiran is an effective and convenient way to tackle the huge burden of dyslipidemia and atherosclerotic cardiovascular disease.[128] The ORION/VICTORIAN clinical trials have shown that inclisiran is both safe and efficient for up to 5 years.[129] Ongoing animal and human studies are testing ASO, siRNA, and miRNA compounds to treat metabolic syndrome, which afflicts a large percentage of the world population.[130] Recently, Nappi *et al.*[131] reviewed the roles of miRNAs in the cardiovascular system, focusing on clinical translation.

Obesity affects one-third of the world's population, including millions of children.[132] This report suggests that "on current trends, overweight and obesity will cost the global economy over US$4 trillion of potential income in 2035, nearly 3% of current global gross domestic product." MASLD affects 25% of the global adult population, and the prevalence of liver steatosis is 76% in obese patients. At present, there is no FDA-approved medication for MASLD treatment.[133] Obesity and MASLD are growing and costly pandemics in need of safe, effective, convenient, and affordable therapies.

Ten years ago, there was little "appetite" to invest in new drugs for obesity and related cardiometabolic disorders due to limited efficacy and serious adverse events leading to drug non-approval or even removal from the market. Regulatory authorities required long and costly outcome studies, causing large pharmaceutical companies to shy away from this market. However, 10 years later, the landscape has changed significantly, thanks to the development of new classes of agents, especially single, double, and triple incretins agonists for obesity and diabetes mellitus, and thyroid hormone receptor β agonists for MAFLD.[134,135] In a phase II clinical trial, the triple-hormone receptor agonist retatrutide achieved up to 24% weight reduction at 48 weeks, comparable to metabolic/bariatric surgery outcomes.[136] In the MAESTRO-NAFLD-1 52-week randomized, double-blind, placebo-controlled phase III trial in adults with NAFLD, the thyroid hormone receptor beta-selective agonist resmetirom was safe and well tolerated.[137] Data from a phase III randomized controlled trial revealed resolution of NASH and/or fibrosis improvement after 52 weeks of treatment with resmetirom.[138,139]

Treatment selection for combating chronic cardiometabolic pandemics should rely on efficacy, safety, convenience, and cost/affordability parameters. The details are further elaborated as follows:

(i) Efficacy: Incretins and metabolic/bariatric surgery have now set the bar for body weight reduction in human subjects at the -24% level that new drug candidates should meet

(ii) Safety and tolerability: Several years of pre- and post-approval monitoring are required to accurately identify the type and incidence of potential adverse events of a given drug.[140]

(iii) Convenience and compliance: Twice-a-year subcutaneous administration of the siRNA inclisiran (Leqvio®) demonstrates improved adherence to therapy and lower low-density lipoprotein cholesterol over time, which in turn is postulated to improve clinical outcomes.[141-143] Such benefits should apply to targeting ONTs developed for combating cardiometabolic pandemics.

(iv) Cost/affordability: Chronic cardiometabolic pandemics affect a large fraction of the world's population. As far as ONTs are concerned, there is relevant experience with

Table 5. Checklist to develop an oligonucleotide therapeutic

Step	Task
1	Choose a relevant disease or unmet medical need
2	Select a specific RNA target and related network of genes and proteins
3	Elect an ONT type (ASO, dsRNA, miRNA agomir or antagomir, aptamer)
4	Incorporate chemical modifications for achieving optimized drug-like properties
5	Pick a targeting agent/delivery method to achieve the best PK/PD profile
6	Validate lead candidate with machine learning, *in vitro*, and *in vivo* studies
7	Optimize and simplify the chemical synthesis of the lead candidate to reduce costs

Abbreviation: PK/PD: Pharmacokinetic/pharmacodynamics.

inclisiran.[144] At the publicly available price of $3,250 per dose, Leqvio® was found to have "an incremental cost-effectiveness ratio (ICER) just above the $50,000 per quality-adjusted life year threshold of $51,686." By comparison, the ICER of the dual GLP-1/GIP analog Tirzepatide® is $70,147 for the 10 mg dose and $54,699 for the 15 mg dose versus the GLP-1 analog semiglutide 2 mg as recently reported in patients with Type 2 diabetes mellitus in the US.[145] In US adult patients with overweight and obesity, semaglutide 2.4 mg was also estimated to be cost-effective compared with no treatment and other anti-obesity medications, with ICERs ranging from $27,113 (vs. no treatment) to $144,296 (vs. phentermine-topiramate).[146]

Thus, an active cellular targeting miRNA antagomir with a PNA backbone produced by solid phase synthesis and a bi-annual administration regimen should be cost-effective, an assumption that will need to be confirmed by clinical studies and cost-effectiveness analyses.

6. Conclusion

ONTs represent a new class of potent therapeutic agents that are now achieving regulatory approvals for use in humans. Within a few years, they have evolved from treating a specific disease of the eyes, muscles, or brain to addressing cardiometabolic pandemics (e.g., inclisiran [Leqvio®] for elevated cholesterol and zilebesiran for arterial hypertension). These developments are the results of improvements in nucleic acid chemical modifications, conjugation to targeting ligands, and/or combination with delivery systems.

The road map to the successful development of an ONT is summarized in Table 5.

Compared to other therapeutic classes, ONTs are reasonably easy to synthesize with high purity at a competitive cost.[147,148] The new generation 2.5 ONTs allow the convenient administration of reduced doses on an extended schedule (e.g., bi-annual for inclisiran [Leqvio®]), which should reduce costs and improve patients' compliance with treatment.

Using the example of miR-22-3p as a therapeutic target, we propose a set of seven criteria to optimize the design and PK/PD profile of active cellular targeting miRNA ONTs. These criteria could be easily adapted to other miRNA targets and human diseases, especially chronic and endemic ones.

Obviously, the validity of such modifications must be confirmed by testing the lead candidate in animal models and, eventually, human subjects.

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Conflict of interest

Marc Thibonnier is the founder and a shareholder of AptamiR Therapeutics, Inc.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data presented in this article can be accessed in our previously published work[53,54,56] or by request to the corresponding author.

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