

Antisense oligonucleotides

A primer

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Abstract

There are few disease-modifying therapeutics for neurodegenerative diseases, but successes on the development of antisense oligonucleotide (ASO) therapeutics for spinal muscular atrophy and Duchenne muscular dystrophy predict a robust future for ASOs in medicine. Indeed, existing pipelines for the development of ASO therapies for spinocerebellar ataxias, Huntington disease, Alzheimer disease, amyotrophic lateral sclerosis, Parkinson disease, and others, and increased focus by the pharmaceutical industry on ASO development, strengthen the outlook for using ASOs for neurodegenerative diseases. Perhaps the most significant advantage to ASO therapeutics over other small molecule approaches is that acquisition of the target sequence provides immediate knowledge of putative complementary oligonucleotide therapeutics. In this review, we describe the various types of ASOs, how they are used therapeutically, and the present efforts to develop new ASO therapies that will contribute to a forthcoming toolkit for treating multiple neurodegenerative diseases.

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Glossary

AD = Alzheimer disease; **ALS** = amyotrophic lateral sclerosis; **ASO** = antisense oligonucleotide; **cET** = constrained ethyl; **DMD** = Duchenne muscular dystrophy; **ESE** = exonic splicing enhancer; **FAP** = familial amyloid polyneuropathy; **FDA** = Food and Drug Administration; **FTD** = frontotemporal dementia; **HD** = Huntington disease; **LNA** = locked nucleic acid; **MOE** = methoxyethyl; **OMe** = *O* methyl; **PD** = Parkinson disease; **PMO** = phosphorodiamidate morpholino; **PNA** = peptide nucleic acid; **PO** = phosphodiester; **PS** = phosphorothioate; **SCA** = spinocerebellar ataxia; **SMA** = spinal muscular atrophy; **SMN** = survival motor neuron; **SSO** = splice-switching oligonucleotide; **STAU1** = Staufen1.

The genetic revolution led to the identification of many neurologic disease genes. The initial hope that finding the mutated protein and placing it into a known cellular pathway would lead to rapid development of therapies has remained largely unfulfilled. The ability to target the disease gene or its encoded messenger RNAs (mRNAs) has opened new opportunities for therapy development. Of the many ways to target the expression of RNA, this review will focus on the use of antisense oligonucleotides (ASOs) for therapy of neurologic diseases.

Therapeutic ASOs range from 18 to 30 base pairs (bp) in length. They modify expression of a target mRNA, by either altering splicing or by recruiting RNase H leading to target degradation. RNase H is a ubiquitous cellular enzyme that recognizes DNA:RNA hybrids and cleaves the RNA in the hybrid. The mode of ASO action depends on the target sequence and the precise ASO chemistry used in the design. An illustration of ASO action depending on target type and ASO chemistry is provided in figure 1.

Leading the way currently in medical use is the ASO drug nusinersen that is approved for treating multiple forms of spinal muscular atrophy (SMA).¹ Other ASO therapeutics that are Food and Drug Administration (FDA) approved include eteplirsen for Duchenne muscular dystrophy (DMD)² and inotersen for familial amyloid polyneuropathy (FAP).³

ASOs targeting *HTT* for Huntington disease (HD),⁴ *SOD1* and *C9ORF72* for amyotrophic lateral sclerosis (ALS),^{5,6} and *MAPT* (TAU) for Alzheimer disease (AD)⁷ are in early-phase clinical trials. Most current ASO therapeutics do not cross the blood-brain barrier and therefore for targets in the CNS have to be delivered by intraventricular injection in mice or lumbar puncture in humans. Eteplirsen and inotersen have targets that are not in the CNS and are delivered by intravenous or subcutaneous injection, respectively. On the other hand, systemic side effects are limited. Uptake into the CNS is an active process and is not uniform for all cell types or neurons. Chemical modifications can dramatically increase the half-life of ASOs and minimize toxicity.

Oligonucleotide chemistry

Natural, or unmodified, nucleic acids are susceptible to nuclease degradation and have poor protein binding and thus inefficient tissue uptake precluding their use as drugs.^{8,9} Multiple types of modifications made to nucleotides, and their

linkages can improve various properties increasing ASO suitability as drugs. Most of these modifications alter pharmacokinetics (improved nuclease resistance resulting in a longer half-life), pharmacodynamics (superior affinity for the target RNA), or endocytic uptake, which is controlled by specific sets of cell surface proteins.^{10,11} But, with the exception of the phosphorothioate (PS) modification to the ASO backbone, most also preclude cleavage by RNase H, which is the desired mechanism of action for many ASOs. Thus, many RNase H ASOs are designed as chimeras, where different bases are a mix of different chemistries, or as gapmers, where some modifications are placed on the “wings” and not the central bases. Yet, for ASOs intended to alter mRNA splicing or translation, chemical compositions that do not support RNase H can be optimal. Thus, ASOs are highly versatile, customizable therapeutic tools. Some of the different ASO compositions and their resultant effects are discussed here, and the relevant structures are presented in figure 2.

Oligonucleotide phosphate linkage modifications

Modifications to the oligonucleotide phosphate linkages predominantly assist in nuclease avoidance. In the phosphorodiamidate morpholino (PMO), the phosphodiester (PO) linkages in the oligonucleotide backbone are replaced with nonionic phosphorodiamidate linkages leading to resistance to PO.¹² Other ASO types have PS modifications that result in resistance to a broad spectrum of nucleases, support RNase H activity, and increase protein binding, which also improves tissue uptake.^{10,11}

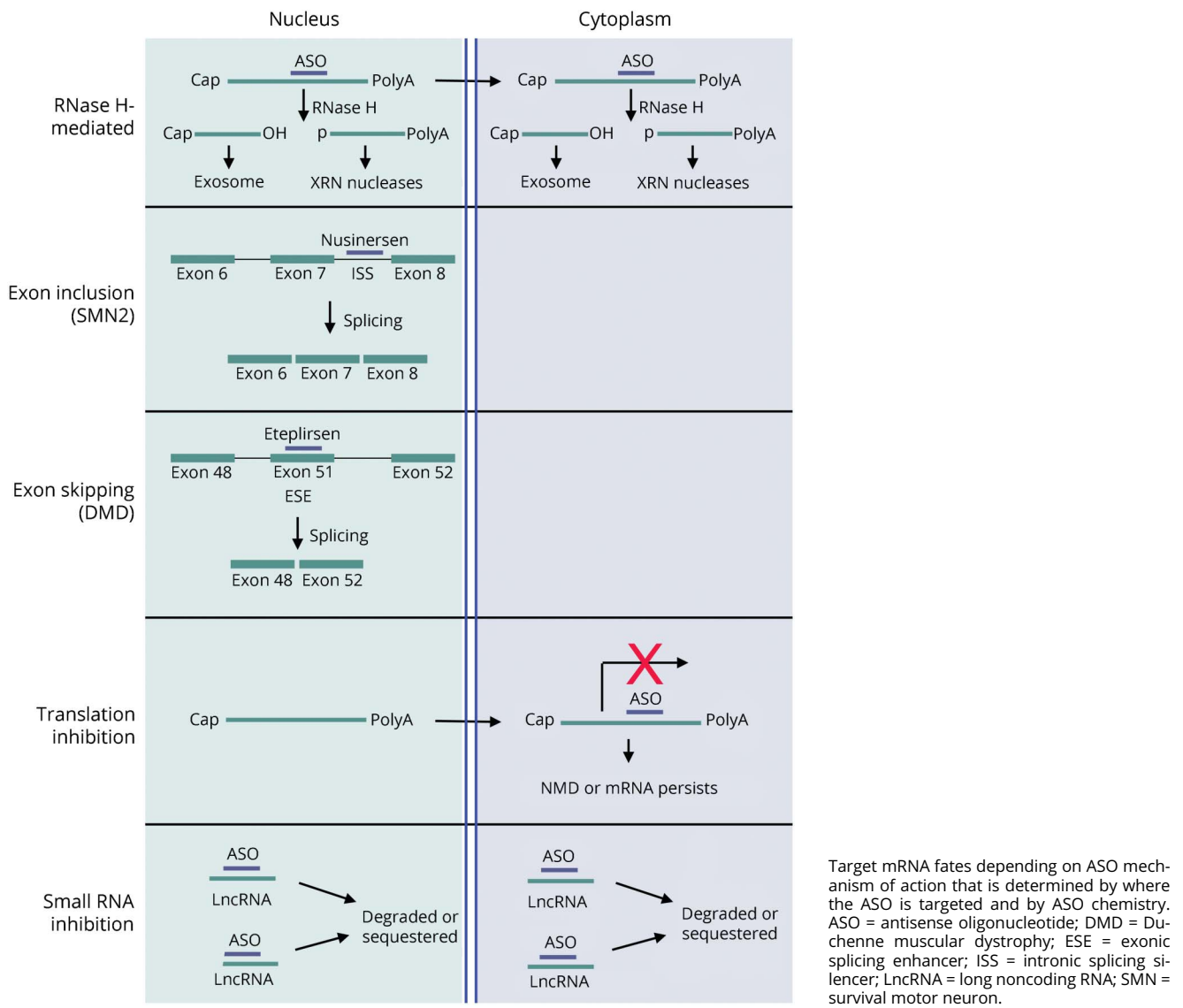
Morpholinos

Morpholinos are oligonucleotides with unique modifications to the ribose sugar that lead to greater target affinity and facilitate nuclease avoidance.¹² This modification reduces oligonucleotide-protein interactions.¹² Eteplirsen, a 30-bp morpholino-based ASO drug for the treatment of Duchenne muscular dystrophy (DMD), represents the only FDA-approved morpholino therapy for a neurodegenerative (or neuromuscular) disease.

Methoxyethyl oligonucleotides

A chemical modification included in most so-called second-generation ASOs is 2' *O*-methoxyethyl (MOE). MOE ASOs have an MOE modification at the 2'-position of the ribose sugar. This change enables enhanced binding affinity to the target mRNA and is considerably less toxic than the 2'-*O*

Figure 1 ASO functions



methyl (OMe) modification. In addition, MOE is sufficiently nuclease resistant that some MOE nucleotides can be synthesized with normal PO linkages so that a mix of PO and PS linkages can be used to fine tune the pharmacokinetics of the ASO. This can facilitate more rapid distribution into tissue while keeping the terminal elimination rate slow.¹³ MOE modifications also reduce plasma protein binding, which seem to shift ASOs away from hepatic metabolism and toward the kidney for excretion in urine.¹³ 2'-MOE PS ASOs delivered to the CSF can have biological half-lives exceeding 6 months.^{50,58}

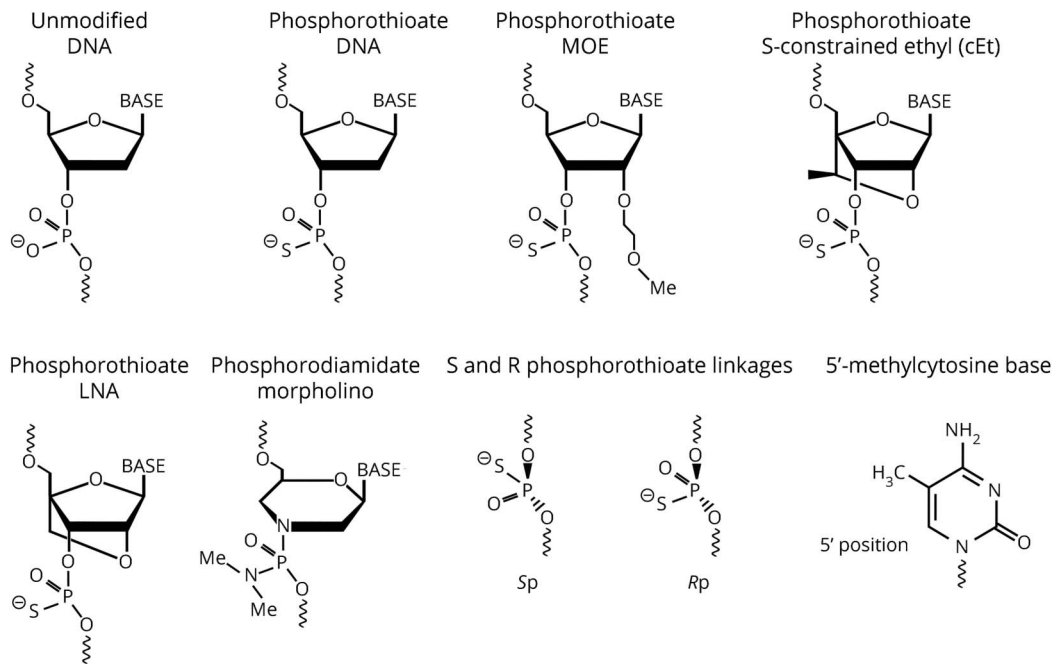
Constrained nucleic acids

Nucleotides that are covalently modified to limit conformation are referred to as constrained or locked. Nucleic acids are considered “locked” when they have a methylene bridge connection made between 2'-oxygen and the 4'-carbon of the

ribose sugar molecule (figure 2). This bond effectively locks the base into a conformation predominantly characterizing the RNA ribose sugar and prevents the conformation characteristic of the deoxyribose sugar.¹⁴ The benefit of locked nucleic acids (LNAs) is that they can produce both increased target specificity and reduced recognition by nucleases. LNAs can hybridize to both DNAs and RNAs forming highly stable double-helix duplexes.¹⁴ LNAs can be incorporated into siRNAs¹⁵ and gapmer ASOs supporting RNase H activity.¹⁶ LNA ASOs can be more potent in vivo than their 2'-MOE analogs.¹⁷

LNAs have been associated with increased liver toxicity¹⁸ but can be used in combination with unmodified bases to reduce toxicity while improving ASO efficacy.^{19,20} To get around hepatotoxicities associated with LNA chemistry, chemists have created a sort of LNA/MOE hybrid by adding a methyl-

Figure 2 Structural elements commonly used in ASOs



cEt = constrained ethyl; MOE = 2' O-methoxyethyl; LNA = locked nucleic acid. Please see the text for descriptions of the structures.

bridge characteristic of LNAs to MOE oligonucleotides. The result is ASOs with reduced liver toxicity and increased potency.²¹ There are multiple types of constrained ethyl (cEt) and MOE oligonucleotides (S-cEt, R-cEt, S-cMOE, and R-cMOE), where S and R refer to the left and right chiral structures, respectively. ASOs with these structures hybridize to target RNAs with affinities like their corresponding LNAs and have improved liver toxicity and increased resistance to nuclease degradation compared with the LNA chemistry.

Stereopure PS ASOs

PS ASOs are usually stereorandom with regard to chiral PS centers, each of which has 2 distinct stereochemical configurations, making 2¹⁹ stereoisomers possible for a 20mer ASO with 19 linkages. Although it is recognized that the 2 stereoisomers (Rp and Sp) differ in their binding affinity and susceptibility to degradation,^{22–24} there are tradeoffs between the two, and it is still debated whether stereopure isomers can be more potent than stereorandom ASOs. Studies in cell culture have failed to show any potency differences between stereopure and stereorandom ASOs. On the other hand, 1 study reported that ASOs with repeated left-left-right (or SSR) chiral PS centers optimized ASO recognition by RNase H and resulted in greater potency in vivo.²² Therapeutic development of stereopure ASOs is the strategy used at WAVE Life Sciences.

Peptide nucleic acid

The peptide nucleic acid (PNA) has a peptide in the position of the ribose sugar. PNAs have typically been used for modulating transcription and other applications not supporting RNase H activity.²⁵ But, PNA gapmers have been shown to support

RNase H cleavage of target mRNAs.²⁶ However, a PNA ASO targeting exon skipping in the *DMD* gene was poorly effective compared with an ASO with the 2'-OMe modification.²⁷

5'-methylcytosine modification

Methylation of cytosines at the 5' position is one way to increase ASO specificity. For example, all cytosines in an ASO against *SOD1* included the 5'-methylcytosine modification.⁵ This inclusion can enhance base pairing by modifying the hydrophobic nature of the ASO. On the other hand, 5'-methylcytosine-thymidine repeats can increase cytotoxicity,²⁸ and CpG motifs can stimulate immunoreactivity that can be at least partially alleviated by including 5'-methylcytosine.^{29,30}

Gapmers, mixed chemistry, and target fate

Gapmer ASOs have “wings” on either side of LNA or MOE modified bases flanking a tract of unmodified bases with PS linkages usually throughout the ASO backbone. Mixed chemistry is intended to maximize resistance to nucleases and minimize toxicity while supporting RNase H activity.³¹ The target mRNAs cleaved by RNase H will be degraded in either the nucleus or the cytoplasm by the exosome complex and XRN exonucleases³² (figure 1). Lead optimization may include a screen for optimal structure activity relationship. For PS ASOs, this may combine cEt, LNA, MOE, cMOE, and 5'-methylcytosine chemistries. Tracts of 4 guanines should be avoided because they can result in complex ASO structures.³⁰ For gapmer ASOs, this may include unmodified bases. Splice-switching oligonucleotides (SSOs) will exclude a gap feature as RNase H activity that could lead to target degradation is unwanted.

Development of ASO therapeutics for neurodegenerative diseases

At the time of writing this review, only 3 ASO therapeutics have been approved by the FDA for neurodegenerative or muscular dystrophy diseases. These are nusinersen for SMA, eteplirsen for Duchenne muscular dystrophy (DMD), and inotersen for FAP. A handful of others are in clinical trials or in preclinical development (table).

Spinocerebellar ataxias

Three Spinocerebellar ataxias (SCAs), all caused by DNA CAG repeat expansion encoding a polyglutamine (polyQ) repeat, have been investigated as diseases targetable by ASO treatment in mouse models. SCA2 is caused by CAG repeat expansion in exon 1 of the *ATXN2* gene.³³ The phenotypes in 2 SCA2 mouse models have been well characterized at the morphologic, physiologic, and transcriptome levels.^{34–36} *ATXN2* mutation is also associated with a substantial rise in the expression of the stress granule protein Staufen1 (STAU1) and production of ATXN2/STAU1/TIA1-positive stress granules.³⁷ After screening for ASOs targeting *ATXN2* in a human cell line, we took the best ASOs reducing expression to in vivo testing by injection into the lateral ventricle of the mouse. These ASOs had a gapmer design. Several ASOs induced a glial or microglial response, a common off-target effect of some ASOs, and were not further evaluated.

The top lead ASO was designated ASO7 and lowered *ATXN2* expression in SCA2 mouse cerebella by >60% for up to 13 weeks, without activating markers of gliosis. When injected

into symptomatic mice, ASO7 improved motor function and restored proteomic and physiologic Purkinje cell abnormalities.^{36,38} The study provided proof of concept of lowering of *ATXN2* for treatment of *ATXN2*-related diseases and the impetus for identifying more efficacious SCA2 ASO for use in humans.

SCA3 (Machado-Joseph disease) is caused by a CAG repeat expansion in exon 10 of the *ATXN3* gene. Different strategies have been used to develop SCA3 ASOs. This has included allele-specific ASOs antisense to the expanded CAG repeat that sterically block translation,³⁹ splice-switching MOE ASOs to exclude exon 10 encoding the expanded repeat,⁴⁰ and MOE gapmer ASOs targeting wild-type and mutant *ATXN3* alleles^{41,42} (figure 1).

SCA7 is characterized by cerebellar ataxia and progressive cone-rod dystrophy and caused by CAG repeat expansion in the *ATXN7* gene. ASO therapies are ideally suited for treating the eye, as they can easily be injected intravitreally. Although no longer in use, 2 decades ago, fomivirsen became the first ever FDA-approved ASO drug, injected into the vitreous of the eye for cytomegalovirus retinitis.⁴³ Niu et al.⁴⁴ developed proof-of-concept data in mice for the use of an ASO therapy for blindness in SCA7. Of note, the ASO therapy was effective in mice with symptomatic eye disease.

Huntington disease

HD is caused by a CAG repeat expansion in an encoded region of the *HTT* gene. PolyQ expansion in huntingtin causes a gain of toxic function. Development of ASOs targeting *HTT* has followed different approaches. One targets

Table ASO therapeutics for neurodegenerative disease

Drug	Indication	Target	ASO chemistry	Status
Nusinersen	SMA	<i>SMN2</i> , exon-7 inclusion	ASO, full 2'-MOE	FDA approved
Eteplirsen	DMD	<i>DMD</i> , exon-51 skipping	Morpholino	FDA approved
Inotersen	FAP	<i>TTR</i> expression	ASO MOE gapmer	FDA approved
WVE-210201	DMD	<i>DMD</i> , exon-51 skipping	Stereopure ASO	Phase 1 clinical trial
RG6042	HD	<i>HTT</i> expression	ASO MOE gapmer	Phase 3 clinical trial
WVE-120101	HD	<i>HTT</i> expression	Stereopure ASO	Phase 1/2 clinical trial
WVE-120102	HD	<i>HTT</i> expression	Stereopure ASO	Phase 1/2 clinical trial
IONIS-MAPTRx	AD	<i>Tau</i> expression	ASO MOE gapmer	Phase 1/2 clinical trial
BIIB078	ALS	<i>C9ORF72</i> expression	ASO MOE	Phase 1 clinical trial
IONIS-SOD1Rx	ALS	<i>SOD1</i> expression	ASO MOE gapmer	Phase 1 clinical trial
ATXN2 ASO	SCA2	<i>ATXN2</i> expression	ASO MOE gapmer	Preclinical development ³⁸
ATXN3 ASO	SCA3	<i>ATXN3</i> expression	ASO MOE gapmer	Preclinical development ⁴²

Abbreviations: AD = Alzheimer disease; ALS = amyotrophic lateral sclerosis; ASO = antisense oligonucleotide; DMD = Duchenne muscular dystrophy; FAP = familial amyloid polyneuropathy; FDA = Food and Drug Administration; HD = Huntington disease; MOE = methoxyethyl; SCA = spinocerebellar ataxia; SMA = spinal muscular atrophy.

both mutant and nonmutant alleles, whereas another approach is allele specific whereby ASOs were screened that target single nucleotide polymorphisms in *HTT* in linkage disequilibrium with expanded CAG repeats.^{45,46} This strategy may potentially target >75% of HD mutation carriers.⁴⁷ Currently, 3 ASOs targeting *HTT* are in clinical trials (table).

Amyotrophic lateral sclerosis

ATXN2 as a target for ALS: Although mutations in several genes cause ALS, a number of studies point to *ATXN2* as a therapeutic target for ALS. Over the past decade, it has become well established that intermediate CAG repeat expansions in the *ATXN2* gene increase the risk of ALS.^{48,49} Intertwined with this discovery was the finding that reducing *ATXN2* expression improved transactive response DNA-binding protein 43 (TDP-43) toxicity in both yeast and flies.⁴⁹ When endogenous *Atxn2* was reduced in TDP-43 transgenic mice by crossing with *Atxn2* knockout mice, survival was significantly improved, TDP-43-positive stress granules were eliminated in motor neurons, and gait scores improved.⁵⁰ Similarly, the survival of TDP-43 transgenic mice was improved by treating with an ASO targeting the *Atxn2* gene.⁵⁰ The effect of targeting *ATXN2* on TDP-43 aggregations might be explained by related effects on *STAU1*.³⁷

Targeting SOD1 for ALS

Approximately 10% of familial ALS cases are caused by mutations in *SOD1* altering its function.⁵¹ Phase I clinical testing of the first-in-human *SOD1* ASO, intended to lower *SOD1* expression, demonstrated that the drug was well tolerated when infused into the CSF, but abundance of the mutant *SOD1* protein in CSF was reduced by only ~12%.⁵² A reformulated version of the drug designated IONIS-SOD1Rx (BIIB067) is presently undergoing phase 1 clinical trials by Ionis Pharmaceuticals and Biogen.⁵³

Targeting C9ORF72 for ALS

GGGGCC repeat expansions in the *C9ORF72* gene are causative of ALS and frontotemporal dementia (FTD).^{54,55} *C9ORF72* repeat expansions result in loss of expression of the normal *C9ORF72* gene and gain of *C9ORF72* mRNA aggregates and repeat associated non-AUG (RAN) translation products. Mice with expanded *C9ORF72* treated by intracerebroventricular injection with ASOs that interfere with translation of GGGGCC expanded *C9ORF72* had reduced mRNA foci and RAN translation products, associated with improved anxiety and cognitive function phenotypes.⁵⁶ With proof of concept established, Ionis Pharmaceuticals and Biogen have undertaken a phase 1 clinical trial of an ASO therapeutic targeting *C9ORF72* for ALS.

Alzheimer disease and tauopathies

Targeting Tau for AD and tauopathies

Lowering Tau abundance by targeting expression of the *MAPT* gene may be therapeutic for AD and FTD. Targeting *Mapt* in mice with a MOE gapmer ASO reduced Tau expression throughout the CNS and protected against seizures

in a mouse seizure model.⁷ Transgenic mice expressing a human P301S mutant Tau that were treated with Tau ASO had reduced neuronal Tau aggregates and prolonged lifespan from 312 to 348 days.⁵⁷ Tau was also reduced in the CNS of cynomolgus monkeys following 6 weeks of Tau ASO treatment delivered intrathecally.⁵⁷ ASOs targeting *MAPT* splicing to exclude a mutant exon also reduced Tau abundance in neuroblastoma cells and in an *MAPT* AD mouse model.⁵⁸ There is another splice-switching ASO strategy for AD that targets the amyloid precursor protein (*APP*) gene to prevent inclusion of its exon 17 to block APP processing and A β production.⁵⁹ And there is yet another, where an ASO is used to correct splicing of the *APOE* gene encoding apolipoprotein E.⁶⁰

Parkinson disease

Reduction of *LRRK2* abundance is predicted to be therapeutic for Parkinson disease (PD). This is supported by observations that disease-causing mutations in *LRRK2* are associated with elevated α -synuclein expression.⁶¹ Targeting *Lrrk2* in wild-type mice was well tolerated supporting that PD is not associated with *LRRK2* loss of function.⁶² MOE-gapmer ASOs reducing *LRRK2* in mice treated with α -synuclein preformed fibrils were also associated with reduced aggregations of phospho(S129)- α -synuclein.⁶³ Some effort has also been made to develop an ASO therapeutic targeting α -synuclein for PD.⁶⁴

Spinal muscular atrophy and nusinersen

SMA is caused by loss-of-function mutations in the *SMN1* gene resulting in the loss of the survival motor neuron (SMN) protein. SMA severity is inversely correlated with copy number of the homologous *SMN2* gene. The cDNA encoded by *SMN2* is identical to that encoded by *SMN1* except for lack of exon 7. The ASO drug nusinersen is an SSO that functions by blocking an intronic splicing silencer element in the *SMN2* intron 7 preventing the spliceosome from excluding exon 7 (figure 1). The result of nusinersen action is expression of the functional, full-length SMN protein from the *SMN2* gene. In *Smn1*^{-/-}; *SMN2*^{+/+} mice, SSOs that restore SMA2 exon 7 splicing also restored tail and ear necrosis phenotypes.⁶⁵ Nusinersen was well tolerated in patients with SMA^{1,66,67} and was approved by the FDA for use in humans for the treatment of SMA in December of 2016.²⁷

Duchenne muscular dystrophy and eteplirsen

DMD is caused by mutation in the *DMD* gene encoding dystrophin, which is one of the largest gene in the human genome. Most of the *DMD* mutations causing DMD result in premature dystrophin truncations. The predominant ASO strategy for treating DMD is employment of ASOs to exclude exons resulting in DMD proteins with partially restored functions. Eteplirsen, an ASO that has a PMO oligomer structure, interacts with the *DMD* pre-mRNA at exon 51 resulting in exon 51 exclusion. As illustrated in figure 1, it is used in DMD patients who have a deletion including exons 49 and 50 resulting in truncated dystrophin; exclusion of exon 51 restores the reading frame from exon 48 to exon 52, partially restoring DMD function.⁶⁸ Approved by the

FDA in 2016 for the treatment of DMD, eteplirsen can be used to treat approximately 14% of DMD cases,⁶⁹ and efforts are ongoing to develop additional ASOs targeting other exons to treat DMD cases caused by other mutations in the dystrophin gene.⁶⁸ Wave Life Sciences has also initiated a phase 1 clinical trial for testing a stereopure ASO for DMD exon 51 exon skipping.

Familial amyloid polyneuropathy and inotersen

Missense mutation in the *TTR* gene encoding transthyretin is the cause of hereditary transthyretin amyloidosis (ATTR). *TTR* mutations result in transthyretin misfolding and progressive accumulation of amyloid deposition in many tissues resulting in polyneuropathy, multiorgan dysfunction, and cardiomyopathy. Multiorgan failure and cardiac arrest pose a greatest risk of death for ATTR patients. Lowering the total expression of TTR at its source in the liver is an effective strategy for ATTR, which is achieved by systemic treatment using the 2'-MOE ASO drug inotersen,³ now approved by the FDA for FAP. Inotersen is treated systemically with delivery made by weekly subcutaneous injections. In the brain, transthyretin is produced by the choroid plexus, and *TTR* mutations cause leptomeningeal amyloidosis that potentially could also be treated by ASO therapy targeting *TTR* expression.⁷⁰

Controlling off-target effects

Ensuring target specificity is a critical step in ASO therapeutic development. One useful approach is to perform quantitative PCR to assess expression of mRNAs with target sequences including mismatches to the ASO candidate to ensure unchanged expression. Another powerful method is to perform transcriptome analysis (RNA-seq) using tissues from mice that are null for the mRNA target, following a treatment trial with the ASO candidate, where differentially expressed genes would indicate off-targets or perhaps incidental cytotoxicity.

A number of chemical modifications have been developed to improve the pharmacokinetic and pharmacodynamic properties of ASOs. PS or PMO backbones are often used. Modifications at the 2' position of the sugar can improve affinity, protein binding, and nuclease resistance. Modifications spanning the 2' and 4' positions to conformationally constrain the sugar can give dramatic improvements in affinity. All modifications except PS prohibit RNase H cleavage, but this can be rescued by a "gapmer" design where the modification is included only in the "wings" of the oligo, and the central bases are just PS. Although there are a number of new ASO therapies for neurodegenerative diseases in preclinical development and in clinical trials, there are only 2 therapies for neurodegenerative diseases that are FDA approved: nusinersen and eteplirsen. However, the success of nusinersen and eteplirsen and promising data from ongoing clinical trials for other ASO therapies to treat HD and ALS predict a robust outlook for new effective treatments for neurodegenerative diseases.

Author contributions

D.R. Scoles wrote the manuscript and produced the figures. E.V. Minikel further contributed to ASO chemistry. S.M. Pulst further contributed to components on movement disorders and neuromuscular diseases.

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