Recent Advances in Antisense Oligonucleotide Therapy in Genetic Neuromuscular Diseases

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Abstract

Genetic neuromuscular diseases are caused by defective expression of nuclear or mitochondrial genes. Mutant genes may reduce expression of wild-type proteins, and strategies to activate expression of the wild-type proteins might provide therapeutic benefits. Also, a toxic mutant protein may cause cell death, and strategies that reduce mutant gene expression may provide therapeutic benefit. Synthetic antisense oligonucleotide (ASO) can recognize cellular RNA and control gene expression. In recent years, advances in ASO chemistry, creation of designer ASOs to enhance their safety and target delivery, and scientific controlled clinical trials to ascertain their therapeutic safety and efficacy have led to an era of plausible application of ASO technology to treat currently incurable neuromuscular diseases. Over the past 1 year, for the first time, the United States Food and Drug Administration has approved two ASO therapies in genetic neuromuscular diseases. This overview summarizes the recent advances in ASO technology, evolution and use of synthetic ASOs as a therapeutic platform, and the mechanism of ASO action by exon-skipping in Duchenne muscular dystrophy and exon-inclusion in spinal muscular atrophy, with comments on their advantages and limitations.

Keywords: Dystrophy, eteplirsen, nusinersen, oligonucleotide, spinal muscular atrophy

INTRODUCTION

First reported by Stephenson and Zamecnik in 1978,[1] synthetic oligonucleotides are shown to be efficient laboratory tools to regulate the expression of specific genes. The early synthetic nucleotides were small in size (15–20 nucleic acid length) and because they were complimentary to sense strand of messenger RNA (mRNA), they were called antisense oligonucleotides (ASOs). It was also surmised earlier on that a synthetic oligonucleotide, if delivered safely to a targeted cellular site in humans, can potentially bind sequence-specifically (Watson-Crick pairing) to the genomic DNA or mRNA site and control the expression of a mutant gene and thus, can be used as a therapeutic agent. In late 1990s, another related technology emerged with the discovery of the small interference RNA (siRNA) pathways that can silence the mutant gene expression and thus the toxic gene product.[2] Duplex siRNAs are potent regulatory agents in laboratories and they also have great potential as RNA-based therapeutic platform. However, the application of siRNA to neuromuscular diseases is not as advanced as ASO technology and will not be discussed in detail in this review.

Although, the potential of ASO as a drug was immediately obvious decades ago, actual development of ASO-based drugs faced major and obvious hurdles.[1,3–8] First of all, nucleic acids are highly susceptible to degradation by endogenous nucleases; ASOs in their native forms have a very short half-life, even before they are filtered out through the kidney. Second, Synthetic ASOs are large (approximately 30 kD) and highly negatively charged molecules and thus do not cross vascular endothelium, dense extracellular matrix and cell and nuclear membranes in order to reach their intracellular DNA or mRNA targets. Third, off-target effect of ASO may lead to devastating adverse reaction. Finally, synthetic ASO can be immunogenic. Synthesizing therapeutic oligonucleotides therefore required historic dozens of incremental chemical steps over the last three decades, each step needing to be almost perfectly efficient, not

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**Design, Development and Mechanism of Action of Antisense Oligonucleotides**

Nucleotides are the basic building blocks of DNA and RNA in all living organisms and plants. A single nucleotide is made of three components: a nitrogen containing base, a five-carbon sugar, and a phosphate group [Figure 1]. The nitrogenous base is either a purine (adenine, guanine) or pyrimidine (thymine, cytosine, uracil). The five-carbon sugar is either a ribose (in RNA) or deoxyribose (in DNA) molecule. In all living species on earth, the diversity in nucleotide sequences in organisms has been the main tenet of species’ organic evolution and differentiation. If foreign naturally occurring nucleotide sequences are introduced in another species, in human for example, they are generally broken down by the abundant nuclease enzymes in body fluids.

Therapeutic ASOs are synthetic single-stranded deoxyribonucleotide analogs, usually 15–30 bp in length. Their sequence (3’ to 5’) is antisense and complementary to the sense sequence of the target nucleotide sequence. As mentioned, unmodified oligonucleotides after quick degradation by circulating nucleases are excreted by the kidney; unmodified oligonucleotides are generally too unstable for therapeutic use. Therefore, chemical modification strategies have been developed to overcome this and other obstacles in ASO therapy program.

The rationale for chemically modifying first-generation ASOs in the 1980s was to reduce the nuclease degradation. This was achieved by replacing one of the nonbridging oxygen atoms in the phosphate group of nucleotide with either sulfur groups (phosphorothioates), methyl groups (methyl phosphonates) or amines (phosphoroamidates) [Figure 1].

![Figure 1: Natural nucleotide and some chemical backbone modifications in synthetic nucleotides. NNT, natural nucleotide; PS, Phosphorothioate; NP, N3'-P5' Phosphoroamidate; OMe, 2'-O-methyl; MOE, 2'-O-methoxy-ethyl; LNA, locked nucleic acid; PMO, phosphoroamidate morpholino](image)

Phosphorothioate substitution (PS) was the earliest and the most commonly used modification that renders the internucleotide linkage resistant to nuclease degradation. In addition to their endogenous nuclease resistance, PS modification has two other distinct advantage. First, it can support endogenous RNase H activity to degrade the target mRNA or mutant toxic mRNA and thereby diminishing its toxic protein product. Second, PS linkages also improve the pharmacokinetic characteristics by their sequence-independent but length-dependent binding with plasma proteins. In this context, protein binding can be favorably exploited to alter the half-life and availability of ASO to the target site. Although substantial advantages are conferred by PS backbone modification of nucleotides, such modifications were shown unfortunately to elicit strong platelet activation, aggregation and thrombi formation in animal models and in vitro experiments using human platelets, raising questions for their safety in humans.

To overcome the various nonsequence-specific side effects of first generation ASOs and to improve further nuclease resistance and target-binding affinity, second generation ASOs were developed. The most commonly used modification in these ASOs is 2’ ribose modifications that include 2’-O-methoxy (OMe), 2’-O-methoxy-ethyl (MOE), and locked nucleic acid (LNA) [Figure 1]. 2’-OMe modifications are commonly used in a ‘gapmer’ design, which is a chimeric oligo comprising a DNA sequence core with flanking 2’-MOE nucleotides that enhances the nuclease resistance, in addition to lowering toxicity and increasing hybridization affinities. However, two ASOs that caused severe thrombocytopenia in recent human trials have this chimeric ASO design, raising doubts about its safety.

Most recently, third-generation ASOs have been developed to enhance their delivery to the target sites. In this technology, the oligo load is covalently bound to a carrier or ligand, such as lipid particles, liposomes, nanoparticles, and more...
recently, the sugar N-acetyl galactosamine to enhance safer delivery to the target site.\cite{4,6,11-19} Recently approved eteplirsen (EtonDys 51) for Duchenne muscular dystrophy (DMD) is the third-generation phosphorodiamidate morpholino ASO. Hybridization of ASO to the intracellular target mRNA can result in specific inhibition of gene expression by two main mechanisms.\cite{13-15} The most common mechanism is by induction of endogenous RNase H activity (ASO-RNase H) that cleaves the mRNA-ASO hetero-duplex, for example. This leads to degradation of the target toxic mRNA while leaving the ASO intact. Such antisense effect is thus catalytic and a single ASO can participate in the destruction of many mRNA molecules. The ASO molecules currently undergoing trials in familial amyotrophic lateral sclerosis (fALS) belong to this category. The second group of ASO mechanism of actions include translational inhibition by steric hindrance, exon skipping, exon inclusion, destabilization of pre-mRNA in the nucleus, or targeting the destruction of microsomal RNAs that control expression of other genes. Strategy utilizing steric hindrance (eteplirsen in DMD) or modulating splice site (nusinersen in spinal muscular atrophy [SMA]) does not utilize RNase H activity and is the main focus of this review.

**Therapeutic Antisense Oligonucleotides in Clinical Trials**

The spectacular laboratory advances in ASO technology in recent decades have led to numerous studies investigating the therapeutic potential of ASO in *in vitro* cell models, animal disease models and in human clinical trials. Interestingly, however, commercial development of early ASO therapy platform was mired with repeated hopeful optimism followed by doubtful downturns chiefly because many clinical studies showed early promise and then faded to disappointment when phase 3 results were revealed. One compound, fomiviren, was approved by the US Food and Drug Administration (FDA) in 1998 for treating cytomegalovirus retinitis by intraocular administration.\cite{16} However, this early success was mitigated by the lack of commercial success because anti-retroviral medications reduced cytomegalovirus retinitis as a major health problem in HIV-infected individuals.

In 2013, US FDA approved two other ASO drugs, lomitapide and mipomersen, in familial hypercholesterolemia. Lomitapide and *mipomersen* target expression of apolipoprotein-\(B\) and lower cholesterol in familial hypercholesterolemia.\cite{17} While lomitapide and *mipomersen* have not been commercial success owing to an overall small patient population and competing statin blockbuster drugs, the demonstration that a systemically administered ASO drug can be successful paved the way to clinical trials of ASO for treatment of neuromuscular and other diseases.

Fully modified ASO technology which has particularly caught recent scientific and media attention includes the manipulation of alternative pre-mRNA splicing where the ASO molecules work as splice switching oligonucleotides (SSOs).\cite{9,13-15,18-21} In this context, ASO can be used to modulate the ratio of splicing variants or correct spicing defects, by either inducing exon-skipping or exon-inclusion.\cite{13-15,18-21} Most notably, these advances have been made for neuromuscular diseases, such as SSO technology in SMA\cite{7,13-15} and DMD.\cite{19-21} These two therapeutic technologies are discussed in the following section. Although significant confusion still exists with regard to the pathogenicity of many fALS-related genes, the use of ASO-RNase H technology is the current area of active research in clinically devastating ALS disease.\cite{22-24}

**Neuromuscular Disease Targets**

**Exon-skipping eteplirsen antisense oligonucleotide:** Eteplirsen in Duchenne muscular dystrophy

DMD is a fatal neuromuscular disorder affecting approximately one in 3500 male births.\cite{25} It is inherited in an X-linked trait and is caused by the loss-of-function mutation in DMD gene that codes for dystrophin.\cite{26} Dystrophin is a cytoskeletal protein which stabilizes the plasma membrane of muscle fibers.\cite{26} The muscle weakness in DMD progresses relentlessly, with boys losing ambulation by 12 years of age. The death often occurs in their 20s, usually from respiratory or cardiac failure.\cite{25} DMD is one of the largest known human genes, spanning 2.4 Mb in chromosomal Xp21 region with 79 exons and producing a large 14 kb transcript.\cite{26-28}

Due to its large size, DMD gene is highly susceptible to deletion mutations. Approximately 70% of DMD cases are due to deletion mutations, and certain DMD gene regions are mutation hot-spots.\cite{27,28} If DMD gene deletion results in “out-of-frame mutation,” it produces none or negligible dystrophin and results in DMD phenotype [Figure 2]. On the other hand, if DMD gene deletion result in “in-frame mutation,” generating a variant transcript that is able to produce functional albeit truncated version of dystrophin, it leads to Becker MD (BMD), a milder dystrophinopathy compared to DMD. Thus, the difference of genetic deletion mutations in DMD and BMD presents an important observation: The nature of the deletion determines the severity of the disorder. It is with this underlying principle that exon skipping ASO has been developed as a therapeutic paradigm for DMD, i.e., conversion of DMD to BMD phenotype [Figure 2].\cite{29}

Eteplirsen is a 30-nucleotide phosphorodiamidate morpholino oligomer type third-generation ASO that hybridizes to exon 51 of DMD Pre-mRNA and causes it to be skipped during splicing;\cite{19-21} this corrects the translational reading frame in certain DMD gene deletions, resulting in the production of shortened but functional dystrophin protein [Figure 2].\cite{19} Eteplirsen ASO therapy is applicable in about 13% cases of DMDs where exon 51 skipping would potentially convert out-of-frame deletion to in-frame deletion in the DMD gene mutations, for example in DMD with exon 50 deletion [Figure 2].\cite{19} A related exon skipping ASO, drisapersen, in earlier DMD clinical trials used second generation 2'-OMe PS type ASO for exon 51 skipping strategy.\cite{6,19}
After obtaining the proof of concept in patient-derived cells and animal models, drisapersen and eteplirsen ASO chemistries were clinically developed for exon 51 skipping in DMD. US FDA rejected drisapersen in 2016 due to safety issues associated with the use of the drug and insufficient evidence of clinical utility. The main difference between drisapersen and eteplirsen is that in the later the nucleotide base is attached to a morpholino moiety which is no longer negatively charged and thus makes it safer to use and deliver to the target.

On September 19, 2016, eteplirsen received accelerated conditional approval by the US FDA for boys with DMD deletion amenable to exon 51 skipping. Eteplirsen (ExonDys 51) is the first approved ASO for DMD and first approved exon skipping ASO to be used in humans. Although its approval is shrouded in some controversy, chiefly from limited data on only 12 cases and from just dystrophin protein as a surrogate marker in muscle biopsies, without proof of clinical improvement, it is hoped that eteplirsen indeed would work and that longer term treatment will reveal a slower disease progression in DMD patients. Several major urgent challenges in DMD ASO therapeutics remain however, and these include, lack of a strategy that can provide a treatment for all patients with DMD, necessity for sustainability and lifelong treatment, and cost of therapy. Eteplirsen (ExonDys 51) is reported to cost in the order of $300,000–400,000/year per patient for life.

**Exon-retention strategy: Nusinersen antisense oligonucleotide in spinal muscular atrophy**

SMA is the most frequent genetic cause of death in children. SMA affects motor neurons in brainstem and spinal cord and causes progressive muscular atrophy and paralysis. SMA is caused by deletions or mutations in the survival motor neuron 1 (SMN1) gene that reduces the level of active SMN protein. Human genome also possesses a second SMN gene centromeric to SMN1 gene on chromosome 5. SMN2 gene has a C to T mutation in exon 7 that does not affect the protein sequence but affects the pre-mRNA splicing and gives rise to an unstable isoform. In fact, SMN2 splices out exon 7 in 90% of transcripts, leading to about 10% of full-length SMN mRNA and low levels of SMN protein. The number of SMN2 copies therefore determines the SMA clinical phenotype, one SMN2 pair gives rise to infantile SMA where the child dies by 2 years of age and more than one SMN2 pair leads to late age-onset SMA (later childhood and adult SMAs, for example). If this splicing error is corrected (inclusion of exon 7 in mRNA), SMN2 gene can produce more active SMN protein and potentially alleviate symptoms of SMA.

Nusinersen is a 2'-OMe phosphorothioate ASO that targets exon 7 within SMN2 pre-mRNA, increasing its inclusion and producing functional SMN protein. Following the proof of concept in cell and animal models, an open-label study of nusinersen in type 1 SMA showed significant divergence from natural history for age on permanent ventilation, for survival and for incremental achievements in motor milestones. Furthermore, placebo-controlled trial in type II SMA patients revealed that nusinersen-treated patients had a significantly higher increase in motor function than placebo-treated patients, thus also meeting the prespecified primary endpoint. Both trials showed a good safety profile and were stopped after interim analysis, enrolling all patents into open-label trials pending regulatory approval. In parallel, an expanded nusinersen open-access program was initiated for type I SMA to expedite and solidify the trial results. After careful analysis of the clinical trial data, US FDA approved nusinersen ASO on December 23, 2016. So far, nusinersen is the only FDA-approved medication for SMA.

ASOs generally do not cross the blood–brain barrier. Therefore, repeated intrathecal nusinersen delivery is required which can be invasive and challenging, especially in young and fragile infants. For older type 1 SMA patients, who are already on permanent ventilation, treatment may prolong survival, but it is currently unknown if improvement in muscle function would occur. Furthermore, duration of clinical research trials has been short and postmarketing studies will need to be done to investigate both the positive and potentially negative effects after years and decades of nusinersen treatment. Finally, an ethical issue that parents and clinicians have to grapple with now includes when to treat and when not to treat infants with significant SMA paralysis. Similar to eteplirsen (ExonDys 51), cost of nusinersen (Spinraza) therapy is high: an estimated $750,000 for the 1st year, followed by approximately $375,000 annually for life.

**Emerging Antisense Oligonucleotide Target in Familial Amyotrophic Lateral Sclerosis**

ALS is currently an incurable and invariably fatal disease characterized by the progressive loss of upper and lower motor...
neurons in cortex, brainstem, and spinal cord. ALS results in progressive paralysis leading to death 3–5 years after disease onset, often from respiratory failure. The vast majority of ALS cases are sporadic (sALS), while 5%–10% are familial (fALS) often with autosomal dominant inheritance involving genes mostly affecting RNA metabolism.\textsuperscript{[38,39]} Although over 31 different genes are now linked to fALS, two genes (SOD1 and C9orf72) account for at least half of these cases.\textsuperscript{[24,38,39]}

Extensive research in murine SOD1 models over the last two decades has shown that overexpressed human mutated SOD1 gene causes progressive motor neuron disease.\textsuperscript{[40]} Although somewhat controversial in SOD1- and C9orf72-linked fALS, toxic gain of function of mutated genes appear to be the proximate cause of neuronal death.\textsuperscript{[22,23,38,40‑42]} Synthetic ASO designed to destroy the toxic SOD1 and C9orf72 gene transcripts can potentially reduce toxic protein and thus be a plausible therapeutic strategy in fALS.\textsuperscript{[22,40,41,43,44]} If successfully and safely delivered to the target site, ASOs can potentially mitigate the toxic effects of SOD1 and C9orf72 transcripts and protein products inside the neurons.

The first successful in vivo oligonucleotide-based study in ALS mouse model used siRNA against human SOD1.\textsuperscript{[44]} Recently, treatment with ASO in in vitro C9orf72 showed a positive effect on toxic RNA foci, causing their reduction in fibroblasts and induced-pluripotent stem cell-derived neuronal cells.\textsuperscript{[40,41]} The first clinical trial of intrathecal delivery of an ASO (ISIS 333611) in 24 patients with SOD1 fALS was well tolerated in a phase 1 study.\textsuperscript{[22]} opening the field to further clinical trials. The function of C9orf72 gene is still largely unknown and therefore the safety of blocking protein expression through ASO-based therapies in C9orf72 fALS cases will have to be carefully researched.

**Conclusions**

The development and recent approval of ASOs that can successfully induce stable exon 51 skipping of dystrophin gene transcript in DMD and alternate splicing of SMN2 in SMA are examples of the potential of ASO technology as a treatment strategy for genetic neuromuscular diseases. Experience with nusinersen demonstrates that ASOs can be safely administered into the central nervous system and it can enter target tissues, modulate the intended target, and produce favorable clinical outcome in SMA patients. Because all nucleotide-based ASO have similar chemical properties, many of the lessons learned during development and refinement of initial ASO therapy platform can be directly transferred to other projects. However, the fact that current ASOs do not readily cross an intact blood–brain barrier limits their application via intrathecal injections for central nervous system diseases. Successful ASO development for other neuromuscular diseases that primarily affect peripheral nerves and skeletal muscles will also require efficient delivery methods. Notwithstanding these and other challenges, ASO-based therapeutics do provide the ability to modulate disease pathways and it is an exciting development for the currently incurable genetic neuromuscular diseases.

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**Conflicts of interest**

There are no conflicts of interest.

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