RNA-targeted drugs for neuromuscular diseases

Alessandra Ferlini[1,3]; Aurelie Goyenvalle[2]; Francesco Muntoni[3,4]

1 Unit of Medical Genetics, Department of Medical Science, University of Ferrara, Italy

[2] Université Paris-Saclay, UVSQ, Inserm, END-ICAP, 78000 Versailles, France

[3] Dubowitz Neuromuscular Centre, UCL Great Ormond Street Institute of Child Health, London, UK

[4] NIHR Great Ormond Street Hospital Biomedical Research Centre, London, UK

Introduction

Neuromuscular diseases (NMD) are common and heterogeneous conditions affecting skeletal muscle, either directly, as in muscular dystrophies, or by targeting motoneurons, peripheral nerves or neuromuscular junctions. The abundance of skeletal muscle and the size of several of the genes responsible for NMD limits the application of adeno-associated viruses gene therapy (*1*). Manipulation of the RNA to correct mutated transcripts has been developed in the last decade with several successful approaches, leading to approved first generation drugs; next generation drugs are now in clinical development. But the development of RNA therapies has also been accompanied by several failures, highlighting problems of safety, efficacy, and tissue targeting that need to be overcome.

Manipulation of mutant RNA can be achieved using synthetic antisense oligonucleotides (AONs), which are short, synthetic, single-stranded DNA analogs, either by modulation of splicing (to induce splice switching) or by inactivation. The first splice-switching approach to arrive in the clinic was exon skipping for Duchenne muscular dystrophy (DMD), an X-linked disorder affecting 1 in 5000 live male births. DMD is characterized by progressive muscle weakness and degeneration. It is caused by mutations in the dystrophin (*DMD*) gene that disrupt the open reading frame (ORF) and thus prevent protein production. Dystrophin is a mechanical and signaling scaffold protein linking the actin cytoskeleton and the extracellular

matrix, a crucial task for maintaining the integrity of the sarcolemma (the membrane of muscle fiber cells) and avoiding muscle degeneration. In the milder dystrophinopathy variant, Becker muscular dystrophy (BMD), the ORF is preserved, leading to a shortened but functional dystrophin.

The exon-skipping approach to DMD treatment uses AONs that mask pre–messenger RNA (pre–mRNA) splicing sites, resulting in removal of one exon from the mRNA, restoration of the ORF, and production of a BMD-like dystrophin (see the figure). AONs with various modifications to the chemical structure of their "backbone" have been developed to protect them from nuclease activity and to increase their stability and affinity to target RNA. Among them, two chemistries were the first used for *DMD* exon 51 skipping: the charged 2'O-methyl-phosphorothiate (2'OMe) and the uncharged phosphorodiamidate morpholino oligomer (PMO). Despite encouraging results in initial clinical studies (*2*), a phase 3 randomized placebo-controlled trial (RCT) with the 2'OMe-modified AON drisapersen delivered subcutaneously failed to demonstrate significant benefit or clear dystrophin production and was associated with toxicities (such as proteinuria and injection site reactions) that prevented further clinical development (*3*). By contrast, the neutrally charged PMO eteplirsen, administered intravenously, induced low but significant levels of dystrophin expression and, in a 4-year study, reduced the risk of becoming wheelchairdependent. In 2016, eteplirsen was granted accelerated approval by the U.S. Food and Drug Administration (FDA), becoming the first approved splice-switching AON and the first approved drug for DMD in the United States.

Other PMO AONs targeting DMD exon 53, golodirsen and viltolarsen, were subsequently approved by the FDA (*4*) (*5*). Despite these successes, the amount of dystrophin in biopsies from PMO-treated patients remained low, and in 2018 the European Medicines Agency (EMA) gave a negative opinion for eteplirsen, judging that the current risk/benefit balance was unknown because efficacy had not been demonstrated in RCTs, and indicating the use of dystrophin as a surrogate biomarker as premature. To provide conclusive evidence of clinical efficacy, phase 3 RCTs are under way with eteplirsen and golodirsen and also with a PMO targeting exon 45, casimersen.

A limiting step of these AONs is their low efficiency at targeting muscle. The muscle uptake of PMOs is dependent on inflammatory foci associated with dystrophic lesions, as well as the fusion of PMO-loaded monocytes and myoblasts into damaged myofibers. The inefficient

targeting of intact muscle fibers also precludes the use of PMOs in conditions with limited muscle damage. Improving AON delivery to muscle is being addressed both with alternative chemistries and new conjugations (*6*). Stereopure AONs were recently developed to optimize delivery and affinity to pre-mRNA targets. Because the phosphorothioate backbone is chiral, a 20-nucleotide phosphorothioate AON is a mixture of 2^{19} different stereoisomers that may not be equally effective. Despite the enhanced potency of the stereopure AON suvodirsen (targeting DMD exon 51) observed in cultured muscle cells, a phase 2 RCT showed no induction of dystrophin expression: Suvodirsen mainly accumulated in the muscle interstitial space, explaining the lack of dystrophin rescue. Clinical development of suvodirsen has been suspended.

Alternative chemistry development includes tricyclo-DNA AONs, a constrained and hydrophobic chemistry allowing higher biodistribution to muscles (*7*). Moreover, tricyclo-DNA AONs can cross the blood-brain barrier (BBB) after systemic delivery in preclinical studies (*7*). This might address DMD comorbidities, such as fear and anxiety, caused by dystrophin deficiency in the brain. Conjugation of AONs to cell-penetrating peptides (CPPs) or antibodies targeting specific receptors (such as the transferrin receptor) is also being investigated. CPPs are short cationic and/or amphipathic peptides that facilitate AON translocation across cell membranes and escape from endosomes. Some CPP-conjugated PMO AONs can also cross the BBB in preclinical models (*8*).

Delivery to the target tissue has been achieved using direct administration in spinal muscular atrophy (SMA). SMA is an autosomal recessive motor neuron disease with an incidence of 1 in 10,000 live births, caused by inactivating mutations in the survival motor neuron 1 (*SMN1*) gene. SMN is a ubiquitous protein involved in transcriptional regulation and intracellular trafficking, and its deficiency results in selective motor neuron death. SMA patients are classified into four groups of clinical severity; the most common is SMA1 (~60%), in which infants never acquire the ability to sit and typically die before the age of 2 years. A primate-specific gene duplication generated a centromeric variant (*SMN2*) that is present in multiple copies in the population, including SMA patients. *SMN2* allows the production of low amounts of SMN protein, and *SMN2* copy number variation accounts for the differences in SMA clinical severity. The *SMN2* sequence differs from that of *SMN1* by a single-nucleotide polymorphism

that weakens its exon 7 splicing enhancer and reduces exon 7 incorporation in the *SMN2* mRNA by 90%, causing the production of an unstable protein.

The splice-switching AON nusinersen enhances *SMN2* exon 7 inclusion, leading to increased production of full-length *SMN2*-derived protein (see the figure). Nusinersen does not cross the BBB and requires direct intrathecal administration [into the cerebrospinal fluid (CSF)]. However, the half-life in CSF is 102 to 111 days, which allows infrequent dosing once a steady state has been achieved. Two phase 3 RCTs have been performed, one in SMA1 and one in the milder SMA2 and SMA3 variants. Nusinersen demonstrated a favourable risk/benefit profile and met the efficacy end points. In SMA1, both survival and acquisition of new motor milestones led to the premature interruption of the RCT, allowing all participants to receive the drug. Similar positive results were obtained in the milder SMA variants. These results prompted FDA and EMA approval (*9*).

Although the response of symptomatic SMA1 patients is robust and clinically meaningful, many of these children have considerable residual disability due to the advanced stage of the disease before treatment initiation. An ongoing phase 2 openlabel trial of nusinersen in presymptomatic infants likely to develop SMA1 or SMA2 found that 88% of those treated achieved walking independently (*9*). In view of these outstanding outcomes, newborn screening has started in many countries, with important implications for translation (i.e., assessment of the long-term therapeutic impact in presymptomatic patients) and for service provision because of the high prevalence of SMA1.

AONs have been successfully used to silence or down-regulate mRNA. This can be achieved by ribonuclease H (RNase H) activation, which recognizes RNA-DNA hybrids, and subsequent targeted RNA degradation. Degradation of mRNA can be allele-specific (targeting the mutated transcript) or biallelic, so the wild-type transcript is also degraded. As a result of nonspecific binding of AONs, allele-specific transcript silencing is rarely selective, except for alleles carrying repeat expansions. Autosomal dominant diseases caused by repeat expansions arise from the propensity to increase the number of triplets during meiosis due to DNA replication and repair errors (10). Myotonic dystrophy type 1 (DM1) is an autosomal dominant disease affecting the skeletal muscle, heart, and central nervous system. It is caused by CUG repeat expansions in the DM1 protein kinase (*DMPK*) gene that form toxic RNA hairpin structures, which accumulate in the nucleus and induce downstream toxic effects, including

sequestration of proteins. AON-mediated silencing can either target and degrade mRNA or inhibit protein binding to the triplets (see the figure). Chimeric AONs, also known as gapmers, induce RNase H–mediated degradation of mRNA containing the CUG repeat expansion, thereby reducing the amount of toxic transcript (*11*). Optimization of gapmers by constrained ethyl modification increases their binding affinity, further reducing *Dmpk* mRNA by 90% in animal models.

Similarly, AON silencing of expanded hexanucleotide repeats is being pursued in a variant of amyotrophic lateral sclerosis (ALS) due to dominant *C9ORF72* mutations. ALS is a late-onset neurodegenerative disorder characterized by involvement of motor neurons, resulting in progressive paralysis and premature death. ALS is genetically heterogeneous, and *C9ORF72* mutations account for >50% of familial ALS cases. Silencing of *C9ORF72* mRNA containing expanded repeats is being studied in human neuronal cells, as well as in transgenic animals after direct intraventricular administration (*12*).

A non–allele-selective silencing approach is being pursued for two other autosomal NMDs: centronuclear myopathy (CNM), caused by mutations in dynamin-2 (*DNM2*), and an ALS variant caused by superoxide dismutase 1 (*SOD1*) mutations. DNM2 is a ubiquitously expressed guanosine triphosphatase (GTPase) mechanosensitive enzyme that is involved in endocytosis, exocytosis, membrane remodeling, and cytoskeletal organization. *DNM2* mutations confer gain of function, and CNM is characterized by the pathological findings of centrally placed nuclei in myocytes, muscle atrophy, and deformed T-tubules. Two different approaches, including AONs, are being used to target both mutated and wild-type *DNM2* RNA (*14*). A reduction of total *DNM2* expression in a mouse model improved outcome, providing proof of principle that reduced *DNM2* expression could be therapeutic in CNM. A phase 1 open-label study targeting *DNM2* with a constrained ethyl gapmer in CNM patients has recently started.

Biallelic silencing is also being pursued in SOD1-related ALS. In this condition, dominant missense variants of SOD1 cause toxic effects in motor neurons by increasing oxidative stress. A phase 1 RCT of an AON targeting SOD1 through intrathecal administration was well tolerated (*15*), and a secondgeneration AON, BIIB067, is entering a phase 3 RCT for SOD1-ALS.

RNA therapies have made impressive progress with the approval of several drugs and further products in the pipeline. Some clinical failures highlight the need to develop alternative

chemistries, conjugates, or delivery systems to improve targeted delivery to muscle. The clinical efficacy of next-generation compounds will be enhanced by better understanding of their uptake and intracellular kinetics. For conditions affecting motor neurons, intrathecal delivery efficiently reaches the brain, although chronic administration of these therapies through this route carries a burden for patients; this could be avoided in the future by AONs that cross the BBB. In AON-mediated silencing approaches, biallelic strategies also raise questions about possible haploinsufficiency-related effects and consequent safety profiles. Studies of these next-generation compounds will clarify the extent of clinical benefit and phenotype reversion in these severe conditions

Acknowledgments

A.F. is principal investigator (PI) of Sarepta clinical trials and is a member of the Sarepta European scientific advisory board (SAB). F.M. is PI of Sarepta, Wave Therapeutics, and Biogen clinical trials. F.M. is a SAB member for Biogen, Pfizer, Sarepta, and Dyne Therapeutics. F.M. also receives funding from Sarepta and Biogen. A.G. is supported by the Institut National de la santé et la recherche médicale (INSERM) and the Association Monegasque contre les myopathies (AMM).

References

1. T. A. Partridge, *Curr. Opin. Neurol.* **24**, 415–422 (2011).
2. N. M. Goemans et al., *N Engl J Med*. **364**, 1513–22 (2011).
3. N. Goemans et al., *Neuromuscul. Disord.* **28**, 4–15 (2018).
4. Y.-A. Heo. *Drugs*. **80**, 329–333 (2020).
5. H. Komaki et al., *Sci Transl Med*. **10** (2018), doi:10.1126/scitranslmed.aan0713.
6. P. P. Seth et al., *J. Clin. Invest.* **129**, 915–925 (2019).
7. A. Goyenvalle et al., *Nature medicine*. **21**, 270–5 (2015).
8. S. M. Hammond et al., *Proc. Natl. Acad. Sci. U.S.A.* **113**, 10962–10967 (2016).
9. D. C. De Vivo et al., *Neuromuscul. Disord.* **29**, 842–856 (2019).
10 Liu and Wilson , Trends Biochem. Sci. 37 (4), 162-172 (2012)
11. T. M. Wheeler et al., *Nature*. **488**, 111–115 (2012).
12. J. Jiang et al., *Neuron*. **90**, 535–550 (2016).
13. H. Tasfaout et al., *Nat Commun*. **8**, 15661 (2017).
14. S. Buono et al., *Proc. Natl. Acad. Sci. U.S.A.* **115**, 11066–11071 (2018).
15. T. M. Miller et al., *Lancet Neurol*. **12**, 435–442 (2013).

FIGURE

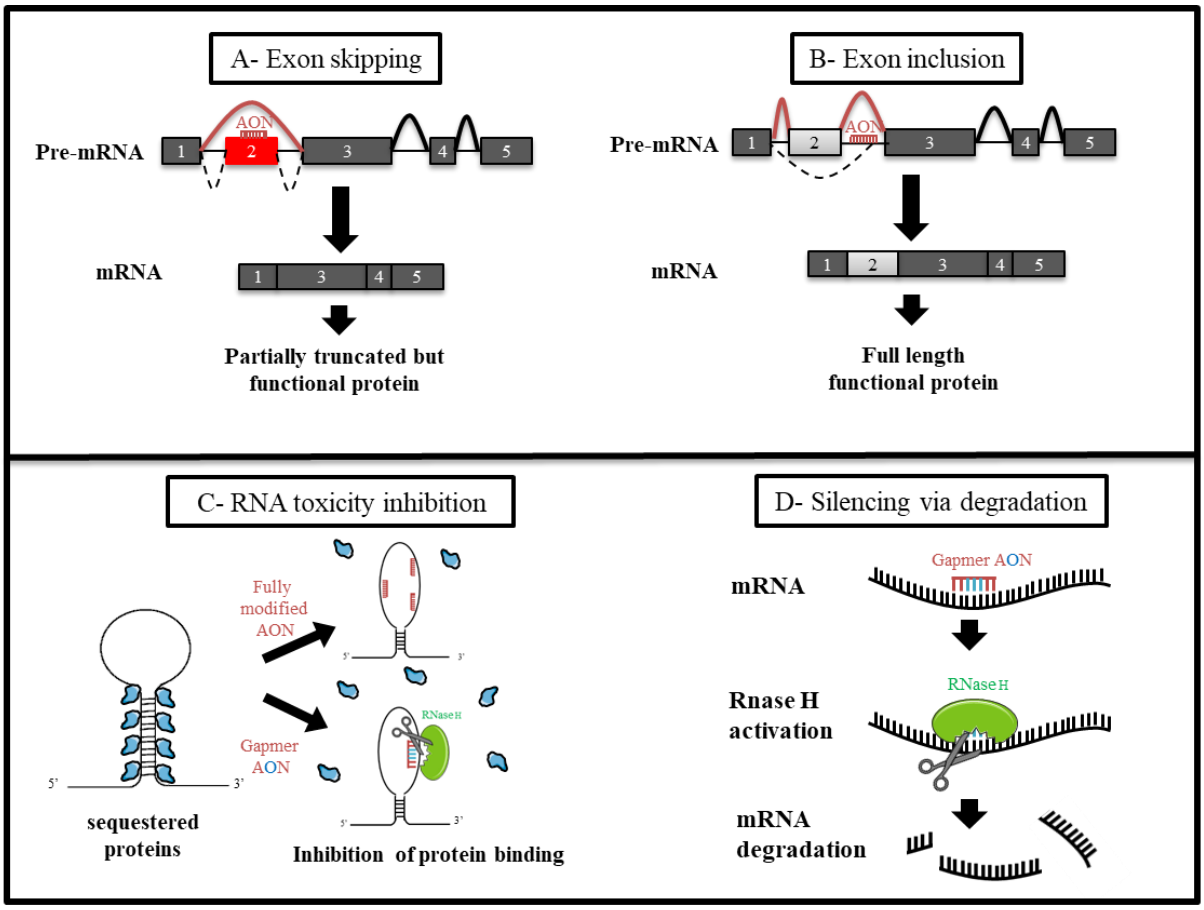

Figure AON therapies for NMD. A) Fully modified AON can modulate RNA splicing to induce exon skipping as in DMD, where the exon-skipping approach aims to omit one exon from the mRNA, to restore the reading frame and induce the expression of a BMD-like dystrophin. B) AON can also force the inclusion of an exon such as in SMA where the re-inclusion of *SMN2* exon 7 allows the production of full length SMN protein. C) Fully modified AON can be used as steric blockers to inhibit RNA toxicity as a therapeutic strategy for DM1. D) Alternatively *DMPK* transcripts mediated toxicity can be inhibited by gapmer AONs which induce mRNA degradation via RNAse H. Similar gapmer AON-mediated silencing approaches are used for CNM and ALS. Gapmer AON are designed with a central core of 8 to 10 consecutive DNA nucleotides (in blue), flanked by modified nucleotides for nuclease resistance (in red).