Developing tissue specific antisense oligonucleotide-delivery to refine treatment for Duchenne muscular dystrophy

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CHAPTER 2

Update on RNA-targeting therapies for neuromuscular disorders

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Abstract

Purpose: Antisense-mediated modulation of transcripts is a dynamic therapeutic field, especially for neuromuscular disorders.

Recent findings: For three diseases, this approach has advanced to the clinical trial phase, i.e. Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA) and myotonic dystrophy (DM). In parallel, numerous proof of concept studies in cell and animal models have been reported for additional neuromuscular disorders.

Summary: This review discusses the most notable advances in preclinical and clinical studies in the past year. For DMD, SMA and DM trials are ongoing to assess safety and efficacy, while in parallel pre-clinical studies are being conducted to identify ways to improve efficiency and delivery. For other neuromuscular diseases, progress is made as well warranting future clinical trials. However, towards clinical trial readiness, it is important not only to optimize the therapy preclinically, but to also develop the infrastructure that is needed to conduct trials.

1. Introduction

Developing therapies for neuromuscular disorders is challenging because affected tissues are difficult to target due to their abundance (skeletal muscle) or inaccessibility (motor neurons). Antisense oligonucleotides (AONs) are small (20-30 nucleotides) single stranded pieces of chemically modified DNA or RNA that can target gene transcripts. Because they are small they can overcome the delivery challenge. Delivery to the nervous system is especially efficient with intrathecal or intraventricular injection. Following delivery in this manner, AONs are rapidly taken up by neurons in the central and peripheral nervous system[1]. As such AONs offer an attractive therapeutic tool for the treatment of neuromuscular disorders, and in fact AONs have progressed to the clinical trial phase for three neuromuscular disorders: Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA) and myotonic dystrophy (DM). In parallel preclinical studies are ongoing to further improve this approach, and reported proof-of-concept studies outline the therapeutic potential of AON therapy for other neuromuscular disorders. This review will focus on the pre-clinical and clinical developments of AON-mediated transcript targeting for neuromuscular disorders of the past 15 months.

2. Clinical trials

2.1 Duchenne muscular dystrophy

AON-mediated transcript targeting has moved into the clinical trial phase for DMD, SMA and DM. For DMD and SMA AONs modulate the pre-mRNA splicing process. DMD is caused by mutations in the *DMD* gene that disrupt the reading frame, leading to premature truncation of protein translation and non-functional dystrophin proteins. Mutations that maintain the reading frame allow the production of an internally deleted, partially functional dystrophin protein. These are associated with a less severely progressive disease, Becker muscular dystrophy. For DMD AON-mediated transcript targeting is exploited with the aim to reframe dystrophin transcripts to allow the production of internally deleted, partially functional dystrophin proteins, as found in Becker muscular dystrophy. This can be achieved by AONs that target specific exons, hiding them from the splicing machinery and causing them to be 'skipped'. This approach is mutation specific, but because skipping a single exon can be applicable to a variety of different mutations, AONs targeting certain exons can be applied to larger groups of patients, e.g. exon 51 skipping applies to 13-14% of all patients[2;3]. Not surprisingly, AONs targeting exon 51 are most advanced in the clinical development, i.e. Drisapersen (2'-O-methyl phophorothioate (2OMePS)) and Eteplirsen (phosphorodiamidate morpholino oligomer (PMO) chemistry).

Eteplirsen has been injected intravenously in weekly doses up to 50 mg/kg in 31 DMD patients[4;5]. The drug was well tolerated, occasionally transient proteinuria was reported. In the most recent study 12 patients received 30 or 50 mg/kg Eterplirsen or placebo for 24 weeks, followed by an open label extension phase where all patients received 30 or 50 mg/kg[5]. Dystrophin restoration was reported for 30-60% of muscle fibers in a biopsy taken after 48 weeks of treatment. For 6 of the patients treated from the onset of the study, the distance walked in 6 minutes (6MWD) remained stable for 120 weeks. Currently, patients have been treated for up to 168 weeks. While there is some decline in 6MWD, the 10 ambulant patients performed better than would be expected from natural history, suggesting that Eteplirsen slowed disease progression (Edward Kaye, personal communication).

Drisapersen has been tested in 4 trials using subcutaneous treatment in ~300 DMD patients[6;7]. While the drug was tolerated well, mild to moderate side effects were reported, involving injection site reactions, proteinuria and in some patients thrombocytopenia[7]. These side effects are commonly found for the 2OMePS chemistry, with injection site reactions observed after subcutaneous but not after intravenous delivery. Drisapersen also appears to slow down disease progression. In an open label extension trial following a dose escalation study in 12 patients, 8 ambulant patients had stable 6 MWD for 177 weeks. Furthermore, treated patients (6 mg/kg subcutaneous weekly or intermittently) outperformed placebo treated patients in the 6MWD in a randomized, double-blind placebo controlled trial involving 54 early stage DMD patients (6-8 years of age)[7]. Two other trials have been completed but are not yet published

(Giles Campion and Tony Hall, personal communication). Early stage patients treated with 6 mg/kg subcutaneous Drisapersen for 24 weeks outperformed patients treated with placebo or 3 mg/kg Drisapersen in a dose comparing trial involving 51 patients. The most recently completed trial involved 186 patients of varying ages (5-16 years) and disease stages. After 48 weeks the treated group did not perform significantly better in the 6 minute walk test than the placebo treated group. Recent natural history data for the 6MWD in DMD patients has revealed that it is very difficult to find significant effects in a population as mixed as the one in the phase 3 trial[8-11]. In young patients the decline in 6MWD is slow and therefore it is challenging to pick up a treatment-induced slowing of disease progression in a 48 week trial. By contrast in patients who are in a more advanced disease state, treatment for more than 48 weeks might be required to induce an discernible effect in the 6MWD, as suggested by data of patients who were treated for an additional 48 weeks in the open label study following the phase 3 trial.

Biomarin/Prosensa has submitted an new drug application (NDA) for accelerated approval of Drisapersen with the Food and Drug Administration in April 2015 and filed for conditional approval of Drisapersen with the European Medicine Agency in June 2015. Sarepta plans to file for accelerated approval using a rolling NDA for Eteplirsen while a phase 3 trial is ongoing. In addition trials are ongoing for exons 44, 45 and 53 skipping (coordinated by Biomarin, Sarepta and Nippon-Shinyaku Co)[12;13].

2.2 Spinal muscular atrophy

SMA is caused by mutations in the *SMN1* gene, leading to death or incomplete development of motor neurons. The *SMN2* gene (only present in humans) can produce functional transcripts, if exon 7 could be retained in the transcripts. Exon 7 possesses a silent mutation and intronic splicing silencers located in the regions flanking exon 7. Using AONs targeting intronic silencers, the level of exon 7 inclusion can be increased, enabling patients to produce more functional *SMN2* transcripts and increased amounts of SMN protein. Extensive preclinical screening has resulted in the identification of ISIS-SMN$_{rx}$, a 2'-O-methoxyethyl phopshorothioate (MOEPS) AON, for which pharmacological properties after central nervous system delivery have been thoroughly investigated in mouse, rats and non-human primates[14]. One exploratory trial with ISIS-SMN$_{rx}$ has been completed by ISIS pharmaceuticals in SMA type 2 and type 3 patients with encouraging preliminary results[15]. Currently, a trial has initiated for severe type 1 patients and additional trials are ongoing in type 2 and 3 patients coordinated by ISIS and Biogen.

2.3 Myotonic dystrophy

For myotonic dystrophy (DM), AONs treatment aims to reduce RNA aggregates formed by the expanded CUG repeat in the *DMPK* gene, which sequester splicing factors, and as such underlie the missplicing pathology observed in DM patients. An MOE gapmer AON to reduce *DMPK* transcript levels (ISIS-DMPK$_{rx}$) has been identified, which normalizes splicing patterns in vitro and in an DM animal model[16]. A dose-finding, safety trial has recently been initiated in DM patients by ISIS pharmaceuticals (https://www.clinicaltrials.gov/ct2/show/NCT02312011).

3. Preclinical studies for DM, SMA and DMD

3.1 Improving chemistry and delivery

In parallel with the clinical trial, preclinical work is ongoing to further optimize the tools and techniques involved. For DM it was shown that a locked nucleic acid targeting the CUG repeat has potential in vitro and in vivo[17]. The locked nucleic acid chemistry has a very high affinity for RNA transcript and very short AONs (8-10-mers) were able to reduce the amount of DMPK aggregates and restore splicing defects. However, with such short AONs there is an obvious risk that CUG repeats in normal transcripts will be targeted as well.

For SMA, it was reported that intraventricular delivery of ISIS-SMN$_{rx}$ AONs normalizes gene-expression levels in the spinal cords of SMA mouse models[18]. Notably, Keil *et al*., published that the phenotype of severe SMA models could be ameliorated with an 8-mer AON[19]. While promising, it is obvious that an 8-mer AON will not be specific for only the *SMN2* gene and comprehensive gene expression analysis as done for the ISIS-SMN$_{rx}$ compound would be warranted to study this AON further.

For DMD efforts focus on improving delivery to heart. The tricyclo-DNA modification resulted in increased exon skipping and dystrophin restoration in heart and even resulted in low levels of exon skipping in the brain[20]. Treatment with this chemistry also rescued the phenotype of a severe mouse model lacking dystrophin and its homologue utrophin (*mdx/utrn-/-* mouse). Clinical trials with this chemistry are planned for DMD. However, as yet safety and tolerability in species other than mice has not been assessed. Another concern is that tricyclo-DNA AONs are shorter than PMO and 2OMePS AONs (15-mer vs 20-30-mers). Therefore there is a risk that the AONs will have (off) target sites in addition to the intended dystrophin exon.

Another way to increase uptake is through addition of peptides [21-23]. Conjugation of short arginine rich cationic peptides with a hydrophobic core increased uptake of phosphorodiamidate morpholino oligomers (pip-PMOs) in heart (as well as skeletal muscle), leading to a slower development of cardiomyopathy[21;23]. Since 2OMePS nucleotides are negatively charged, it is not possible to conjugate positively charged peptides to these AON. However, conjugation of a 7-mer peptide identified from a phage display peptide library resulted in increased uptake and exon skipping levels[24].

3.2 Applicability for different mutations

DMD exon skipping is a mutation specific approach. Thus far the focus has been on skipping single exons for deletion mutations, while ~12% of patients have a duplication of 1 or more exons. Exon skipping for duplications is challenging, since the AONs will target both the original and the duplicated exon and generally both exons will be skipped, resulting in an out-of-frame transcript[25]. However, it was recently reported that exon skipping may be possible for the most common single exon duplication, i.e. an exon 2 duplication[26]. Here, if exon skipping is too efficient and both exons 2 are omitted, the resulting transcript (deletion of exon 2) can still give rise to functional dystrophin through the activation of an internal ribosomal entry site in exon 5. Skipping multiple exons exon 2-7) was reported as an alternative approach to restore an exon 2 duplication[25]. This approach involves a combination of multiple AONs. Multiexon skipping has also been proposed as a 'cocktail' approach to induce skipping of exon 45-55 (i.e. the mutation hotspot)[27;28], which would in theory be therapeutic for a large group of patients (~40-60% depending on which mutation database is queried[2;29]) and produce a highly functional dystrophin. It was recently shown to be feasible to skip exon 45-51 and exon 53-55 and restore dystrophin expression in a mouse model lacking mouse dystrophin exon 52 using a combination of 10 vivo-PMOs (PMOs with an oligodendrimer group for enhanced tissue uptake). While multiexon skipping is feasible in animal models, developing an approach involving 11 individual AONs poses regulatory challenges[30].

3.3 Timing, duration and effects of AON treatment

Preclinical studies have revealed insight on the duration of treatment and the impact of treatment initiation. While 2OMePS AON levels and exon skipping transcripts decline and were barely detectable 12 weeks after treatment was stopped, dystrophin protein levels continued to increase for another 8-12 weeks and were still detectable 24 weeks after treatment termination in *mdx* mice[31]. An elegant study from Wu *et al.,* used peptide conjugated PMOs to restore high levels of dystrophin in the severe *mdx/utrn-/-* model. The level of benefit critically depended on the time of intervention. Animals treated before 4 weeks of age had increased lifespan, normal posture, limited muscle wasting and very limited kyphosis, while no benefit was observed for animals that were treated in ~6-7 week old animal with advanced pathology, despite high levels (>50% of normal) of dystrophin restoration[32].

Multiple studies have focused on the effect of systemic versus CNS treatment in SMA mouse model [21;33-36]. It appears that the combination of CNS and systemic delivery results in a markedly improved increase in survival compared to only CNS or systemic delivery. Since in newborn mice the blood-brain-barrier is not yet mature, systemic treatment with high doses of AON will result in body-wide delivery, including the central nervous system. In order to investigate the effect of treatment of only peripheral tissues in new born mice, Hua et al used a decoy AON (i.e. a sense oligo that can sequester an AON, rendering it ineffective[34]. New-born mice were injected with AONs postnatally and received an injection with the decoy AON through intraventricular injection. As such SMN was restored only in the periphery, which resulted in rescue of ear and tail necrosis in a mild SMA model and increased survival in a severe model. It is not yet clear whether the importance of peripheral SMN restoration is specific for mouse or whether it translates to the human situation. For now, clinical trials for SMA patients involve only intrathecal delivery.

RNA targeting therapies for other neuromuscular disorders

Antisense-mediated transcript targeting has been reported for multiple neuromuscular disorders (Table 1). Here, we will focus only on reports of the past 15 months.

Table 1. Overview of transcript targeting antisense approaches for neuromuscular

Disease	Mechanism	Reference
Amyotrophic lateral sclerosis and frontotemporal degeneration	Reduced levels of mutated transcript (C9ORF72)	[37;38]
Congenital myosthenia	Normalize CHRNA1 splicing	[39]
Duchenne muscular dystrophy	Reading frame restoration	[5;6]
Dysferlinopathies	Bypassing mutation in in-frame exon	[40]
Fukuyama Muscular Dystrophy	Skipping of cryptic retrotransposon exon	[41]
Laminopathies	Bypassing mutation in in-frame exon 5	[42]
Myotonic Dystrophy	Reduced levels of mutated transcript	[16]
Oculopharyngeal muscular dystrophy	Polyadenylation site usage	[43]
Pompe Disease	Reduced levels of glycogen synthase	[44]
Spinal muscular atrophy	Exon inclusion	[45;46]

4.1 Oculopharyngeal muscular dystrophy

Oculopharyngeal muscular dystrophy (OPMD) is caused by an alanine expansion in the polyadenyline binding protein 1 (PABPN1), that makes the PABPN1 protein prone to aggregation. In OPMD lower amounts of soluble PABPN1 protein lead to a genome-wide shift of proximal rather

than distal polyadenylation site usage. This deregulation of gene expression is proposed to underlie the premature aging observed in affected OPMD muscles. ARIH2 E3-ligase is one of the genes misregulated in OPMD. Notably, ARIH2 protein levels positively regulate the amount of total and soluble PABPN1. As such restoring the normal polyadenylation site usage for ARIH2 could also lead to increased levels of PABPN1. Indeed, AONs targeting the proximal polyadenylation signal of ARIH2 resulted in increased levels of ARIH2, higher levels of PABPN1 and improved differentiation of OPMD-derived cell cultures in vitro[43].

4.2 Laminopathies

Mutations in the lamin A/C encoding *LMNA* gene underlie multiple disorders, including neuromusclar disorders such as Emery-Dreifuss muscular dystorphy, limb-girdle muscular dystrophy type 1B and congenital muscular dystrophy. In an effort to identify exons encoding redundant parts of lamin A, a recent study studied expression vectors of lamin A cDNA lacking selected exons. This work revealed that cDNA without the in-frame exon 5 resulted in a lamin protein that was localized normally, could rescue the abnormal nuclear phenotype of laminopathy-patient-derived cells and did not have dominant negative effect in control cells[42]. AONs to induce *LMNA* exon 5 skipping in vitro have been identified[42]. This approach might have therapeutic potential for laminopathy patients with mutations in exon 5, although further studies are required in cell and animal models to fully evaluate the potential.

4.3 Pompe disease

Pompe disease is characterized by progressive accumulation of undegraded glycogen, due to mutations in the *GAA* gene that encodes for acid alpha-glucosidase (GAA), an enzyme involved in breaking down glycogen to glucose. Infusion with recombinant human GAA is an approved treatment for Pompe patients. However, GAA infusion generally is insufficient to completely abolish all aspects of the disease. In an effort to develop an add-on therapy, Clayton *et al*., tested peptide-conjugated PMOs targeting an out-of-frame exon of the glycogen synthase enzyme as a way to reduce glycogen production. Treatment with these PMOs resulted in lower levels of glycogen synthase and lower amounts of glycogen accumulation in a Pompe animal model[44].

4.4 Congenital myasthenic syndrom*s*

Congenital myasthenic syndroms (CMS) are caused by mutations in genes encoding proteins required for neuromuscular junction formation, maintenance and regulation. The *CHRNA1* encodes the alpha subunit of the nicotinic acetylcholine receptor (nAChR), which is expressed in the post synaptic membrane of the neuromuscular junction. In humans, the *CHRNA1* gene produces two transcripts that either include or exclude exon

P3A. Only the transcript lacking exon P3A encodes a functional protein. Mutations have been identified in CMS patients that result in increased levels of P3A inclusion. Tei *et al.*, have identified AONs that could induce P3A exon skipping in minigenes containing mutated sequences and in wild type cells[39]. Further work is needed to assess the potential of this approach in patient-derived cells and animal models.

5. Forward look

It is clear that antisense-mediated transcript targeting is a dynamic field for the neuromuscular disease space: proof-of-concept studies accumulate and the approach is taken into preclinical and clinical development for some neuromuscular disorders. However, it has become apparent that it is crucial to develop tools and infrastructure to conduct clinical trials in parallel with the development of the potential therapy, rather than at the initiation of the clinical trial phase. In addition to patient registries and trial sites, this involves developing functional and molecular outcome measures to be used in trials as well as natural history data for these measures. When these tools are in place this will allow better trial design and hopefully faster clinical evaluation of potentially therapeutic compounds. In addition, good communication with key regulatory agencies is critical to move these therapies towards clinical practice.

6. Conclusion

RNA targeting is a promising therapeutic approach for multiple neuromuscular disorders. However, in addition to proof-of-concept in cell and animal models, clinical infrastructure is required to further the clinical development of this approach towards clinical application.

RNA targeting is a promising therapeutic approach for neuromuscular disorders

RNA targeting is tested in clinical trials for DMD, SMA and DM

Proof-of-concept has been shown for multiple neuromuscular disorders

Infrastructure for clinical trial needs to be in place for each neuromuscular disorder to facilitate and accelerate clinical development of (RNA targeting) therapies

7. Conflict of Interest

8. Acknowledgements

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