

Antisense Oligonucleotide-Mediated Exon Skipping for Duchenne Muscular Dystrophy: Progress and Challenges

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Abstract: Duchenne muscular dystrophy (DMD) is the most common childhood neuromuscular disorder. It is caused by mutations in the *DMD* gene that disrupt the open reading frame (ORF) preventing the production of functional dystrophin protein. The loss of dystrophin ultimately leads to the degeneration of muscle fibres, progressive weakness and premature death. Antisense oligonucleotides (AOs) targeted to splicing elements within *DMD* pre-mRNA can induce the skipping of targeted exons, restoring the ORF and the consequent production of a shorter but functional dystrophin protein. This approach may lead to an effective disease modifying treatment for DMD and progress towards clinical application has been rapid. Less than a decade has passed between the first studies published in 1998 describing the use of AOs to modify the *DMD* gene in mice and the results of the first intramuscular proof of concept clinical trials. Whilst phase II and III trials are now underway, the heterogeneity of DMD mutations, efficient systemic delivery and targeting of AOs to cardiac muscle remain significant challenges. Here we review the current status of AO-mediated therapy for DMD, discussing the pre-clinical, clinical and regulatory hurdles and their possible solutions to expedite the translation of AO-mediated exon skipping therapy to clinic.

Keywords: Antisense oligonucleotides, clinical trials, duchenne muscular dystrophy, becker muscular dystrophy, dystrophin, exon skipping, RNA therapy

Introduction

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a fatal, X-linked, neuromuscular disorder that affects 1 in 3,500 newborn boys. Patients are typically diagnosed as toddlers; they develop progressive muscle weakness and cardiomyopathy and lose the ability to walk by their early teens. Unless appropriate standards of care (including non-invasive ventilation,

glucocorticoid and cardio-protective treatment) are implemented, premature death by cardiac or respiratory failure occurs in the second decade of life (1-3). DMD is caused by mutations in the *DMD* gene that disrupt the open reading frame (ORF) thus aborting the full translation of its protein product, dystrophin (4, 5). The *DMD* gene comprises 79 exons and the majority (~65%) of mutations responsible for DMD are out-of-frame deletions,

although duplications (~10%), small mutations including non-sense and splice site changes (~22%) and deep intronic mutations (~3%) are also documented (6, 7). Some *DMD* deletions are more frequent than others and the gene has two deletion hotspots (6): the most commonly mutated region is exons 45–55 followed by exons 2–19.

Dystrophin is located underneath the sarcolemma and connects the sub-sarcolemmal cytoskeleton to the extracellular matrix by binding N-terminally to cytoskeletal F-actin and to β -dystroglycan via a cysteine rich domain near the C-terminus (8) (Top panel, Figure 1). It contains four main functional units: an N-terminus, a central rod domain, a cysteine rich domain and a C-terminal domain. The central rod domain consists of 24 spectrin-like repeats and four hinge domains (9). Dystrophin interacts with actin at both its N-terminus and via spectrin-like repeats 11-17; the C-terminus has also recently been shown to allosterically affect actin binding (10). The cysteine rich domain binds to β -dystroglycan (BDG) (11-15) and the C-terminal domain is required for binding to syntrophin (16) and dystrobrevin (17). These and other sarcolemmal proteins such as the sarcoglycans are components of the dystrophin associated glycoprotein complex (DGC). Dystrophin and the DGC play an important role in stabilising the muscle fibre against the mechanical forces of muscle contraction by providing a shock-absorbing connection between the cytoskeleton and the extracellular matrix.

Loss of dystrophin leads to disruption of the complex, which results in inflammation, increased intracellular calcium influx, muscle degeneration and replacement of muscle with adipo-fibrous tissue (4). In addition, dystrophin plays a role in signalling and is associated with members of the stretch-activated calcium channels; their mislocalisation and dysfunction in dystrophic muscle contributes to disease progression (18). Spectrin repeats 16 and 17 within the central rod domain, encoded by exons 42–45, are also required for binding to neuronal nitric oxide synthase (nNOS) (11, 12, 19). nNOS regulates the blood flow in skeletal muscle (20); disruption of this pathway may contribute to *DMD* pathogenesis by inducing paradoxical vasoconstriction during exercise (21).

Naturally-occurring dystrophin positive “revertant fibres” (isolated or less commonly small clusters of fibres strongly positive for dystrophin) and “traces” (fibres expressing very low levels of dystrophin at the sarcolemma) occur in more than 50% of the muscle biopsies of *DMD* patients (22). Revertant fibres represent a very small percentage of the total fibres, in which somatic mutations or stochastic alternative splicing events of the dystrophin pre-mRNA lead to exon skipping, the restoration of the ORF and consequent expression of dystrophin (23, 24). Revertant dystrophins are correctly localised to the sarcolemma and associate with other DGC proteins, suggesting a retained function (25, 26). Revertant fibres have been well characterised in the

mouse model of DMD, the *mdx* mouse (24, 27, 28). Whilst traces have not been described in the *mdx* mouse, they are present in approximately a third of DMD patients (22) and may represent up to 25% of the total muscle fibres (29). The molecular mechanism of trace dystrophin expression remains to be elucidated, but it is thought to be at least in part different from that of revertant fibres. For example, traces can express different dystrophin epitopes than the surrounding revertant fibres (22).

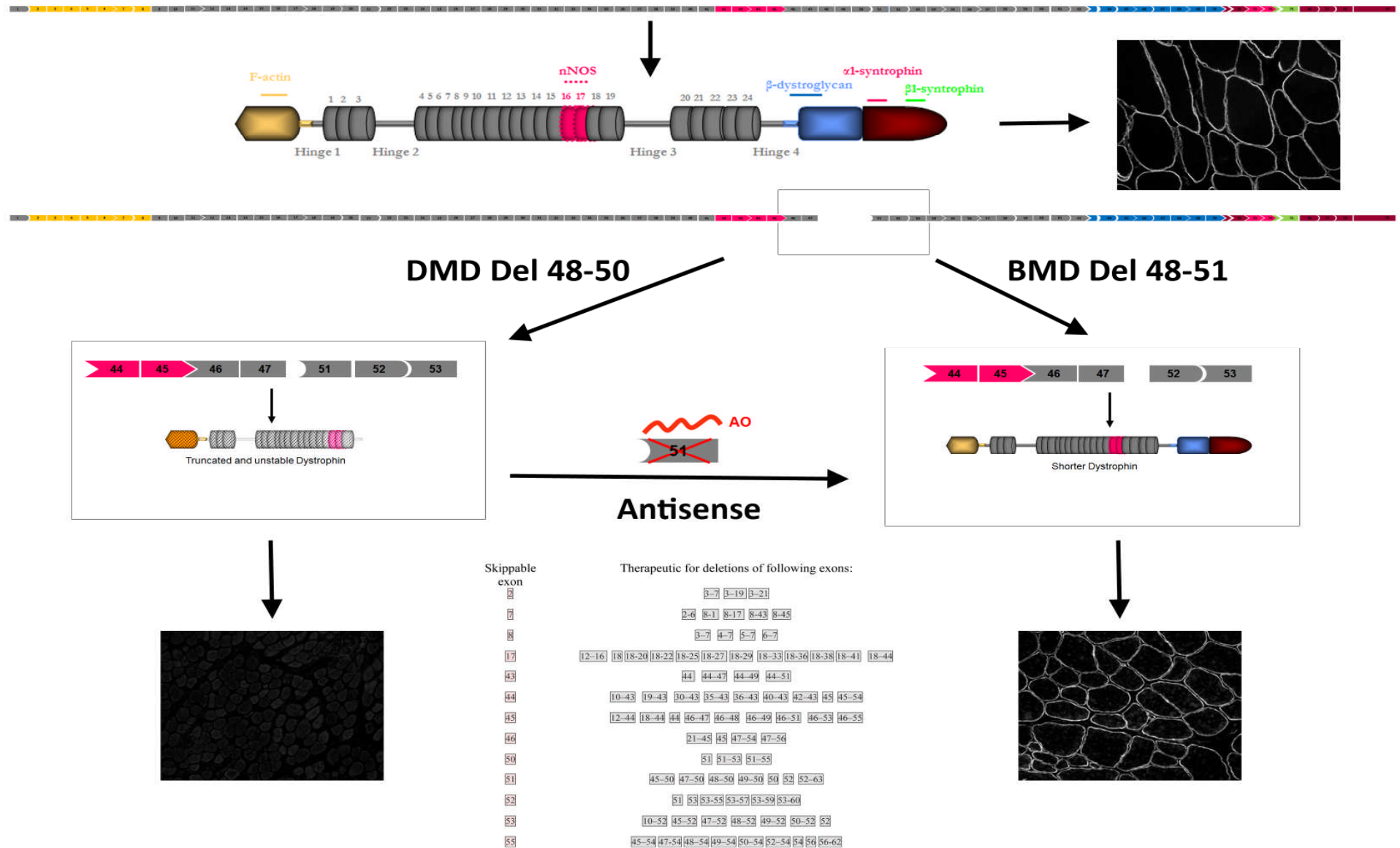
Becker muscular dystrophy

Mutations in the *DMD* gene are also responsible for a milder disorder, Becker muscular dystrophy (BMD), a disease with an extremely variable spectrum of severity ranging from patients with walking difficulties in their late teens or early twenties, to the majority of individuals in whom ambulation is preserved into late adulthood and who have an essentially normal lifespan (30). DMD and BMD mutations differentially affect the *DMD* gene: in DMD the mutations *disrupt* the reading frame, while mutations that cause BMD *maintain* the ORF (31, 32) leading to the production of an internally deleted dystrophin protein. The size of the deletion does not correlate with the severity of the disease, as long as the reading frame rule is maintained (33-40), and provided crucial domains of dystrophin such as the β -dystroglycan binding site are not removed by the deletion. While the central and distal rod domain is less vital for function

(35), (some patients missing these domains only have very mild disease manifestations such as myalgia and muscle cramps, and mild weakness), in frame deletions that affect the binding of dystrophin to other proteins such as cytoskeletal actin or β -dystroglycan result in a severe phenotype (41, 42).

The existence of revertant fibres in DMD and the occurrence of mildly affected BMD individuals with in-frame deletions suggest that it is feasible to modify splicing by exon skipping (Figure 1) and induce the production of functional dystrophin in DMD patients, as long as crucial domains of dystrophin are not disrupted. Artificially restoring the ORF in this way is thus an attractive therapeutic strategy for DMD, as approximately 70% of DMD patients have mutations amenable to exon skipping (43).

Figure1 Exon skipping principle. (Next page) Upper panel: Schematic representation of dystrophin mRNA (in-frame exons are represented as square boxes, out-of-frame exons round or arrow boxes). Normal splicing of these exons produces dystrophin protein (pictured immediately below) retaining functional protein-binding domains and correctly localised to the sarcolemma (see section of control muscle stained with anti-dystrophin antibody Dys2). **Lower panel:** representation of dystrophin pre-mRNA highlighting the differences in splicing between a Del48-50 DMD patient (left) and a Del48-51BMD patient (right). The DMD deletion disrupts the open reading frame (ORFs) which results in unstable mRNA and the absence of functional dystrophin protein in muscle sections. In the BMD patient the deletion maintains the ORF and generates the production of an internally deleted dystrophin isoform that retains the critical amino and carboxyl terminals and Cysteine-rich domains. The ORF can be corrected by forced skipping of exon 51 by directing antisense oligonucleotides to sequences within exon 51 or to neighbouring intronic regions. Exon 51 skipping restores the ORF, generating a dystrophin equivalent to that of the BMD patient. **Insert table:** Theoretical applicability of single exon skipping in a series of DMD deletions.



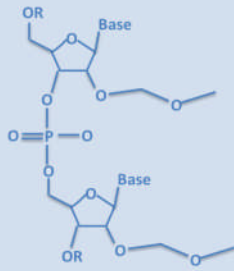
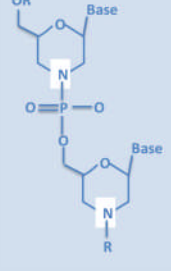
	PRO051/GSK2402968	AVI-4658/ETEPLIRSEN		
Company	Prosensa/GlaxoSmithKline	AVI-BioPharma		
Type	2'O-methyl phosphorothioate (2'OMe)	Phosphorodiamidate morpholino oligomer (PMO)		
Backbone structure				
Size and sequence	20 mer (TCAAGGAAGATGGCATTCT)	30 mer (CTCCAACATCAAGGAAGATG GCATTCTAG)		
Delivery	SC	IV		
Plasma protein binding	Backbone binds to serum proteins	No		
Serum half life	<4 h to 28 days (44, 45)	1.62 to 3.60 hours (46)		
Max non-toxic dose in patients:	Proteinuria seen in all patients at 6mg/kg (44)	Not reached (46)		
Max tested non-toxic dose in mice:	?	960mg/kg (47)		
Max tested non-toxic dose in primates:	?	320mg/kg (47)		
Orphan drug	Yes	Yes		
SYSTEMIC TRIAL REPORTED RESULTS*				
Total number of patients	12 (44)		19 (46)	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Maximum reported dystrophin-positive fibres	Not done	100%	5%	55%
Maximum dystrophin signal intensity to control muscles by immunofluorescence	Not done	15.6%	11%	27%
Maximum dystrophin protein level to control muscles by western blotting	Not done	Not done	5%	18%

Table 1 Comparison between the two leading candidates for exon 51 skipping. SC = subcutaneous; IV = intravenous; IM = intramuscular

Antisense oligonucleotide-mediated exon skipping

Antisense oligonucleotides (AOs) targeted to splicing elements represent the most clinically advanced therapeutic tools developed to induce dystrophin exon skipping (48, 49). AOs are typically 20–30 nucleotides in length, and complementary in sequence to regions of the pre-mRNA transcript (50). While several AO chemistries exist, the two AOs in clinical development for DMD are 2'-O-methyl phosphorothioate oligoribonucleotide (2'OMe) and phosphorodiamidate morpholino oligomers (PMO), (Table 1) and (51, 52) for a detailed review. 2'OMe AOs bind to albumin, showing high plasma concentrations and long half-lives (45); this might be an advantage as PK studies indicate a longer persistence in blood compared to PMO (up to 28 days as opposed to less than 4 hours); however binding to protein has been shown to trigger activation of the immune system, anaphylaxis, hypotension, or antiarrhythmic effects in preclinical and clinical studies (53). PMOs are not metabolised and are resistant to endonucleases (54); they are rapidly eliminated from the bloodstream as they are uncharged and do not bind serum proteins, which is likely why they have not been associated with the side effects mentioned for the 2'OMe clinical studies. Both AOs have proven successful in pre-clinical mouse models (55-58) and as far as the PMO is concerned, also the more severe dog model (59), in which systemic delivery has resulted in dystrophin protein

production (60, 61) and physiological improvement (58) of skeletal muscle.

Clinical progress

Both PMO (AVI-4658/eteplirsen) and 2'OMe (GSK-2402968) AOs targeting exon 51 (which will restore the ORF in the largest group of DMD patients (13%)) have proven successful at inducing local dystrophin expression in pivotal proof-of-concept intramuscular clinical trials (62, 63). Recently, systemic studies using the two different AO chemistries have been completed (Table 2) (44, 46), demonstrating that AO therapy for DMD is indeed safe and well tolerated with no significant drug-related adverse events. Both studies reported significant dystrophin restoration in a dose-dependent manner as determined by western blotting and immunohistochemistry, with levels of dystrophin approaching 20% of normal levels in the PMO study.

The outcome of randomised placebo-controlled studies of both eteplirsen and GSK-2402968 is expected in 2012 and further studies are planned. Table 2 summarises the design of both completed and ongoing ClinicalTrials.gov registered studies correct at the time of publication. In addition, AOs for exons 45, 52, 53 and 55 are undergoing pre-clinical development by GSK whilst AVI BioPharma is developing PMOs targeting exons 45, 50 and 53. Plans to extend trials of systemically-delivered AOs to non-ambulant boys are also underway for exon 51 skippable patients (both with eteplirsen and GSK2402968).

	Completed				Ongoing						
	Intra-muscular		Systemic		Systemic						
Study drug	AVI-4658 (Eteplirsen)	PRO051 (GSK-2402968)	AVI-4658 (Eteplirsen)	PRO051 (GSK-2402968)	AVI-4658 (Eteplirsen)	PRO051 (GSK-2402968)	PRO051 (GSK-2402968)	PRO051 (GSK-2402968)	PRO051 (GSK-2402968)	PRO051 (GSK-2402968)	PRO044
ClinicalTrials.gov identifier	NCT00159250	Netherlands trial register: NTR712	NCT00844597	EudraCT number: 2007-004819-54	NCT01396239	NCT01153932	NCT01462292	NCT01254019	NCT01128855	NCT01451281 (parent study: NCT01462292)	NCT01037309
Phase	I/II	I/II	I/II	I/II	II	II	II	III	I	n/a	I/II
Study design	Single-blind, placebo-controlled, dose-escalation	Single dose	open-label, dose-escalation	open-label, dose-escalation	Randomised, double-blind, placebo-controlled, multiple Dose	Randomised, double blind, placebo controlled	Randomised, double blind	Randomised, double blind	Double-blind, escalating dose, randomized, placebo-controlled	n/a	Non-randomised, open label
Chemistry	PMO	2'OMe	PMO	2'OMe	PMO	2'OMe	2'OMe	2'OMe	2'OMe	2'OMe	2'OMe
Number of patients	7	4	19	12	12	54	54	180	32	85	18
Target exon	51	51	51	51	51	51	51	51	51	51	44
Ambulant/Non Ambulant	Ambulant	Ambulant	Ambulant	Ambulant	Ambulant	Ambulant	Ambulant	Ambulant	Non-ambulant	Ambulant	Ambulant
Delivery	IM (EDB)	IM (TA)	IV	Subcutaneous	IV	Sub-cutaneous	Sub-cutaneous	Sub-cutaneous	Subcutaneous	Sub-cutaneous	Subcutaneous & I.V (1.5, 5mg/kg)
Dose	0.09 and 0.9 mg	0.8 mg	0.5, 1, 2, 4, 10, and 20 mg/kg body weight	0.5, 2, 4 and 6 mg/kg body weight	30, 50 mg/kg body weight	6 mg/kg body weight	3, 6 mg/kg body weight	6 mg/kg body weight	3, 6, 9 & 12 mg/kg body weight	3, 6 mg/kg body weight	0.5, 1.5, 5, 8, 10, 12 mg/kg body weight
Frequency of administration	Single	Single	Weekly	Weekly	Weekly	Weekly & twice weekly	Weekly	Weekly	Single	Weekly	Weekly
Duration	3-4 weeks	4 weeks	12 weeks	12 weeks	24 weeks	24-48 weeks	24 weeks	1 year	5 weeks	24 weeks	5 weeks
Primary outcome measure	Safety	Adverse events	Safety	Safety	Dystrophin positive fibers	Efficacy	6 minute walk distance test	Efficacy	Pharmacokinetics	MRI changes in skeletal muscle	Safety, tolerability, pharmacokinetics & dystrophin expression
Start Date	October 2007	2006	January 2009	2008	July 2011	September 2010	October 2011	December 2010	July 2010	September 2011	December 2009
Completion date	March 2009	2007	December 2010	2011	June 2012 (estimated)	September 2012 (estimated)	April 2013 (estimated)	December 2012 (estimated)	November 2011	2012 (estimated)	December 2012 (estimated)

(Previous page) Table 2 Summary of completed and on-going exon skipping clinical trials for DMD

IV = intravenous; IM = intramuscular

Current challenges

In-vitro optimisation of novel AOs

Systematic screening for AO targets has already identified targets for most of the 79 dystrophin exons (64-66), but there is variability in the processes used to design and evaluate target AOs. For example, variations in the cell type for in vitro studies, transfection reagents, time of evaluation and quantification of skipped product (64, 67-69) make inter-study comparisons difficult. Despite the fact that bioinformatic tools (70-73) can provide optimal target areas for AO binding, and help rank AO sequences according to their predicted bioactivity (43, 65), empirical analysis in-vitro is always necessary to confirm the suitability of the sequence (69). Importantly, restoration of dystrophin expression can only be shown in differentiated myotubes derived from patients' cells as dystrophin is only expressed in myotubes and not in myoblasts. Ideally, cells from several patients holding different amenable deletions should be used to test the efficacy of an AO, as the intronic breakpoints differ between patients and might affect splicing efficiency (74). Primary myoblasts derived from DMD muscle biopsies can be difficult to expand in culture (75, 76) and the extent of myogenic differentiation of DMD myoblasts is often low (77, 78). Similar

levels of differentiation would be required for quantitative comparison of the efficacy of the same AO on cells from patients with different mutations. In order to improve the proliferative capacity of human myoblasts so that large numbers of cells are available for replicate experiments, techniques to immortalise human myoblasts have been developed (79).

A less invasive alternative to the use of muscle biopsies to prepare satellite cell-derived myoblasts, are fibroblasts prepared from a skin biopsy. Fibroblasts can be induced to differentiate into myotubes by forced expression of the myogenic regulatory factor MyoD (80). However, the levels of *DMD* transcripts in myotubes derived from fibroblasts can be low and the variable extent of myogenic differentiation should be controlled for in comparative experiments. Transgenic mice, harboring the entire human *DMD* locus, may be used to test antisense oligonucleotides (64, 67, 81-83). However, these mice also carry the mouse dystrophin gene and have no skeletal muscle pathology.

Outcome measures

A validated set of clinical outcome measures for ambulant DMD patients is in use in the ongoing phase II and III clinical trials. Future trials on non-ambulant patients pose a further challenge where robust measurements of upper limb strength and function in late disease stage are required. While clinical outcome measures are needed to demonstrate

functional improvement, biochemical outcome measures (BOMs) are required to monitor AO efficacy. However, critical differences in the methodology used between the different research centres are of concern. Standardised BOMs are essential in order to reliably compare the efficacy of the different chemistries and dosing regimens. Specifically, the most reliable methods for quantification of both exon skipping and dystrophin restoration must now be established through initiatives such as the TREAT-NMD registry of outcome measures for neuromuscular disorders (www.researchrom.com/) and from ongoing international collaborative studies aiming to cross-validate standard operating procedures. The outcomes of these should be the standardisation of methodology across centres that could be presented to regulatory authorities as the preferred BOMs in future clinical trials.

The efficacy of exon skipping is measured at both the RNA and protein level. Nested RT-PCR is traditionally used to assess and quantify (semi-quantitatively) AO efficacy at the RNA level (84, 85). To detect transcripts using this method, it is necessary to use up to 70 PCR cycles after which linearity is lost and it is therefore not possible to accurately quantify the percentage of exon skipping. Thus several quantitative methods are currently in development such as qRT-PCR using highly specific TaqMan assays for skipped and total dystrophin targets. The advent of digital PCR and micro fluidic technology enables the high throughput analysis of

patient RNA. For example, exon skipping could be assessed by measuring changes in mRNA decay pre and post treatment using TaqMan assays that cover all 79 dystrophin exons. Such a platform, the FluidDMD, has recently been described which simultaneously analyses 85 TaqMan assays recognising 76 out of 78 DMD exon junctions (86).

As the aim of AO-mediated exon skipping is to restore dystrophin production, reliable methods to quantify dystrophin expression are vital. The presence of dystrophin traces and revertant fibres in DMD muscles (22) makes it essential to compare treated muscles with a pre-treatment biopsy of the same patient, in order to accurately distinguish and quantify AO-mediated dystrophin protein production (87). The two most commonly used methods are western blotting and immunostaining (46, 87, 88). Consideration should be given to the antibodies to be used, which must be sensitive, specific and have an epitope appropriate for the dystrophin exons retained following exon skipping.

As muscle biopsies are invasive and sample a single muscle, there are limitations in their use to monitor response to therapy and efforts are being made to identify non-invasive biomarkers to monitor DMD disease progression. Studies aimed at validating the role of magnetic resonance imaging (89) and spectroscopy as well as serum or urine biomarkers such as small non coding RNAs (such as miRNA) are currently underway both in animal models and in clinical

studies; it is hoped that these will reduce the need for muscle biopsies (90).

Functionality of the de novo dystrophin protein

Whilst the primary outcome measure of the completed systemic trials was clinical safety, the GSK-2402968 study also reported a modest improvement in the 6-minute walk test which is encouraging (44). From a biochemical perspective, data from both the intramuscular and systemic eteplirsen trials further indicate that the internally deleted dystrophins generated by exon skipping in different patients are indeed functional, as they led to the restoration of proteins of the DGC (46, 91). Additional evidence of functional improvement is provided by a reduction in cytotoxic T cells within treated muscle biopsies (46); this is promising considering that the pre-symptomatic induction of inflammatory cascades and the invasion of muscle by immune cells is one of the earliest pathways induced in dystrophin deficient muscle and is thought contribute to DMD pathology (92, 93). However, the possibility of an immunological reaction both against revertant and novel dystrophin epitopes remains a possibility (94) and presents a new issue to address in future clinical trials that will require the assessment of any pre-existing immunological response to dystrophin epitopes in patients prior to their inclusion in a clinical trial, as well as any post-treatment response to the newly-generated dystrophin protein (94).

Some mild or asymptomatic BMD patients naturally express the dystrophin proteins that we aim to produce by exon skipping (32). A recent study correlated dystrophin and dystrophin-associated protein expression with disease severity in a cohort of BMD patients (26). The amount of dystrophin, nNOS and BDG correlated to clinical severity and BMD patients with deletions equivalent to those created by exon 51 skipping have higher dystrophin levels than either those with large multi-exon deletions, or those harbouring exon 53 skippable deletions (26). These findings demonstrate the therapeutic potential of the protein that will be generated by exon 51 skipping trials whilst the functionality of other dystrophins, especially those with larger internal deletions, is less clear (43, 95-97).

Variability of response

The completed AO-mediated exon skipping clinical trials have revealed a high degree of variability in patient response, even between patients harbouring the same deletions (46). These findings suggest that the variability is unlikely to be due to inter-patient differences in stability of the resultant protein, immunological response, or the pharmacodynamics of the PMO (46). However, it has been suggested (46) that differences in the genetic background, such as intronic deletion breakpoints, differences in the efficiencies of mRNA splicing, or differences in the vascular access of the AO to individual muscles may contribute to the variable response.

An important future goal must be to understand the mechanisms behind this variability and why some patients respond better to treatment than others. Interestingly studies in the *mdx* mouse with both PMO and 2'OMe and in the GRMD dog using PMO have identified similar variability in response, even in the same animal, when different muscles were studied (61, 98-100). This indirectly points towards stochastic events involved in delivering the AO to skeletal muscle rather than a genetic difference, although more studies are needed to elucidate the mechanism responsible for the observed variability and whether this variability may be reduced after long-term treatment as indicated by studies on the *mdx* mouse (98).

Next generation AOs

Although extremely high-doses of PMO without modification can induce dystrophin rescue in *mdx* cardiac muscle (101), unmodified AOs are largely unsuccessful at inducing exon skipping in the heart and they do not cross the blood brain barrier (56, 58). This is important given that cardiac complications are observed in up to 90% of DMD patients (102) and that 1/3 of DMD patients suffer cognitive impairment related to the deficiency of dystrophin in the brain (103, 104). One approach to improve AO targeting to cardiac muscle is the direct conjugation of cell penetrating peptides to AOs which improves AO delivery to skeletal (105-112) and cardiac *mdx* mouse muscles (111, 113-115); however the

toxicology of these conjugates has yet to be ascertained. The fact that the dystrophin protein is thought to have a long half-life should increase the possibility of achieving and maintaining therapeutically-relevant dystrophin protein levels with weekly or longer dosing intervals

Regulatory hurdles

The regulatory process for developing AOs to skip other dystrophin exons is at present cumbersome as each new AO is considered a novel drug and requires the full battery of genotoxicity, rodent and non-human primate acute and chronic toxicity studies (reviewed in (116)). This stringent assessment of safety is of paramount importance, considering that there has been little experience in dosing individuals with AOs at high doses and for durations exceeding 1 year (and theoretically for a lifelong therapy). Nevertheless, the current studies have not reported severe drug related adverse events; in addition most of the toxicity related to AOs derives not from the individual sequences but from the chronic chemical load which is therefore largely backbone but not sequence specific. It is hoped that the positive clinical experience gained from the exon 51 skipping studies and hopefully also from other exons will allow us to gather additional information so that in the future these compounds could obtain class approval and follow a more informed and streamlined regulatory process.

Conclusions

Preclinical and clinical studies using two different chemistries have demonstrated the potential of antisense oligonucleotide-mediated *DMD* exon skipping to modify the progression of DMD. If progress in this field continues at the pace of the last decade, treatment for common DMD mutations may soon be feasible. If no sequence-specific toxic effect is found, treatment of rare mutations could follow as regulatory hurdles are overcome. Furthermore this approach could treat nonsense mutations or other frame-shifting mutations located in in-frame exons that could be removed by skipping a single exon. The clinical development of next generation AOs that effectively target cardiac as well as skeletal muscle will provide a significant quality of life improvement for patients.

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