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Personalized exon skipping strategies to address clustered non-deletion dystrophin mutations

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Abstract

Antisense oligomer induced exon skipping is showing promise as a therapy to reduce the severity of Duchenne Muscular Dystrophy. To date, the focus has been on excluding single exons flanking frame-shifting deletions in the dystrophin gene, however, a third of all Duchenne Muscular Dystrophy causing mutations are more subtle DNA changes. Forty-five dystrophin exons are potentially frame-shifting and mutations in these will require the targeted removal of exon blocks to generate in-frame transcripts. We report that clustered non-deletion mutations in the dystrophin gene respond differently to different antisense oligomer preparations targeting the same dual exon block, the removal of which bypasses the mutation and restores the open reading-frame. The personalized nature of the responses to antisense oligomer application presents additional challenges to the induction of multi-exon skipping with a single oligomer preparation.

Keywords

Duchenne muscular dystrophy; exon skipping; antisense oligomers; non-deletion mutations; personalized genetic therapy; splice-switching

Introduction

Antisense oligomer (AO) induced exon skipping is being evaluated as a therapy to reduce the severity of Duchenne Muscular Dystrophy (DMD) and has rapidly progressed from *in vitro* and preclinical animal studies to completion of proof of concept in human clinical trials [1,2]. Given the incidence and severity of DMD, affecting approximately 1:3500 live male births [3], it is important to develop therapies that are directly translatable to improve muscle function and quality of life for those affected by DMD [4].

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This work was performed in Perth, Western Australia

DMD is caused by mutations in the dystrophin gene that result in the absence of functional dystrophin, most commonly through premature protein truncation [5-7]. Dystrophin is an integral member of the dystrophin-glycoprotein complex, linking cytoskeletal actin to the extracellular matrix [8-10]. In the absence of functional dystrophin, the mechanical link is lost and sarcolemmal integrity is compromised, leading to muscle wasting and premature death as a result of respiratory or cardiac failure before the third decade of life [11,12].

Becker muscular dystrophy (BMD) is caused by in-frame mutations in the dystrophin gene producing an internally deleted but partially functional protein [13,14]. BMD patients present with a wide spectrum of disease severity, ranging from severe BMD patients in whom ambulation is lost in the late teens or early 20s, to patients essentially asymptomatic, in whom the only marker of the genetic defect is persistently elevated serum creatine kinase levels, depending upon the nature and location of the dystrophin mutation [6,11,14-16]. Individuals lacking up to 67% of the central rod domain have been described with a mild BMD phenotype [17,18], indicating that certain dystrophin domains are not essential for near-normal function [17,19,20]. It has been estimated that selected exon exclusion to bypass protein truncating dystrophin mutations is a potential therapy for up to 80% of DMD cases, however, this will inevitably require one or more AOs for each target exon, and will be a very personalized therapy [4].

Much effort to date has focused on the application of AOs targeting single exons [21,22] flanking deletions in the dystrophin gene, particularly in the two deletion hotspots [23-25]. Excision of exon 51 could be of benefit to 13% of all DMD patients [26,27] and by only 12 mono-skipping events it would be possible to convert approximately 75% of the DMD mutations to their closest BMD counterpart [28,29].

However, a third of all DMD mutations are more subtle DNA changes such as duplications, nonsense mutations, splice defects and small intra-exonic indels spread across the entire dystrophin gene. These will require targeting of the mutation-bearing exons, often in addition to normal flanking exons, in order to remove the mutation and maintain the reading frame [30].

We have developed and optimized a series of AOs targeting exons 2 to 78 of the normal dystrophin gene transcript [22]. Forty-five dystrophin exons are potentially frame-shifting when deleted and intra-exonic mutations in these will require the targeted removal of exon blocks to generate in-frame transcripts [28]. Furthermore, multi-exon skipping can potentially increase the scope of each AO therapy by using a limited number of oligomer preparations to address all dystrophin mutations that do not occur in functionally critical domains [22,28]. It is anticipated that clustered deletions will respond similarly when targeted by an AO optimized to the normal dystrophin transcript. However, it has not been established whether the same oligomer will be as effective when directed to a mutated exon, where the primary gene lesion may influence splicing or secondary structure.

Here, we by-pass the protein-truncating dystrophin gene lesions in cells from three “non-deletion” DMD patients. Four individual oligomers, 2 non-overlapping compounds directed at exon 17, and 2 partially overlapping oligomers targeting exon 18 had been developed and optimized to excise each of the target exons. In the cases described here, the mutation could only be by-passed using oligomer cocktails that induce removal of exons 17 and 18. We show that four different oligomer cocktails, that were optimized against the normal dystrophin gene transcript and induce similar levels of exon 17 and 18 skipping in wild-type cells, show disparate efficiencies in the patient cells. It would appear that these clustered but distinct gene lesions subtly influence dystrophin pre-mRNA processing and consequently the efficiency of targeted exon skipping. Treating a cluster of DMD-causing gene defects

with a single oligomer preparation may not be ideal and optimization for individual mutations is indicated.

Methods

AO design and synthesis

Oligomers consisting of 2'-O-methyl modified bases on a phosphorothioate backbone were designed to excise human dystrophin exons 17 and 18, and synthesized on an Expedite 8909 synthesizer, as described by Adams *et al.* [31]. Antisense oligomer sequences, nomenclature and cocktail composition are shown in Table 1.

Cell Culture and Transfection

Normal human myoblasts were prepared from de-identified muscle biopsies, obtained during elective surgery at the Department of Neuropathology at Royal Perth Hospital, with informed consent, essentially as described by Rando *et al.* [33]. DMD patient cells, donated after informed consent, were prepared at the University College of London Institute of Child Health and sent to our laboratory with the approval of the host institution. The use of human tissue was approved by the University of Western Australia Human Ethics Committee (approval number RA/4/1/2295).

Myogenic cells from normal and DMD muscle biopsies were proliferated and differentiated as described previously by Harding *et al.* [34]. Lipoplexes of Lipofectamine 2000® and AO were transfected (1:1w/w) in serum-free media, opti-MEM (Invitrogen, Melbourne Australia). The oligomer cocktails consisted of equimolar amounts of both AOs directed to exons 17 and 18, and concentrations indicated represent the total AO concentration, eg 200 nM contains 100 nM of each AO.

RNA extraction and nested RT-PCR

Total RNA was harvested 24 h after transfection from duplicate wells using TRIzol® (Invitrogen) according to the manufacturer's instructions, and resuspended in 30 µL of RNase free water. Approximately 50 ng of total RNA was used as template for primary amplification using Superscript® III One-step RT-PCR system with Platinum Taq (Invitrogen) to amplify exons 12 to 20. After 35 cycles a 1 µL aliquot was removed and subjected to nested PCR to amplify exons 13 to 19 for 30 cycles, using AmpliTaq Gold (Applied Biosystems, Melbourne, Australia). Details of amplification primers (Geneworks, Hindmarsh, South Australia) used to amplify the dystrophin gene transcript are shown in Table 2.

Gel Analysis, Imaging and Sequencing

PCR products were resolved on 2% agarose gels in TAE buffer and relative exon skipping efficiency was estimated by densitometry of the full length and induced shortened PCR products on images captured by the Chemi-Smart 3000 system (Vilber Lourmat, Marne-La Vallee, France) as described previously [31]. The identity of induced transcripts was confirmed by band stab [35] and sequencing using BigDye terminator chemistry (Applied Biosystems).

Results

The nested amplification conditions described here are only qualitative, but reflect amounts of the AO induced skipped transcripts, relative to the full-length product, in a dose dependant manner. Nested amplification was necessary because the myogenicity of the patient cultures was low, with less than 5% of cells staining positive for desmin (data not

shown). There was sufficient dystrophin expressed in the wild-type treated and untreated myogenic cells to allow densitometric analysis of the fractionated primary PCR products, but nested PCR was required to generate consistent products from the patient cultures (data not shown).

RT-PCR analysis of dystrophin transcripts shows the variable efficiency of exon 17 and 18 excision from the dystrophin gene transcripts expressed in normal and DMD patient myogenic cells. While all AO cocktails resulted in similar levels of exon skipping during expression of the normal dystrophin transcript (Figure 1a), these oligomer combinations elicited different responses in cells from the various DMD patients (Figure 1b-d). Transfection and transcript analysis was repeated ($n=3$) to obtain the average titration profiles shown in Figure 2. Densitometry analysis was repeated 3 times and was highly reproducible, thus providing further validation of the technique (data not shown).

In cells from the patient with exon 17 nonsense mutation, all cocktails induced substantial exon 17 and 18 skipping at the high transfection concentration, with cocktails 3 and 4 inducing the most pronounced response at the lower transfection concentrations (Figure 2b). In cells from the patient with a 20 base deletion in exon 18 (Figure 2c), cocktails 1 and 2 show similar dose response trends to those observed in transfected normal human myoblasts (Figure 2a). Conversely, cocktails 3 and 4, which included a different AO targeting exon 17, were less effective in cells from this patient, and only approximately 40% exon skipping was induced at the highest transfection concentration (Figure 2c). When targeting the exon 18 duplication, all four cocktails induced substantial exon 17 and 18 excision, with cocktail 4 inducing the most efficient exon skipping, most evident at the lower AO transfection concentrations (Figure 2d).

The nonsense mutation and 20 base deletion do not occur within the AO annealing regions, and while AO binding should not be directly affected, it is possible that dystrophin pre-mRNA splice motifs or secondary structures are altered, which in turn influences oligomer efficiency. The splice motif predictor programs, ESE finder [36], and Human Splicing Finder [ref] were used to predict putative exonic splicing enhancers (ESEs), and a comparison of normal dystrophin exonic sequence for exon 17 and 18 with those in the DMD patients showed that some SR binding sites are altered or lost as a consequence of the nonsense mutation in exon 17 and the 20 base deletion in exon 18 (Figure 3). Both ESE Finder and Human Splicing Finder indicated the exon 17 nonsense mutation ablated an SF2/ASF SR binding motif, while creating a SRp55 binding motif (Figure 3b). Both these programs were consistent in suggesting the 20 base deletion in exon 18 removed a cluster of ESEs upstream from the donor splice site (Figure 3c and d).

Dystrophin sequences for exons 17 and 18 were imported into M-fold [37] along with 50 bases of flanking intronic sequence to predict secondary structures of the wild-type and DMD pre-mRNAs (Figure 4). The nonsense mutation in exon 17 was predicted to occur at the base of an open circle in the wild-type sequence, and this change induced potential loop-outs, neither of which altered the structure of the AO binding sites in this configuration. However, the titration studies show that cocktails containing H17A(+61+86), which is closest to the nonsense mutation performed better than cocktails containing the AO targeting a sequence further from the mutation.

The M-fold [37] prediction of differences in exon 18 structure, as a result of the 20 base deletion, also shows no direct impact on the AO binding sites (Figure 4c and d). Although this deletion was predicted to remove a cluster of ESEs (Figure 3c and d), this loss had minimal influence on the activity of the two oligomers directed at exon 18. Remarkably, this

small deletion appeared to influence the efficiency of oligomers targeting exon 17, indicating unforeseen complexities in induced exon skipping.

Discussion

Exon skipping as a therapy for DMD has focused on excluding single exons in the mutation-prone deletion hotspots [1,38,39], although some deletions will need more than one exon targeted to restore the reading frame. However, at least one third of all DMD mutations are not whole exon deletions [24], and about one half of these will require removal of at least 2 exons to by-pass the gene lesion and produce an in-frame transcript [26,28,40]. Consequently, the development of combinations of AOs to excise in-frame exon blocks could address clustered dystrophin mutations and reduce the number of oligomer cocktails needed in the clinic. It has been proposed that multi-exon skipping across exons 45-55 would address approximately two thirds of all DMD mutations [41]. Removing a large exon block is technically challenging at this time, although we have been able to skip 7 consecutive exons from the mouse dystrophin pre-mRNA [42]. Specific multi-exon was induced, but the overall efficiency was poor, as only low levels of the in-frame shortened product was generated. Others have reported similar shortcomings in the induced excision of large exon blocks [43]. Nevertheless, we show here that 2 or 3 exons can be removed at high efficiency from dystrophin gene transcripts.

This is the first report of clustered, non-deletion mutations being treated using a single splice intervention strategy, that is removal of exons 17 and 18. Here, we report that three clustered mutations were targeted with four different oligomer cocktails, to remove exons 17 and 18, which were optimized against the normal human dystrophin transcript. The oligomers in these cocktails were optimized individually against the normal dystrophin gene transcript, and as such, induced out-of frame transcripts, which should be subjected to nonsense mediated decay (NMD).

However, when applied to the wild type dystrophin pre-mRNA, each of the cocktails excised both target exons with similar efficiency, and the induced in-frame transcripts would not be susceptible to NMD. When the cocktails were evaluated in cells from the different DMD patients, we found generally higher levels of induced exon skipping, presumably due to the induced in-frame transcripts escaping NMD, but nevertheless reproducible differences in exon skipping efficiency were apparent. It appears that mutations within exons alter the recognition of splicing motifs and/or secondary structure of the exon and thus influence the response to AOs. *In silico* analysis indicates that putative SR binding sites are altered or deleted by two of the mutations discussed here, which may account for the varying efficiencies of the AO cocktails.

The processing of a pre-mRNA requires recognition of various enhancer and silencer elements, in addition to the consensus splice motifs at the branch-point, acceptor and donor splice sites (for review see [44,45]). Clearly splicing requires a delicate balance of cis and trans interactions and some changes may have unexpected consequences. There are natural precedents where some dystrophin single base changes, in some cases nonsense mutations, alter exon recognition sites and promote exon skipping [46-49]. Similarly, a high proportion of oligomers designed to induce dystrophin exon skipping have been shown to exert some effect, albeit at variable efficiencies [21,22].

Although the nonsense mutation in exon 17 did not appear to substantially alter the removal of the target exons after transfection at high concentrations, it appears that this bases change influences the response to the cocktails, so that those preparations containing the exon 17 oligomer annealing closer to the mutation were more effective, independent of the exon 18

oligomer used. *In silico* ESE mapping and RNA folding failed to reveal any obvious structural correlations that may account for the variation in cocktail efficiencies.

While it was reasonable that a mutation in exon 17 that ablated two potential SF2/ASF binding motifs and created a SRp55 motif would alter oligomer targeting of that exon, we did not anticipate that a 20 base deletion in exon 18 would influence AO targeting and excision of the preceding exon. Remarkably, the loss of a cluster of putative ESEs from exon 18 did not seem to influence the activity of either exon 18 oligomer, whereas this deletion resulted in oligomer annealing closest to the exon 17 acceptor site being in the most efficient oligomer cocktails for exon 17 and 18 skipping. A recent report focused on five patients with nonsense or frameshifting mutations also demonstrated that in some cases, skipping efficiencies for mutated exons were quite dissimilar when compared with the respective wild-type exons [50].

The exon skipping profiles in the cells containing the exon 18 duplication resembled those seen with cocktail transfection in normal myogenic cells, although cocktail 4 appeared more efficient as determined by exon skipping levels induced after transfection at lower concentrations. It is not surprising that the profiles generated were similar between this duplication and normal cells, as normal exons 17 and 18 are targeted in both cases. The duplication of exon 18 will result in the first exon 18 donor site being followed by an exon 18 acceptor site, rather than the normal exon 19. However, in this case it appears although the exon 18 donor: acceptor was out of context at the duplication junction, this arrangement did not appear to affect oligomer activity.

Although we cannot be certain that inter-patient variation in exon skipping across the dystrophin gene be common, other studies have already indicated inter-patient variation [50]. In the first proof-of-concept study using a direct injection of a phosphorodiamidate morpholino oligomer to excise exon 51 from the dystrophin gene transcript, the mean random intensity measurements of dystrophin positive fibres varied from 22% to 32% in the five patients receiving the high dose [Kinali].

In summary, we have demonstrated that some intra-exonic dystrophin gene mutations that lead to DMD can influence the ability of oligomers, designed against the normal dystrophin gene transcript, to excise target exons. We propose that demonstrating accurate dose responses to induced exon skipping will allow discrimination of the more efficacious oligomer preparations. Clearly, an oligomer, or oligomer combination, capable of inducing robust (greater than 50% *in vitro*) exon skipping after transfection at 25nM will be more appropriate for clinical application than preparations that show equivalent levels of exon skipping at higher concentrations. This study does not indicate any obvious parameters for identifying the best oligomer preparation for a particular mutation in the target exons. It would be of interest to establish how these oligomer cocktails perform in cells from DMD patients carrying deletions of either exon 17 or 18, but we do not currently have access to these cell cultures.

We have long maintained that for targeted exon skipping to become widely available and effective to all amenable mutations, it should be developed as a personalized medicine, although the use of oligomer cocktails targeting in-frame exon blocks may reduce the number of preparations ultimately used to address a range of different mutations. While cocktail 4 appeared most effective in restoring the dystrophin reading frame in cells with an exon 17 nonsense mutation and an exon 18 duplication, this same preparation performed poorly in cells with an exon 18 twenty base deletion. It will have to be determined if it is better to develop one preparation for appropriately clustered mutations, or if exon skipping should be a truly personalized therapy and preparations are optimized for each patient.

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Figure 1.

Representative (n=3) RT-PCR analysis of dystrophin transcripts expressed in wild-type myogenic cells (a) and cells from DMD patients with (b) exon 17 nonsense mutation, (c) 20 base intra-exonic deletion in exon 18 and (d) exon 18 duplication after transfection with AO cocktails (Table 1), designed to induce exon 17 and 18 exon skipping. L2K: treated with Lipofectamine 2000 (Invitrogen) only; UT: untreated; -ve: no template control. The full-length product amplified from the normal transcript is 799 bp, and the product missing exons 17 and 18 is 499 bp.

Figure 2.

Relative exon skipping expressed as a percentage of total transcript product, determined by densitometry of fractionated PCR products from three independent transfections of wild-type myogenic cells (a) and cells from DMD patients with (b) exon 17 nonsense mutation, (c) 20 base intra-exonic deletion in exon 18 and (d) exon 18 duplication.

Figure 3.

SR binding sites for (a) normal human dystrophin exon 17, (b) exon 17 containing a nonsense mutation, (c) normal human dystrophin exon 18 and (d) exon 18 carrying a 20 base deletion, as predicted by ESE finder 3.0. (Red - SF2/ASF, pink - SF2/ASF (igM-BRCA1), blue - SC35, green - SRp40 and yellow - SRp55). Oligomer binding sites are indicated below the X-axis and the mutation details are shown.

Figure 4. Secondary structure of (a) normal and (b) mutated dystrophin exons 17, and (c) normal and (d) mutated exons 18, with 50 bases of flanking intronic sequence as predicted by M-Fold [37]. The locations of the oligomer annealing sites and mutations are indicated.

Table 1

AO sequences, annealing coordinates and cocktail composition. Each cocktail consists of equimolar amounts of the individual oligomers. Oligomer nomenclature is based upon annealing position in the pre-mRNA, as described by Mann *et al.*, 2002 [32].

Sequences shown 5'-3'	H17A(+10+35) AGUGAUGGCUGAGUGGUGGUGACAGC	H17A(+61+86) UGUCCCCUUGUGGUCACCGUAGUUAC
H18A(+24+53) CAGCUUCUGAGCGAGUAAUCCAGCUGUGAA	Cocktail 1	Cocktail 3
H18A(+31+61) CUGCAACACAGCUUCUGAGCGAGUAAUCCAG	Cocktail 2	Cocktail 4

Table 2

Sequences of amplification primers used to study the dystrophin transcript.

Nomenclature	Sequence (5'-3')
HDysEx12F	TAATGGATCTCCAGAATCAG
HDys20R	CAGTTAAGTCTCTCACTTAGC
HDysEx13F	CACGCAACTGCTGCTTTGGAAG
HDysEx19R	ACGTTCCACCAGGGCCTGAG
HGAPDH9F	ATGCTGGCGCTGAGTACGT
HGAPDH9R	TTCACCCCATGACGAACATG
HDysEx27F	GGCTGAATGGGAAATGCAA
HDysEx28R	AAGTATGACAATAACTCATGCCAACAT