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Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human *DMD* gene: Implications for future clinical trials

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ABSTRACT

Duchenne muscular dystrophy (DMD) is caused by the lack of functional dystrophin protein, most commonly as a result of a range of out-of-frame mutations in the *DMD* gene. Modulation of pre-mRNA splicing with antisense oligonucleotides (AOs) to restore the reading frame has been demonstrated in vitro and in vivo, such that truncated but functional dystrophin is expressed. AO-induced skipping of exon 51 of the *DMD* gene, which could treat 13% of DMD patients, has now progressed to clinical trials. We describe here the methodical, cooperative comparison, in vitro (in DMD cells) and in vivo (in a transgenic mouse expressing human dystrophin), of 24 AOs of the phosphorodiamidate morpholino oligomer (PMO) chemistry designed to target exon 53 of the *DMD* gene, skipping of which could be potentially applicable to 8% of patients. A number of the PMOs tested should be considered worthy of development for clinical trial.

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle-wasting disease, affecting 1:3500 live male births, caused by the lack of functional dystrophin protein in skeletal muscles, as a result of frame-disrupting deletions or duplications or, less commonly, nonsense or missense mutations in the *DMD* gene [1]. Mutations that maintain the reading frame of the gene and allow expression of semi-functional, but internally-deleted dystrophin are generally associated with the less severe Becker muscular dystrophy (BMD) [1,2].

Transforming an out-of-frame DMD mutation into its in-frame BMD counterpart with antisense oligonucleotides (AOs) is the basis of the potentially exciting exon skipping therapy for DMD (reviewed by Muntoni and Wells) [3]. The hybridization of AOs to specific RNA sequence motifs prevents assembly of the spliceosome, so that it is unable to recognise the target exon(s) in the pre-mRNA and include them in the mature gene transcript [4,5]. AOs have been used to induce skipping of specific exons such that the reading frame is restored and truncated dystrophin expressed in vitro in DMD patient cells [6,5,7–9], and in animal models of the disease in vivo [4,10–13].

Initial proof-of-principle clinical trials, using two different AO chemistries (phosphorothioate-linked 2′-*O*-methyl modified bases (2′OMePS) [14] and phosphorodiamidate morpholino oligomer (PMO) [15]) for the targeted skipping of exon 51 of the *DMD* gene after intramuscular injection, have been performed recently with encouraging results. While both chemistries have excellent safety profiles [16,17], PMOs appear to produce more consistent and sustained exon skipping in the *mdx* mouse model of DMD [18–20], in human muscle explants [21], and dystrophic canine muscle cells in vitro [22]. However, for some human exons, 2′OMePS and PMO AONs performed equally well [17]. Since the mutations that cause DMD are so diverse, of those DMD patients with genomic deletions, skipping of exon 51 would have the potential to treat only 13% of such patients on the Leiden DMD database [23], and 15% of such patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO). Although any predictions on the frequency of mutations and percentage of skippable patients should be viewed with caution, it is undeniable that the continued development and analysis of AOs for the targeting of other DMD exons is vital.

Here we report the comparative analysis of a series of PMOs targeted to exon 53, skipping of which would have the potential to

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treat a further 8% of DMD patients with genomic deletions on the Leiden database [23], and a further 13.5% of patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO). PMOs designed and previously tested in normal human skeletal muscle cells (hSkMCs) for the targeting of exon 53 [24] were further studied here in cells from a DMD patient with a relevant deletion (del 45–52). These PMOs were directly compared to a PMO based on a AO previously identified as being the most bioactive by Wilton et al. [25]. Time-course studies were performed to evaluate the persistence of skipping and dose–responses were examined. Findings from these experiments were supported by in vivo studies in a mouse model transgenic for the entire human dystrophin locus [12]. Collectively, this work reports a number of PMOs able to produce targeted skipping of exon 53 to levels that would suggest them worthy of consideration for upcoming PMO clinical trials.

2. Materials and methods

2.1. AO design

All AOs were synthesized as phosphorodiamidate morpholino oligomers (PMOs) by Gene Tools LLC (Philomath OR, USA).

2.2. DMD patient primary myoblast culture

Skeletal muscle biopsy samples were taken from a diagnostic biopsy of the quadriceps from a DMD patient with a deletion of exons 45–52. Informed consent was obtained before any processing of samples, and all work was carried out with the approval of the institutional ethics committee. Muscle precursor cells were prepared from the biopsy sample by sharp dissection into 1 mm^3 pieces and disaggregated in solution containing HEPES (7.2 mg/ml), NaCl (7.6 mg/ml), KCl (0.224 mg/ml), glucose (2 mg/ml), Phenol red (1.1 μg/ml), 0.05% Trypsin–0.02% EDTA (Invitrogen, Paisley, UK) in distilled water, three times at 37 °C for 15 min in Wheaton flasks with vigorous stirring. Isolated cells were plated in non-coated plastic flasks and cultured in Skeletal Muscle Growth Media (Promocell, Heidelberg, Germany) supplemented with 10% Foetal Bovine Serum (PAA Laboratories, Yeovil, UK), 4 mM L-glutamine and 5 μg/ml gentamycin (Sigma–Aldrich, Poole, UK) at 37 °C in 5% CO_2.

2.3. Nucleofection of DMD primary myoblasts

Between 2×10^5 and 1×10^6 cells/ml were pelleted and resuspended in 100 μl of solution V (Amaxa Biosystems, Cologne, Germany). The appropriate PMO to skip exon 53 was added to the cuvette provided, sufficient to give the concentrations described, followed by the cell suspension, and nucleofected using the Amaxa nucleofector 2, program B32. Five hundred microliters of medium was added to the cuvette immediately following nucleofection [26]. This suspension was transferred to a 6 well plate in differentiation medium. Nucleofected cells were maintained in differentiation media for 3–21 days post treatment before extraction of RNA or protein. Transfections were performed blindly and in each experiment in triplicate. Each experiment was repeated at least once to ensure reproducibility of results.

2.4. Lactate dehydrogenase cytotoxicity assay

A sample of medium was taken 24 h post-transfection to assess cytotoxicity by release of lactate dehydrogenase (LDH) into the medium, using the LDH Cytotoxicity Detection Kit (Roche, Burgess Hill, UK), following the manufacturer's instructions. The mean of three readings for each sample was recorded, with medium only, untreated and dead controls. The readings were normalised for background (minus medium only) and percentage toxicity expressed as [(sample-untreated)/(dead-untreated) × 100].

2.5. Transgenic human DMD mice

A transgenic mouse expressing a complete copy of the human *DMD* gene has been generated [12,27]. Experiments were performed at the Leiden University Medical Center, with the authorization of the Animal Experimental Commission (UDEC) of the Medical Faculty of Leiden University as described previously [9]. Twenty micrograms of each PMO was injected once into two gastrocnemius muscles, pretreated with cardiotoxin. Mice were sacrificed 1 week after the injection, and RNA harvested from the isolated gastrocnemius muscles and analysed by RT-PCR.

2.6. RNA isolation and reverse transcription-polymerase chain reaction analysis

RNA was isolated and analysed by RT-PCR, as described previously [9]. Primer sequences and detailed PCR protocols are available on request. PCR products were analysed on 1.5% (w/v) agarose gels in Tris–borate/EDTA buffer. Skipping efficiencies were determined from gel images by comparing induced shortened dystrophin mRNAs to the intact transcript of the full length using densitometric analysis with Image J software (for patient samples) or by quantifying the skipped products with DNA 1000 LabChip Kit on the Agilent 2100 bioanalyzer (Agilent Technologies, USA) (for hDMD mouse samples). Skipping percentages were calculated as the amount of skip transcripts relative to the total transcripts (skip and full length). Equal amounts of the induced and intact transcripts would be regarded as representing 50% efficiency, while an estimate of 25% exon skipping would be represented by the intact transcript being three times more abundant than the band representing the induced transcript. Likewise, if the induced transcript was present at three times the level of the intact transcript, the exon skipping efficiency would be assessed to be 75%. Where appropriate, the two-tailed student's *t*-test was used to determine the statistical strength of the skipping efficiencies produced.

2.7. Sequence analysis

RT-PCR products were excised from agarose gels and extracted with a QIAquick gel extraction kit (Qiagen, Crawley, UK). Direct DNA sequencing was carried out by the MRC Genomics Core Facility.

2.8. Western blot analysis of dystrophin protein

DMD patient cells, transfected as described and cultured in differentiation medium, were harvested 7, 14 or 21 days post-transfection. Cells (4×10^5) were pelleted and resuspended in 50 μl of loading buffer (75 mM Tris–HCl pH 6.8, 15% sodium dodecyl sulphate, 5% ß-mercaptoethanol, 2% glycerol, 0.5% bromophenol blue and complete mini protease inhibitor tablet). Samples were incubated at 95 °C for 5 min and centrifuged at 18,000*g* for 5 min. Twenty microliters of sample was loaded per well in a 6% polyacrylamide gel with 4% stacking gel. Protein from CHQ5B cells differentiated for 7 days was used as a positive control for dystrophin. Gels were electrophoresed for 5 h at 100 V before blotting on nitrocellulose membrane at 200 mA overnight on ice. Blots were stained with protogold to assess protein loading, then blocked in 10% non-fat milk in PBS with 2% Tween (PBST) for 3 h. Blots were probed with antibodies to dystrophin, NCL-DYS1 (Vector Labs, Peterborough, UK) diluted 1:40 and to dysferlin, Hamlet1 (Vector Labs)