Comparative Analysis of Antisense Oligonucleotide Sequences for Targeted Skipping of Exon 51 During Dystrophin Pre-mRNA Splicing in Human Muscle

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ABSTRACT

Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene that result in the absence of functional protein. In the majority of cases these are out-of-frame deletions that disrupt the reading frame. Several attempts have been made to restore the dystrophin mRNA reading frame by modulation of pre-mRNA splicing with antisense oligonucleotides (AOs), demonstrating success in cultured cells, muscle explants, and animal models. We are preparing for a phase I/IIa clinical trial aimed at assessing the safety and effect of locally administered AOs designed to inhibit inclusion of exon 51 into the mature mRNA by the splicing machinery, a process known as exon skipping. Here, we describe a series of systematic experiments to validate the sequence and chemistry of the exon 51 AO reagent selected to go forward into the clinical trial planned in the United Kingdom. Eight specific AO sequences targeting exon 51 were tested in two different chemical forms and in three different preclinical models: cultured human muscle cells and explants (wild type and DMD), and local in vivo administration in transgenic mice harboring the entire human DMD locus. Data have been validated independently in the different model systems used, and the studies describe a rational collaborative path for the preclinical selection of AOs for evaluation in future clinical trials.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is caused by nonsense or frame-shifting mutations in the DMD gene, which results in nonfunctional dystrophin proteins (Hoffman et al., 1987). At the same time, interstitial mutations at the DMD locus that maintain the dystrophin mRNA open reading frame give rise to internally deleted but semifunctional dystrophins and the milder Becker muscular dystrophy (BMD) (Hoffman et al., 1987; Monaco et al., 1988). Internally truncated but at least partially functional dystrophins are also expressed in so-called revertant fibers, individual dystrophin-positive fibers found in 50% of DMD patients and in mdx mice (Nicholson et al., 1989; Hoffman et al., 1990; Burrow et al., 1991; Fanin et al., 1992; Sherratt et al., 1993; Yokota et al., 2006). Revertant fibers arise via some alternative splicing mechanism occurring within dystrophin pre-mRNAs, skipping of frame-shifting exons to remove protein-truncating mutations, and restoration of the dystrophin open reading frame (Lu et al., 2000). These revertant dystrophins lack exon domains flanking the gene lesion in DMD patients (Fanin et al., 1995; Thanh et al., 1995) and the mutated exon 23 in the mdx mouse (Lu et al., 2000). Despite being internally truncated, dystrophin molecules found in BMD patients can be functional, as demonstrated by several families with in-frame deletions in the DMD gene, associated with elevated serum creatine kinase but displaying no clinical myopathy (e.g., deletions of exons 32–44, 48–51, or 48–53 [Melis et al., 1998], exon 48 [Morrone et al., 1997], exons 48–51 or 50–53 [Beggs et al., 1991], exons 45–55 [Beroud et al., 2007], or exons 50–51 [Lesca et al., 2007]). The efficacy of internally

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truncated dystrophins lacking an appreciable portion of the rod domain has also been demonstrated in transgenic mdx mice, and exploited to design so-called microdystrophins compatible with delivery by AAV vectors. These engineered recombinant microdystrophins have been shown to restore normal expression of the dystrophin-associated protein complex (DPC), improve sarcolemmal stability, and prevent myofiber degeneration in the mdx mouse model (Wang et al., 2000; Fabb et al., 2002; Harper et al., 2002; Gregorevic et al., 2006).

The first suggestion that a functional, truncated dystrophin molecule could be created in DMD patients originally came from Takeshima et al. after skipping exon 19 in vitro in control cells (Takeshima et al., 1995; Pramono et al., 1996). Shortly after, restoration of the reading frame in dystrophic cells was shown by Dunckley et al. (1998). These authors, and others immediately afterward, proposed the use of antisense oligonucleotides to modulate dystrophin mRNA splicing to enlarge out-of-frame DMD mutations into the nearest in-frame BMD-like mutation and produce an internally deleted functional dystrophin protein (Dunckley et al., 1998; Wilton et al., 1999; Takeshima et al., 2001; van Deutekom et al., 2001).

The mechanism of antisense oligonucleotide (AO) modulation of dystrophin pre-mRNA splicing involves hybridization to specific motifs involved in splicing and exon recognition in the pre-mRNA. This prevents normal spliceosome assembly and results in the failure of the splicing machinery to recognize and include the target exon(s) in the mature gene transcript (Mann et al., 2001; Aartsma-Rus et al., 2003).

In this way one or more exons and their flanking introns are removed during splicing of the pre-mRNA. In the case of the dystrophin gene deletions, selective removal of specific flanking exons should result in in-frame mRNA transcripts that may be translated into an internally deleted, BMD-like and functionally active dystrophin protein with predictable therapeutic activity (Wilton et al., 1999; van Deutekom et al., 2001).

In DMD research, the potential clinical use of AOs has evolved from studies in vitro on cultured mdx mouse muscle cells (Dunckley et al., 1998; Mann et al., 2001, 2002) and human DMD muscle cells (van Deutekom et al., 2001; Aartsma-Rus et al., 2002, 2003, 2004a) to in vivo studies in mdx and GrMD (golden retriever muscular dystrophy) animal models (Lu et al., 2003, 2005; Fletcher et al., 2006; McClurey et al., 2006) (Table 1). Direct in vivo AO-induced exon skipping in humans, however, has yet to be demonstrated and this proof of principle, along with clinical safety, is the crucial aspect of initial clinical studies. Preparations have been reported for two parallel clinical trials of AO therapeutics in DMD patients that will target dystrophin exon 51. Exclusion of exon 51 is predicted to allow the restoration of the dystrophin open reading frame (ORF) in ~17% of DMD deletion patients (deletions of exons 45–50, 47–50, 48–50, 49 and 50, 50, 52, and 52–63; van Deutekom and van Ommen, 2003). Although these two clinical trials will both target exon 51, each will evaluate different AO chemistries: 2′-O-methyl-modified ribose moieties on a phosphorothioate backbone (2-OMe AOs) (completed by Leiden University Medical Center [LUMC] and Prosensa [Leiden, The Netherlands]) (van Deutekom et al., 2007) and phosphorodiamidate morpholino oligomers (PMOs) (http://clinicaltrials.gov/ct/show/NCT00159250; in progress in the United Kingdom [Muntoni et al., 2005]).

MATERIALS AND METHODS

AO design

Eight different 2′-O-methyl AOs to human dystrophin exon 51 (A20, B30, and C20–H20) were designed on the basis of ES-Finder analysis (Smith et al., 2006), and relative to previously published sequences (Fig. 1A). Splice site AOs (C20 to H20) were designed on the basis of work performed in our laboratory on the mdx mouse, published previously (Graham et al., 2004). To avoid differences in synthesis conditions and concentrations all AOs were purchased from Eurogentec (Seraing, Belgium), diluted to the same concentration in water by the same operator, aliquoted, and stored at ~80°C. Three further AOs (A25, B30, and I25; Fig. 1A) were synthesized as phosphorodiamidate morpholino oligomers (PMOs) by Gene Tools (Philomath, OR). To facilitate introduction into cultured cells, the uncharged PMOs were hybridized to phosphorothioate-capped DNA leashes, based on a previous design (Gebski et al., 2003), and stored at 4°C.

Cell culture and AO transfection

Transfections of AOs were performed at two separate institutions, Imperial College (IC, London, UK) and Royal Holloway (RH, London, UK), using normal primary human skeletal muscle cells from different sources: human fetal muscle cells obtained from the Medical Research Council (MRC) Tissue Bank (London, UK) and human primary skeletal muscle cultures were
<table>
<thead>
<tr>
<th>Model</th>
<th>Tissue</th>
<th>Study</th>
<th>AO</th>
<th>Administration</th>
<th>Result</th>
</tr>
</thead>
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<td><em>mdx</em> mouse</td>
<td>Myoblast culture</td>
<td>Dunckley <em>et al.</em> (1998)</td>
<td>2-OMe</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining)</td>
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<td>Wilton <em>et al.</em> (1999)</td>
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<td>Mann <em>et al.</em> (2001, 2002)</td>
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<td></td>
<td>Myoblast culture</td>
<td>Gebski <em>et al.</em> (2003)</td>
<td>PMO plus leashes</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in Western blot</td>
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<td>Graham <em>et al.</em> (2004)</td>
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</tr>
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<td><em>In vivo</em></td>
<td>Myoblast culture</td>
<td>Mann <em>et al.</em> (2001)</td>
<td>2-OMe</td>
<td>Intramuscular</td>
<td>Exon skip (RT-PCR); dystrophin in muscle sections (immunostaining and Western blotting)</td>
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<td>PMO plus leashes</td>
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<td>Exon skip (RT-PCR); dystrophin in muscle sections (immunostaining)</td>
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<tr>
<td></td>
<td>In vivo</td>
<td>Graham <em>et al.</em> (2004)</td>
<td>2-OMe</td>
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<td>Exon skip (RT-PCR); dystrophin in muscle sections (immunostaining); dystrophin in Western blots</td>
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<tr>
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<td>2-OMe</td>
<td>Intramuscular</td>
<td>Dystrophin in muscle sections (immunostaining and Western blotting)</td>
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<td>Adjei <em>et al.</em> (2003)</td>
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<td>Systemic</td>
<td>Dystrophin in muscle sections (immunostaining); dystrophin in Western blots</td>
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<td>Gebski <em>et al.</em> (2005)</td>
<td>2-OMe and PMO</td>
<td>Systemic and intramuscular</td>
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<td>Aartsma-Rus <em>et al.</em> (2005)</td>
<td>2-OMe</td>
<td>Systemic</td>
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<td></td>
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<td>Exon skip (RT-PCR)</td>
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<td>Dystrophin in muscle sections and by Western blotting; body-wide functional levels of dystrophin</td>
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<tr>
<td>Model</td>
<td>Tissue</td>
<td>Study</td>
<td>Chemicals</td>
<td>Method</td>
<td>Transfection</td>
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<td>Canine model MD (GRMD)</td>
<td>Myoblast culture</td>
<td>McClorey <em>et al.</em> (2006b)</td>
<td>2-OMe</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in Western blot. 2-OMe chemistry was effective only for short-term induction of corrected transcript and could not induce detectable dystrophin protein.</td>
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<td>van Deutekom <em>et al.</em> (2001)</td>
<td>2-OMe</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining).</td>
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<td>de Angelis <em>et al.</em> (2002)</td>
<td>Retroviral construct</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in Western blot.</td>
</tr>
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<td>Aartsma-Rus <em>et al.</em> (2003)</td>
<td>2-OMe</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining); dystrophin in Western blot.</td>
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<td>Human</td>
<td>Myoblast culture</td>
<td>Aartsma-Rus <em>et al.</em> (2004a)</td>
<td>2-OMe</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining); dystrophin in Western blot.</td>
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<td>Aartsma-Rus <em>et al.</em> (2004b)</td>
<td>2-OMe, PMO, LNA</td>
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<td>Exon skip (RT-PCR); comparison of AO analogues.</td>
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<td>Myoblast culture</td>
<td>Surono <em>et al.</em> (2004)</td>
<td>2-OMe plus ENA</td>
<td>Transfection</td>
<td>Exon skip (RT-PCR); dystrophin in cultured cells (immunostaining).</td>
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<tr>
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<td>In vivo</td>
<td>Takeda <em>et al.</em> (2006)</td>
<td>2-OMe</td>
<td>Systemic</td>
<td>In one patient, exon skip after third injection (RT-PCR); faint dystrophin immunoreactivity in biopsy.</td>
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</table>

*Abbreviations:* 2-OMe, 2′-O-methyl; AO, antisense oligonucleotide; ENA, ethylene-bridged nucleic acid; GRMD, golden retriever muscular dystrophy; LNA, locked nucleic acid; PMO, phosphorodiamidate morpholino oligomer; PMO-Pep, peptide-linked PMO; RT-PCR, reverse transcription-polymerase chain reaction.
Explants were undertaken at concentrations of 100 and 300 nM. Dose–response correlations, whereas time course experiments ranging from 50 to 500 nM according to the manufacturer’s instructions. AO concentrations of the AOs diluted in a final volume of 500 μl of OptiMEM (Invitrogen) plus penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Forty-eight hours after the infusion, 500 μl of DMEM plus 5% horse serum was added to each well. Cells were cultured and transfected as described above. One week after transfection, cells were harvested (four wells per experiment) and washed with phosphate-buffered saline (PBS) and protease inhibitors. Samples were denatured at 95°C for 5 min before being loaded in 5% 2-mercaptoethanol, 2% glycerol, bromophenol blue, and protein extracts were isolated directly in 50 μl of loading buffer (75 nM Tris-HCl [pH 6.8], 15% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 2% glycerol, bromophenol blue, and protease inhibitors). Samples were denatured at 95°C for 5 min and centrifuged at 18,000 × g for 5 min before being loaded in a 6% polyacrylamide gel with a 4% stacking gel. Gels were electrophoresed for 4 hr at 100 V and blotted to a nitrocellulose membrane overnight at 200 mA. Blots were blocked for 1 hr with 10% nonfat milk in PBS–Tween (PBST) buffer and Western blot analysis of dystrophin protein

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dystrophin protein was detected by probing the membrane with NCL-DYS1 primary antibody (Vision BioSystems, Newcastle upon Tyne, UK) diluted 1:40 in 3% milk. A biotinylated secondary anti-mouse IgG antibody (diluted 1:1000; GE Healthcare, Little Chalfont, UK) and a streptavidin/horseradish peroxidase (HRP)-conjugated antibody (diluted 1:5000; Dako, Carpinteria, CA) allowed visualization in a luminol–HRP chemiluminescence reaction (ECL-Plus; GE Healthcare) on film (Hyperfilm; GE Healthcare) exposed at 20-sec intervals (20 sec to 2 min, with occasional longer exposures).

Transgenic human DMD mice

Work with transgenic human (h)DMD mice was performed at the LUMC, where a transgenic mouse expressing a complete copy of the human DMD gene was generated (Bremmer-Bout et al., 2004). The experiments were authorized by the Animal Experimental Commission (UDEC) of the Medical Faculty of Leiden University. Mice (approximately 5 weeks of age) were anesthetized by intraperitoneal injection of a 1:1 (v/v) solution of Hypnorm (fentanyl and fluanisone; Janssen Pharmaceutica, Berchem, Belgium) and Dormicum (midazolam; Roche, Mid drecht, The Netherlands). The muscles were pretreated to induce muscle degeneration and regeneration by injection of 3.6 nmol of each PMO per injection in a final volume of 40 μl, using a Hamilton syringe with a 22-gauge needle. Mice received two injections at 24-hr intervals and were then killed. This muscle was isolated and frozen in liquid nitrogen-cooled 2-methylbutane (Bremmer-Bout et al., 2004).

Results

Exon-internal 2-OMe AOs are more efficient than those targeting the 5’ splice site

AO sequences targeting exon 51 were designed using different strategies. One strategy, based on previous results obtained in mouse dystrophin exon 23 (Graham et al., 2004), was that of designing an overlapping stepped array of 2-OMe AOs complementary to the 5’ (donor) splice site of intron 51. The other strategies examined other potential splice motifs across exon 51, including the 3’ (acceptor) splice site and the branch point, as well as exon-internal sequences (Aartsma-Rus et al., 2004b; Wilton et al., 2007). After initial characterization in each partner laboratory (Table 2) (Aartsma-Rus et al., 2002, 2005; Harding et al., 2007), eight AOs, six targeting the 5’ splice site designed in the first partner laboratory (RH) (C20 to H20; Fig. 1A) plus two overlapping AOs (A20 and B30; Fig. 1A), designed independently in the other two partner laboratories (LUMC and the University of Western Australia [UWA], Perth, Australia), were then all compared in parallel in a fourth laboratory (IC), at a concentration of 100 nM on cultured differentiated human skeletal muscle cells. RNA from these cultures was analyzed blindly by RT-PCR, the results of which are shown in Fig. 1B. Whereas the AOs targeting the 5’ splice site (C20 to H20; Fig. 1A) were confirmed, surprisingly, as being largely ineffective at inducing skipping of exon 51 (Fig. 1B, lanes 2 to 7), those against the exon-internal sequences, AOs A20 and B30 (Fig. 1A), were capable of inducing exon 51 skipping at a level approaching 50% (Fig. 1B, lanes 1 and 8).

Table 2. Preliminary Comparison Between Candidate Antisense Oligonucleotides

<table>
<thead>
<tr>
<th>Institution</th>
<th>AO name</th>
<th>Sequence</th>
<th>Compared in this study</th>
<th>Chemistry</th>
</tr>
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<tr>
<td>Leiden University</td>
<td>h51AON1</td>
<td>UCAAGGAAGAUGGCAUUUCU</td>
<td>A20</td>
<td>20Me</td>
</tr>
<tr>
<td>Medical Center, The Netherlands</td>
<td>h51AON24</td>
<td>GAAAGCCAGUGGCAUUUCU</td>
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</tr>
<tr>
<td>Western University of Australia</td>
<td>H51A (+61+90)</td>
<td>ACCAGAAGAAGAUGGCAUUUCU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>H51A (+68+95)</td>
<td>CUCCAACCAUGGAAGAUGGCAUUU</td>
<td>B30</td>
<td>20Me/PMO</td>
</tr>
<tr>
<td>Royal Holloway University of London, United Kingdom</td>
<td>DMD51 (-12+8)</td>
<td>UAGUUGCUUCUCUACCUACUCU</td>
<td>C20</td>
<td>20Me</td>
</tr>
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</table>

*aUndertaken in the partner laboratories (Leiden University Medical Center, Leiden, The Netherlands: University of Western Australia, Perth, Australia; Royal Holloway-University of London, Egham, UK) (Aartsma-Rus et al., 2004b; Harding et al, 2007) and a selection was further confirmed in a fourth laboratory (Imperial College, London, UK).
Exon-internal 2-OMe AOs induce efficient and persistent exon 51 skipping

The two most efficient AOs (A20 and B30) were compared in terms of dose response in normal human skeletal muscle cells, to determine the concentration necessary to induce exon skipping, and in a time course study, to determine how long after transfection the effect lasted in culture. AO concentrations ranging from 50 to 400 nM were used, and the results shown in Fig. 2A demonstrate that skipping of exon 51 was achieved at the lowest dose used for both AOs, as judged by the size difference (212 bp) between the skipped and full-length bands. Precise exon 51 skipping was confirmed by direct sequencing of the lower band (Fig. 2C). Transfection of AOs A20 and B30 into normal human skeletal muscle cultures at concentrations of 100 and 300 nM, followed by harvesting of RNA at various time points, demonstrated that skipped products were still detectable by RT-PCR 10 days after transfection (Fig. 2B).

The same experiments were repeated in differentiated muscle cultures derived from DMD patients carrying deletions, which could be amenable to exon 51 skipping to restore the reading frame. Two patients carrying different deletions (Δ48–50 and Δ50) were used for these experiments. Cultured myogenic cells from the DMD patient with a deletion of exons 48 to 50 were used to perform a dose–response study of the two 2-OMe AOs. This demonstrated that, whereas both AOs were able to induce exon skipping at doses as low as 50 nM, AO B30 was much more efficient at inducing exon 51 skipping, even at low concentrations, as can be appreciated by the relative intensities of the full and the skipped bands (Fig. 3A). Direct sequencing of the lower RT-PCR product showed an accurate junction between exons 47 and 52 (Fig. 3B), confirming exon 51 skipping.

Furthermore, protein samples isolated from similarly transfected cultures from this patient were analyzed by Western blotting, and demonstrated the restoration of dystrophin protein at levels that correlate with the relative degree of exon skipping observed by RT-PCR (compare lanes A20 and B30; Fig. 3C). The levels of dystrophin are, however, rather low, as compared with an equivalent loading of protein extract from normal human cells (Fig. 3C, lane C). No dystrophin is detectable in a protein extract of nontransfected cells from this patient (Fig. 3C, lane Mock).

Cells derived from the DMD patient with a deletion of exon 50 were used to study the persistence of the skip over time. At concentrations of 100 nM, it was evident that AO B30 (+66+95) was substantially more effective at inducing the skipping of exon 51 for as long as 9 days after transfection (Fig. 3D).

Exon skipping in response to low doses of PMO in human fetal muscle cells and DMD cells

The PMO backbone presents certain advantages for uptake by skeletal muscle after systemic injection in vivo (Sazani et al., 2002) but its uptake in vitro is relatively poor unless either high concentrations are used, or scrape loading is employed to enhance cellular uptake (Summerton and Weller,

**FIG. 2.** (A) Normal hSkMCS were transfected with increasing concentrations of the two 2-OMe AOs. Both AOs achieved the correct skip at low concentrations, with AO B30 (+66+95), showing more consistent results. (B) Normal hSkMCS were transfected with two concentrations (100 and 300 nM) of the two AOs and studied 1, 3, 7, and 10 days after transfection. It was possible to confirm the presence of a fragment of the expected size 10 days after transfection in each case. (C) The bands were analyzed by sequencing, confirming that the correct exon skip had been achieved. In both experiments, whereas the skip achieved with AO A20 was approximately 40% of the total, the skipped band present when cells were transfected with AO B30 was approximately 60%. Note that additional fragments, slightly shorter than the wild-type products, are visible in some analyses. This is due to heteroduplex formation and has been described previously (Aartsma-Rus et al., 2003).
DYSTROPHIN EXON 51 AO COMPARISON

However, we have used the observations of Gebski et al. (2003) to design mixed backbone DNA leashes, which can complex with transfection reagents and thus act as carriers for PMOs into cultured cells. We tested four different, commercially available transfection reagents for their ability to introduce a leashed PMO variant of AO B30 into normal human skeletal muscle cells in culture, at a relatively high concentration of 500 nM. Although all of the reagents tested were capable of achieving a degree of transfection, as judged by the presence of exon 51 skipping in the usual RT-PCR assay (Fig. 4), it was evident that the use of Lipofectin (Invitrogen), at a ratio of 4 μl/μg of leashed PMO, was the most effective in these cultures. Other ratios of Lipofectin were also tested but found to be less effective (data not shown). We subsequently used these conditions to directly compare three PMOs in normal human skeletal muscle cells (B30, A25, and I25; Fig. 1A) in two of the research laboratories (IC and RH). I25 was included, despite the fact that the 2-OMe tested had not been effective at achieving exon skipping when targeting the 5′ splice site, because it targeted the 5′ splice site, which was shown previously to be effective in mouse exon 23 (Mann et al., 2002; Graham et al., 2004). In addition, when these experiments were designed it was not clear whether one could extrapolate results between sequences when different chemistries were used. The leashed B30 PMO was clearly the most efficient, demonstrating efficacy at the lowest concentration tested in both laboratories (50 nM) (Fig. 5A and B): A25 showed less efficient results than B30 at IC (Fig 5A), whereas at RH, the two leashed 25-mer PMOs (A25 and I25) appeared incapable of inducing skipping of exon 51 to any substantial level at either 100 or 500 nM (Fig. 5B). The leashed PMOs A25 and B30 were also transfected at IC into cells from the DMD patient with a dele-
tion of exon 50, demonstrating skipping by RT-PCR (Fig. 5C) and *de novo* production of dystrophin protein at low levels (Fig. 5C and D).

**AO B30 is able to induce exon 51 skipping in DMD muscle explants**

Once results had been confirmed in cultured cells from DMD patients, we wished to determine whether muscle fragments retaining some of their original three-dimensional properties would be able to produce the same exon skipping when AOs were applied. We tested the PMO B30 on several muscle explants derived from a DMD patient with a nonrelevant deletion (Δ3–11), as a proof of principle. The correct-sized RT-PCR product was observed, consistent with accurate exon 51 skipping (Fig. 6A, lane AO B30), which was subsequently confirmed by direct sequencing of the RT-PCR product (Fig. 6B).

**Blind comparison of several AOs in the hDMD mouse**

The hDMD mouse offers the possibility of studying processing of the human DMD gene in an *in vivo* setting, providing a model to test the behavior of the AO reagents *in vivo* before clinical testing in patients. PMOs A25, B30, and I25 were injected into the gastrocnemius muscle of hDMD mice, and RNA extracts from treated muscles were analyzed, in a blinded fashion, 2 weeks later for exon 51 skipping. The hierarchy of efficacy of PMOs was the same as that demonstrated *in vitro*, with
the highest percentage of skipping being achieved with PMO B30, +66+95 (Fig. 7, lane 4).

DISCUSSION

DMD is a fatal neuromuscular disorder that affects 1 in 3500 male newborn boys (Moser, 1984). There is no cure for DMD, but a putative treatment may lie in the correction of the reading frame to generate semifunctional dystrophins (Wilton et al., 1997). The possibility of inducing exon skipping by blocking recognition sites required for the correct splicing of RNA has advanced enormously since it was first tested in lymphoblastoid DMD cells (Pramono et al., 1996) and mdx muscle cells (Dunckley et al., 1998), with promising results in human muscle cultures and more recently in the mdx mouse model in vivo (Table 1).

The therapeutic use of AOs has advanced enormously: previously, DNA AOs had been used to downregulate the expression of specific genes involved in cancer and inflammatory diseases in cell cultures (Zamecnik and Stephenson, 1978) and were used in an in vitro assay to modulate splicing by targeting splice sites in a mutated β-globin gene associated with β-thalassemia (Dominski and Kole, 1993). These splicing modulation approaches were later reproduced in human cell lines (HeLa and NIH 3T3) stably expressing the β-globin gene carrying the thalassemic mutation IVS2-654 (Sierakowska et al., 1996). The method was also tested in human and mouse cell lines stably expressing mutated genes associated with cystic fibrosis (Friedman et al., 1999). The modulation of splicing with AOs has also been shown to be useful in changing the ratio of alternative spliced transcripts, for example, from an antiapoptotic to a proapoptotic isoform (Dominski and Kole, 1993; Mercatante et al., 2002).

In most cases, AOs designed to modulate splicing target the splice donor, acceptor, or branch point sequence. However, exon skipping can also be induced with AOs targeting exonic splicing enhancer (ESE) motifs (Aartsma-Rus et al., 2005). These weakly defined motifs are bound by a subfamily of splicing factors, the so-called SR proteins, which facilitate splicing by recruitment of splicing effectors U1 and U2AF to the donor splice site and the polypyrimididine tract (reviewed in Cartegni et al., 2002). Interestingly, for human DMD exons there is a correlation between exon “skippability” and the predicted strength of the acceptor splice site and ESEs but not with the predicted strength of the donor (5') splice site (Aartsma-Rus and van Ommen, 2007).

However, several years after the first attempts at dystrophin exon skipping with AOs, there are still no clear rules to guide investigators in their design, and in mouse and human muscle cells in vitro there is great variability for different targets and exons. The consensus sequences at the intron–exon boundaries that are involved in splice site selection are only poorly conserved, and the ESEs that are involved in exon definition are themselves of multiple motifs and their identification is complex. Until these key elements are better understood only length and target region seem to be important when designing exon-skipping AOs for the DMD gene (Adams et al., 2007).

As different approaches in AO design lead to different results, it was clear to us that it was necessary to test a wide range of AOs to select the best possible sequence for the trial planned in the United Kingdom. The independent design of AOs by three research groups (at RH, UWA, and LUMC) guaranteed as many different approaches to AO design as groups and, while an independent group (IC) validated the efficacy of the best sequences; the final results were eventually cross-checked by experiments performed by each group.

FIG. 6. (A) Muscle explants from a DMD patient (Δ3–11) were immersed in medium containing a 400 nM concentration of PMO AO B30. AO B30 was successful at achieving the skip in the muscle explant. (B) The skipped band was analyzed by sequencing analysis, showing the correct skip.

FIG. 7. Three different PMOs were injected, in a blinded experiment, into the gastrocnemius muscle of hDMD mice. Quantification of the PCR products, using a DNA LabChip, gave the following results: lane 1 (AO I25, −7+18), 1.6%; lane 2 (Invert control), 0%; lane 3 (AO A25, +63+87), 5.6%; lane 4 (AO B30, +66+95), 22.1%.
A similar strategy was followed when the model in which to test the AOs was selected: the very nature of this targeted exon skipping approach makes it impossible to test many of the specific sequences to be used in humans in healthy volunteers, because of the potential for disrupting the intact dystrophin open reading frame, leading to nonfunctional dystrophins. Therefore, to select the best possible AO to be used in a clinical trial, we have used several models in an attempt to combine their respective advantages and limitations to produce a reliable and coherent picture. Human myoblasts allowed us to test the sequences in a controlled in vitro setting, in which we were able to show the presence of the correct skipping event both in normal cells and cells from two DMD patients with different deletions. The RNA results were further confirmed by the presence of dystrophin protein in myoblasts from patients carrying these two different deletions treated with both the 2-OH AOs and PMO AOs. DMD muscle explants provided the cells with a three-dimensional structure more similar to the natural environment (McClorey et al., 2006a) and again we were able to show exon 51 skipping in this situation. To assess the results in an in vivo model before testing the AOs in patients, we used a mouse encoding the human DMD gene. Again, we were able to show the correct exon exclusion.

The comparison has been systematic and the results have been validated by different laboratories within the collaborating institutions. The strength of this collaborative approach derives both from the unbiased and blinded analysis of different sequences and from the multiple independent approaches used, which took advantage of patient samples with the appropriate deletions, but also in vivo assessment of efficacy in the hDMD mouse model. A similar collaborative approach could be used to validate future target sequences in DMD.

As the results of our study all indicated the superiority of the AO targeting the sequence (+66+95) in exon 51, this has been selected as the reagent that will be used in the first U.K. clinical trial. This trial will evaluate the efficacy of this sequence on a PMO backbone. Although both 2-OH and PMO AOs show promising results in vitro and in vivo, PMOs have the advantage of having been tested for other purposes in human patients and are currently in use in clinical trials for restenosis, cancer, and viral infections (Kipshidze et al., 2005; Nikravesh et al., 2007). The U.K. trial will be a phase I/IIa study on the effect of the intramuscular injection of this AO in DMD patients with relevant deletions. If this first study is successful, a second clinical trial is planned in which the same AOs will be administered systemically, as this approach holds promises of functional benefit for DMD patients.

ACKNOWLEDGMENTS

The authors thank their coordinator, Jenny Versnel, and the remaining members of the UK Muscular Dystrophy EXon skipping (MDEX) consortium for helpful discussion (Prof. D. Wells, Prof. K. Bushby, Prof. V. Straub, and Dr. M. Wood) and the Charities Muscular Dystrophy Campaign, Parent Project UK, DMD Support Group, Muscular Dystrophy Ireland, and participating patients for the support to our study. This work was funded by the Department of Health (UK). J.E.M. is funded by an MRC collaborative career development fellowship in stem cell research. The authors also thank J.R. Christensen and Dr. P. O’Hanley for the support of AVI Biopharma in the supply of the PMO AOs.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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Received for publication May 24, 2007; accepted after revision August 9, 2007.

Published online: August 31, 2007.