20 (2) 95–154
ISSN 0960-8966

Neuromuscular Disorders

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Official Journal of the World Muscle Society

Neuromuscular Disorders

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This international, **multidisciplinary** journal covers all aspects of neuromuscular disorders in childhood and adult life (including the muscular dystrophies, spinal muscular atrophies, hereditary neuropathies, congenital myopathies, myasthenias, myotonic syndromes, metabolic myopathies and inflammatory myopathies).

The Editors welcome original articles from all areas of the field:

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- **Basic scientific studies** of relevance to the clinical syndromes, including advances in the fields of molecular biology and genetics;
- Studies of **animal models** relevant to the human diseases.

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Printed by Polestar Wheatons Ltd, Exeter, UK

Neuromuscular Disorders

Editor-in-Chief
V Dubowitz

Volume 20 No. 2 (2010)

ELSEVIER
Amsterdam - London - New York - Oxford - Paris - Shannon - Tokyo

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Publication information: *Neuromuscular Disorders* (ISSN 0960-8966). For 2010 Volume 20 is scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where air delivery by SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be make within six months of our publication (mailing) date.

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USA mailing notice: Neuromuscular Disorders (ISSN 0960-8966) is published monthly by Elsevier B.V., Radarweg 29, 1043 NX Amsterdam, the Netherlands. Periodical postage paid at Rahway NJ and additional mailing offices.
USA POSTMASTER: Send change of address to *Neuromuscular Disorders*, Elsevier Customer Service Department, 3251 Riverport Lane, Maryland Heights, MO 63043, USA.
AIRFREIGHT AND MAILING in USA by Mercury International Limited, 365 Blair Road, Avenel, NJ 07001.

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Neuromuscular Disorders 20 (2010) 102–110

Contents lists available at ScienceDirect

Neuromuscular Disorders

journal homepage: www.elsevier.com/locate/nmd

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

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ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 20 October 2009
Accepted 27 October 2009

Keywords:
Exon skipping
Duchenne muscular dystrophy
Antisense oligonucleotides
Phosphorodiamidate morpholino oligomer

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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doi:10.1016/j.nmd.2009.10.013

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 18,000g 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)

diluted 1:300 in 3% non-fat milk/PBST. An anti-mouse, biotinylated secondary antibody (diluted 1:2000; GE Healthcare, Amersham, UK) and streptavidin/horse radish peroxidise conjugated antibody (1:10,000; Dako, Ely, UK) allowed visualisation in a luminol-HRP chemiluminescence reaction (ECL-Plus; GE Healthcare) on Hyperfilm (GE Healthcare), exposed at intervals from 10 s to 4 min.

2.9. Statistical analysis

For the blind comparison at 300 nM in DMD patient cells, data from two separate experiments performed in duplicate and triplicate respectively were pooled and compared by two-tailed student *t*-test. Dose–response and time-course experiments were compared by two-tailed, paired *t*-test.

3. Results

Twenty-four AOs designed to target exon 53 of the *DMD* gene have been previously tested in normal human skeletal muscle cells (hSkMCs) [24,25]. Table 1 summarises the names and target sequence characteristics of these AOs (shown in bold), and % skipping produced by each in normal hSkMCs. However, studies in normal hSkMCs are limited as they do not allow assessment of the therapeutic effect at the protein level (i.e. dystrophin restoration). Further studies have therefore been performed here to elucidate and confirm which AO(s) would have the potential as a treatment for patients with an eligible deletion. AOs, whose target sites are within the sequence +29 to +74 of exon 53, the region previously shown to be in open conformation, binding to which interferes with spliceosome-mediated pre-mRNA splicing, such that exon 53 is skipped [24,25], were directly compared in exon 53-skippable patient cells (at UCL), and in the humanised DMD (hDMD) mouse (at LUMC). The AOs were all synthesized as PMOs to allow direct comparison of skipping efficacy. While PMOs were hybridized to mixed-backbone DNA leashes in the previous study [24], the nucleofection method used here was performed on unleashed PMOs.

3.1. Comparison of PMOs to exon 53 in DMD patient cells

Our comparative evaluation of PMO-induced exon skipping efficiencies was performed in a blinded fashion. All transfections were performed in triplicate and repeated at least once to ensure uniformity of results. Skipping efficiencies were determined from RT-PCR gel images by comparing induced shortened dystrophin mRNAs to the intact full length transcript using densitometric analysis, as described previously [25]. Sequencing of RT-PCR products confirmed the targeted skipping of exon 53 (results not shown). For quantification, the skip-products were analysed using densitometric analysis with Image J software. This technique for quantifying skipping efficiencies of AOs targeted to the *DMD* gene has been published previously [9,17]. Real-time PCR quantification of intact and induced transcripts has proven to be impossible due to a number of obstacles (variation of amplification efficiencies of each transcript, possible interference of intact and induced transcript primers/probes with each other) (results not shown). No DMD exon skipping studies thus far reported have included real-time PCR quantification of AO efficacy, and we believe we have used the best method available for quantification. Skipping efficiency is given as the percentage of skip transcript over the total amount of transcript (skip and full length). AOs were sub-divided on the basis of their skipping efficiency. PMOs that produced over 50% exon skipping were designated as Type 1, those that produced between 25% and 50% exon skipping were described as Type 2, while those that produced less than 25% as Type 3. Where appropriate, the two-tailed student's *t*-test was used to assess significant differences between AOs.

The 13 PMOs, whose target sites are within the sequence +29 to +74 of exon 53, were compared directly at a 300 nM dose by nucleofection [26]. This dose was selected for comparison, since such concentrations of AOs have been used in numerous previous exon skipping studies in DMD [5,6,9]. PMOs-G, -H and -A were the most efficient, producing a mean of 73% (±4.10%), 68% (±4.77%) and 68% (±4.14%) exon skipping respectively (classified as Type 1) (Fig. 1). The other PMOs tested produced the following exon skipping levels: PMO-I, 63% (±7.5%); PMO-B, 56% (±6.29%); PMO-M, 52% (±10.78%) (all classified as Type 1); PMO-J, 37% (±4.95%); (classified as Type 2). All other PMOs tested gave exon skipping at levels of between 15% and 26%. When compared by two-tailed student *t*-test, PMO-G (the most efficient PMO) gave significantly higher levels of exon skipping than PMOs -C ($p < 0.0001$), -D ($p < 0.0001$), -E ($p < 0.0001$), -F ($p < 0.0001$), -J ($p = 0.0005$), -K ($p = 0.0002$) and -L ($p < 0.0001$), but was not significantly more effective than the other PMOs tested. The more efficacious PMOs should produce sustained and pronounced exon skipping when applied at lower concentrations. Therefore, the six most effective PMOs (i.e. Type 1) (-A, -B, -G, -H, -I and -M) were selected for dose–response and time-course experiments.

When the concentration dependence of exon skipping was examined for the most efficient PMOs, skipping levels approaching 30% were evident for the Type 1 PMOs -G and -H at concentrations as low as 25 nM (Fig. 2a, b). The other PMOs classified as Type 1 (PMOs -A, -B, -I and -M) did not induce such levels of exon skipping when used at lower concentrations. Similar levels of skipping (30%) were only achieved by PMO-A, PMO-B and PMO-M at 100 nM, while PMO-I needed to be present at 200 nM to produce over 30% exon skipping (Fig. 2a, b). This is why the concentration dependence of exon skipping is a valuable tool in ascertaining the most efficient AO(s).

The exon skipping produced by the six Type 1 PMOs was shown to be persistent, lasting for up to 10 days after transfection, with over 60% exon skipping observed for the lifetime of the cultures for PMOs -A, PMO-G and PMO-H (Fig. 3a, b). When compared by two-tailed, paired *t*-test across all time-points, PMO-G gave significantly higher levels of skipping than PMOs -B, -H, -I and -M ($p = 0.0004$, 0.0126, 0.0008 and 0.0004, respectively) and bordered on significance for PMO-A ($p = 0.0550$). Three of the most effective Type 1 PMOs (-A, -G and -H) were also compared in a longer time-course experiment up to 21 days after transfection (Fig. 3c, d). PMO-G gave sustained high levels of exon skipping (over 60%) for the 21 days whereas skipping by PMOs -A and -H had fallen to 48% and 46%, respectively by day 17. When compared by two-tailed, paired *t*-test across all time-points, PMO-G gave significantly higher levels of skipping than PMOs -A and -H ($p = 0.0422$ and 0.0231, respectively). There was no evidence that any of the PMOs tested caused cellular cytotoxicity relative to mock-transfected controls, as assessed by visual inspection, and lactate dehydrogenase release into culture medium (results not shown). The relative efficacy of the six Type 1 PMOs in the direct comparison, dose–response and time-course assays is summarised in Fig. 4a. Exon 53 skipping by PMO-G is used as the baseline set at 100%. This clearly shows the PMO-G outperforms the other Type 1 PMOs in vitro in patient cells. However, it should also be noted that a number of the other Type 1 PMOs, namely PMO-A and PMO-H, also appear to perform very well across these three comparative tests.

The most effective Type 1 PMO (PMO-G) from the time-course experiments was compared to the most effective PMO to skip exon 51 that has been taken forward to clinical trial, in DMD patient cells with an amenable mutation (del 45–52 for exon 53 skipping and del 48–50 for exon 51 skipping) in a dose–response experiment from 25 to 400 nM (see Fig. 4b). PMO-G gave higher levels

Table 1
Table summarising the characteristics of PMOs used. Characteristics of the PMOs and their target sites listed (taken from [24]).

	PMO	% Skip	Position		%GC	Exon-PMO binding energy	PMO-PMO binding energy	% Open[a]	Ends in open loops[a]	% Overlap with hybrid. peak
			Start	End						
(a)										
A	h53A1	12.7	+35	+59	52	−38.6	−17.4	50	2	92
B	h53A2	9.7	+38	+62	56	−36.1	−17.4	46.7	1	100
C	h53A3	2.0	+41	+65	56	−36.7	−13.7	36.7	0	0
D	h53A4	10.5	+44	+68	48	−34.3	−8.5	20	0	100
E	h53A5	9.0	+47	+71	48	−35.5	−8.5	43.3	2	100
F	h53A6	0.3	+50	+74	48	−35.3	−8.5	43.3	2	92
N	h53B1	0	+69	+93	28	−22.1	−12.1	53.3	1	0
O	h53B2	0.6	+80	+104	48	−30.1	−11.3	23.3	1	0
P	h53B3	3.0	+90	+114	48	−34.5	−5.5	48	2	0
Q	h53C1	0	+109	+133	48	−32.4	−9.8	46.7	2	0
R	h53C2	0	+116	+140	56	−31.3	−12.7	33.3	1	0
S	h53C3	0	+128	+152	60	−34.6	−13.7	26.7	1	0
T	h53D1	0	+149	+173	52	−34.1	−13.4	30	1	0
U	h53D2	0.9	+158	+182	48	−36.5	−14.5	40	2	0
V	h53D3	3.7	+170	+194	36	−34.3	−11.2	40	1	0
W	h53D4	12.3	+182	+206	32	−30.9	−9.2	63.3	1	0
X	h53D5	7.9	+188	+212	36	−31.5	−3.3	66.7	1	0
G	h53A30/1	52.4	+30	+59	50	−48.1	−17.4	56.7	1	92
H	h53A30/2	87.2	+33	+62	53	−45.1	−17.4	63.3	1	100
I	h53A30/3	80.1	+36	+65	53	−44.6	−17.4	53.3	1	100
J	h53A30/4	38.6	+39	+68	50	−43.4	−17.4	43.3	1	100
K	h53A30/5	9.4	+42	+71	47	−42.4	−11.3	46.7	1	100
L	h53A30/6	35.9	+45	+74	47	−42.3	−8.5	56.7	1	100
M	H53A	N/D	+39	+69	52	−48.5	−17.4	48.4	2	100

	PMO	# Rescue ESE sites	% Overlap with Rescue ESE	% Overlap with		ESE finder values over threshold[b]						
				PESE	PESS	SF2/ASF	BRCA1	SC35	SRp40	SRp55	Tra2B	9G8
(b)												
A	h53A1	7	56	84	0	6.58	7.26	0	3.12	0	24.04	19.02
B	h53A2	4	32	72	0	6.58	7.26	0	3.12	0	7.25	19.02
C	h53A3	3	32	60	0	6.58	7.26	0	3.12	0	7.25	11.9
D	h53A4	4	28	48	8	6.58	7.26	0	3.12	0	7.25	11.9
E	h53A5	3	36	36	20	6.58	7.26	0	3.12	0	7.25	11.9
F	h53A6	2	36	28	32	6.58	7.26	0	0	0	7.25	11.9
N	h53B1	5	56	40	40	0	9.26	3.62	10.66	0	5.06	1.1
O	h53B2	5	60	60	0	0	9.26	3.62	4.73	0	5.06	8.28
P	h53B3	8	72	64	0	3.49	9.26	3.44	4.73	0	24.04	28.68
Q	h53C1	6	52	72	0	4.19	6.72	0	2.04	0	24,04	28.68
R	h53C2	1	24	60	0	4.19	6.72	10.2	4.38	0	0	8.28
S	h53C3	1	24	32	0	3.49	6.41	10.2	4.38	6.86	0	14.18
T	h53D1	4	40	32	0	0.52	0	18.68	0	6.86	0	12.71
U	h53D2	6	44	32	0	0.52	1.8	18.68	0.42	0	0	12.71
V	h53D3	9	64	0	0	0	1.8	0	6.95	0	24.04	10.49
W	h53D4	16	96	24	0	8.5	11.95	0	7.67	0.33	24.04	7.14
X	h53D5	14	92	44	0	8.5	11.95	0	7.67	0.33	24.04	7.14
G	h53A30/1	9	60	86	0	6.58	7.26	0	3.12	0	24.04	19.02
H	h53A30/2	8	53	77	0	6.58	7.26	0	3.12	0	24.04	19.02
I	h53A30/3	6	43	67	0	6.58	7.26	0	3.12	0	24.04	19.02
J	h53A30/4	4	43	57	7	6.58	7.26	0	3.12	0	7.25	11.9
K	h53A30/5	5	47	47	17	6.58	7.26	0	3.12	0	7.25	11.9
L	h53A30/6	5	48	37	27	6.58	7.26	0	3.12	0	7.25	11.9
M	H53A	4	45	58	10	6.58	7.26	0	3.12	0	7.25	11.9

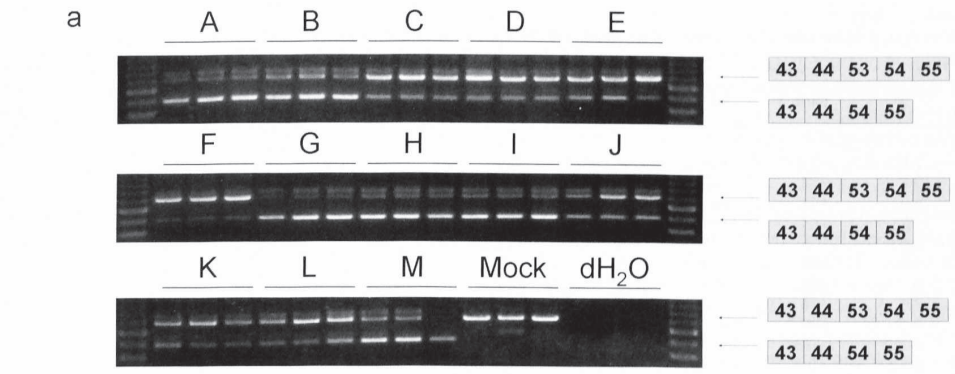
Alphabetical codes shown in bold represent the PMOs compared in this present study.
[a] Calculated as % of PMO target site in open structures on predicted RNA secondary structure obtained using MFOLD analysis. The position of the PMO target sites relative to open loops in the RNA secondary structure is listed (0, no ends in open loops; 1, one end in an open loop; 2, both ends in open loops).
[b] In our analyses, SR binding sites were predicted using splice sequence finder (http://www.umd.be/SSF/) software. Values above threshold are given for PMOs whose target sites cover 50% or more of potential binding sites for SF2/ASF, BRCA1, SC35, SRp40, SRp55, Tra2β and 9G8.

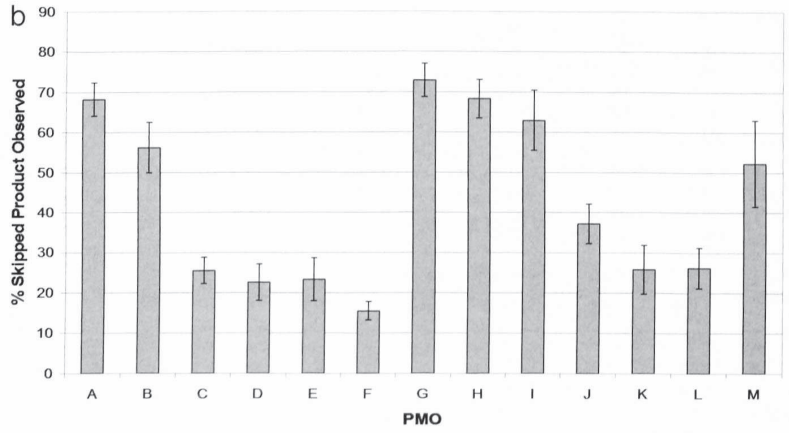
of skipping at all doses tested. When compared by two-tailed, paired *t*-test, PMO-G across all concentrations used, gave significantly higher levels of skipping than h51A ($p = 0.0033$).

Western blot analysis of DMD patient (del 45–52) cell lysates, treated in culture with the Type 1 25mers (PMOs -A and-B) and 30mers (-G, -H, -I and -M) is shown in Fig. 5a. De novo expression of dystrophin protein was evident with all six PMOs, but was most pronounced with PMOs -H, -I, -G and -A, producing 50%, 45%, 33% and 26% dystrophin expression, respectively, relative to the positive control, and seemingly weakest with PMO-B and PMO-M (11% and 17% dystrophin expression respectively, relative to the positive control). Although there are limitations to quantifying Western blots of this nature, the qualitative importance of the data holds.

3.2. Comparison of PMOs to exon 53 in humanised DMD mouse

The hDMD mouse is a valuable tool for studying the processing of the human *DMD* gene in vivo, and as such provides a model for studying the in vivo action of PMOs, prior to clinical testing in

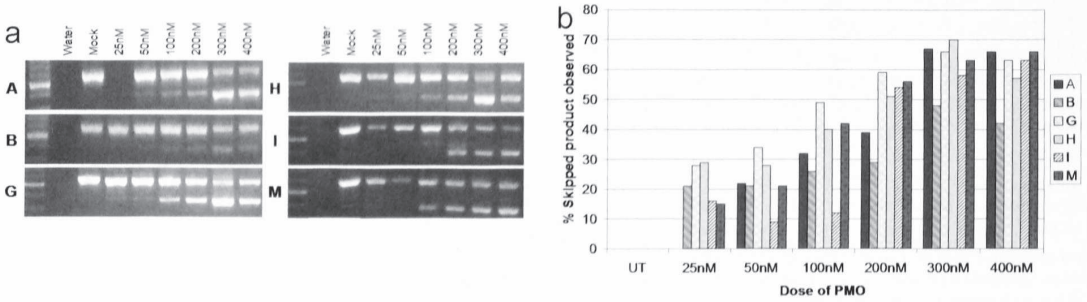




c

PMO	P-value
G vs A	0.4496
G vs B	0.0577
G vs C	<0.0001*
G vs D	<0.0001*
G vs E	<0.0001*
G vs F	<0.0001*
G vs H	0.4935
G vs I	0.2799
G vs J	0.0005*
G vs K	0.0002*
G vs L	<0.0001*
G vs M	0.1124

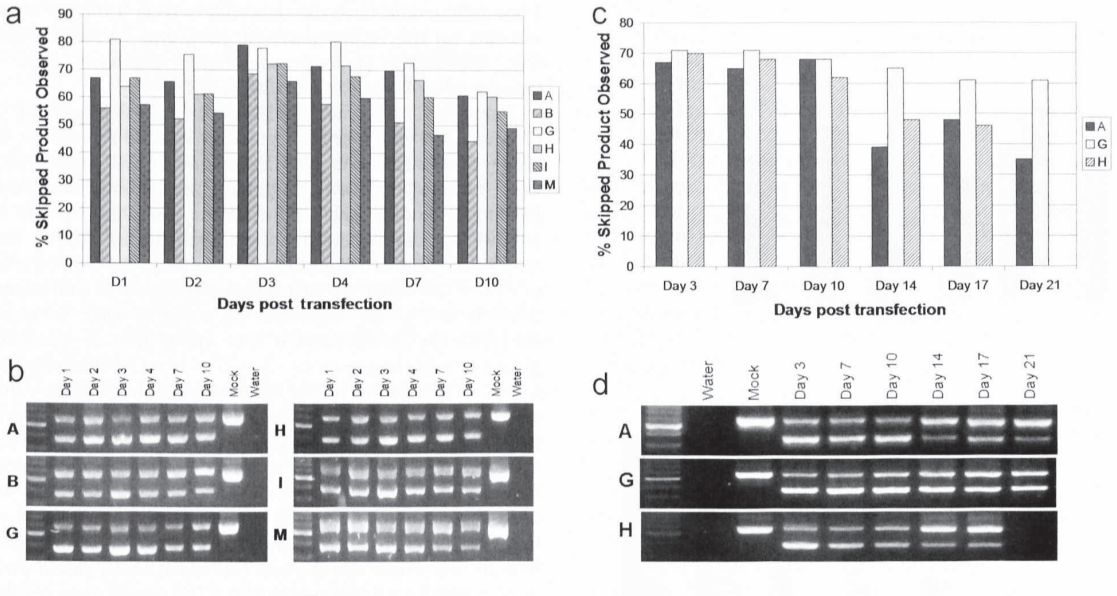
Fig. 1. Blind comparison of 13 PMO oligonucleotide sequences to skip human exon 53. Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected by nucleofection with each of the PMOs (300 nM) in triplicate. RNA was harvested 3 days following transfection, and amplified by nested RT-PCR. (a) Bars indicate the percentage of exon skipping achieved for each PMO, derived from Image J analysis of the electropherogram of the agarose gel (b). Skipped (477 bp) and unskipped (689 bp) products are shown schematically. The larger full length amplicon is often seen and is due to carry over of primers from the first round of the PCR into the second. (c) Efficacy of PMOs was compared by two-tailed, student *t*-test. PMO-G gave significantly higher efficacy of exon skipping than PMOs C, D, E, F, J, K and L ($^*p < 0.05$), but not significantly higher than PMOs A, B, H, I and M.



c

PMO	P-value
G vs A	0.0491*
G vs B	0.0022*
G vs H	0.0941
G vs I	0.0527
G vs M	0.0759

Fig. 2. Dose–response of the six best-performing PMOs. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected with the six best-performing PMOs by nucleofection, at doses ranging from 25 to 400 nM. RT-PCR products derived from RNA isolated from cells 3 days post-transfection were separated by agarose gel electrophoresis. (b) The percentage of exon skipping observed is expressed for each concentration of each PMO as a comparison of the percentage OD of skipped and unskipped band, as measured using Image J. (c) Data for each PMO over the range of doses were pooled and compared by two-tailed, paired student *t*-test. PMO-G gave significantly higher efficiency than PMOs A and B ($^*p < 0.05$) but did not give significantly higher efficacy than PMOs H, I and M.



e

PMO	P-value (10 days)	P-value (21 days)
G vs A	0.0550	0.0422*
G vs B	0.0004*	-
G vs H	0.0126*	0.0231*
G vs I	0.0008*	-
G vs M	0.0004*	-

Fig. 3. Persistence of dystrophin expression in DMD cells following PMO treatment. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected by nucleofection with each of the six best-performing PMOs (300 nM), and were cultured for 1–10 days before extracting RNA. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (b). (c) Long term exon skipping up to 21 days after transfection with PMOs A, G and H. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (d). (e) Data for each PMO over the time-course experiments were pooled and compared by two-tailed, paired student *t*-test. Over the 10 day time-course experiment, PMO-G gave significantly higher efficiency than PMOs B, H, I and M ($^{*}p < 0.05$) but did not give significantly higher efficacy than PMO A. Over the 21 day time-course experiment, PMO-G gave significantly higher efficacy than PMOs A and H ($^{*}p < 0.05$).

patients. The Type 1 PMOs (-A, -G, -H, -I and -M) (20 μg) were injected into the gastrocnemius muscle of hDMD mice in duplo. RNA extracted from the muscles was analysed for exon 53 skipping by RT-PCR (Fig. 5b). For quantification, the skipped products were analysed using the DNA 1000 LabChip Kit on the Agilent 2100 bioanalyzer, which, unlike densitometry, corrects for fragment length. Skipping percentages were calculated as the amount of skip transcripts relative to the total transcripts (skipped and full length). Skipping of exon 53 was evident for each of the PMOs tested; average skipping seen in both legs was 8% for PMO-A, 7.6% for PMO-I, 7.2% for PMO-G, but a slightly lower level of 4.8% for PMO-H. PMO-M produced exon skipping levels of less than 1%, which is the detection threshold for the system used.

4. Discussion

We describe here the comparative analysis of PMOs designed to target exon 53 of the human *DMD* gene and thereby induce its skipping. Previously, a series of PMOs had been designed and their exon skipping efficacy investigated in normal human skeletal muscle cells [24]. These were directly compared to a PMO based on an AO previously identified as being the most bioactive by Wilton et al. [25]. Skipping efficiencies of the PMOs were compared here by two independent groups in two different systems (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [9]. The use of primary human myoblast cultures allowed controlled in vitro comparison of PMO sequences, and confirmation of skipping of exon 53 at the RNA level by PMOs in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent confirmation of optimal sequence(s) for the targeted skipping of exon 53.

The putative use of AOs to skip the exons which flank out-of-frame deletions is fast becoming a reality in the treatment of DMD boys. Indeed the restoration of dystrophin expression in the TA muscle of four patients, injected with a 2′OMePS AO optimised to target exon 51 of the *DMD* gene, has been reported recently [14]. Moreover a clinical trial using a PMO targeting exon 51 has recently been completed in seven DMD boys in the UK [15]. However, the targeted skipping of exon 51 would have the potential to treat only 13% of DMD patients on the Leiden database with genomic deletions [23]. There is therefore a definite requirement for the optimisation of AOs to target other exons commonly mutated in DMD. The targeted skipping of exon 53 of the human

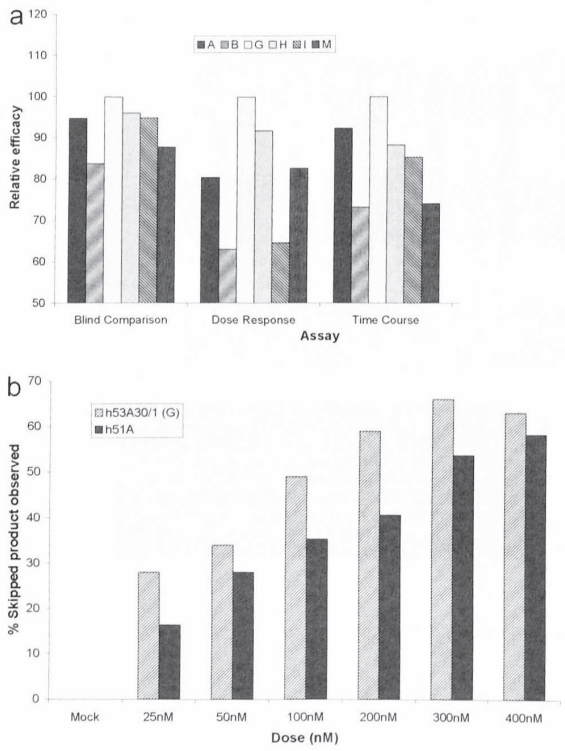


Fig. 4. Relative efficacy of Type 1 PMOs for the targeted skipping of exon 53. (a) The relative efficacy of the 6 most effective PMOs compared in the different assays. PMO-G was used as a baseline set at 100%. (b) The most effective PMO (G) compared to the most effective PMO to skip exon 51 in amenable DMD patients cells at doses ranging from 25 to 400 nM. PMO-G gave significantly higher levels of skipping ($p = 0.0033$ compared by two-tailed, paired t-test).

DMD gene would have the potential to treat a further 8% of DMD patients on the Leiden database [23], and 13.5% of patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO).

There have been many large screens of AO bioactivity in vitro [24,25,28,29] which provide guidelines to aid AO design. The targeting of AOs to exonic splicing enhancer (ESE) motifs [25,28,29], RNA secondary structure, target site accessibility and strength of AO-target binding are all important predictors of AO efficacy [24,29]. Although there are tools available to aid the design of AOs for the targeted skipping of *DMD* exons, the empirical analysis of AOs is still required. Hence the importance of this study in the development of AO sequences as potential gene therapy drugs for DMD. The data presented here would indicate that PMOs targeting within the sequence +30+65 of exon 53 (namely PMO-A, -G and –H) produce levels of exon skipping that may be considered effective (over 50% exon skipping). There remains however the possibility that a stepped base-by-base screening of AOs across the entirety of exon 53 and some indeterminate distance into the flanking intronic sequences might reveal an AO with a better dose–response and longevity of action profile. Sequence +30+65 has been shown to be accessible to binding on hexamer hybridization array analysis and in open conformation by mfold prediction of pre-mRNA secondary structure [24]. These Type 1 PMOs can therefore bind more strongly since they can access their target site more directly. These thermodynamic considerations have also been reported in a complementary study of 2′OMePS AOs [29]. Indeed, the fact that the 30mer PMOs (-G, -H) were more bioactive than 25mer PMO counterpart (-A) targeted to the same open/accessible sites on the exon, would suggest that strength of binding of PMO to the target site may be the most important factor in determining PMO bioactivity. The influence of AO length on bioactivity has been reported elsewhere [9,30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart. Previous studies by the Leiden

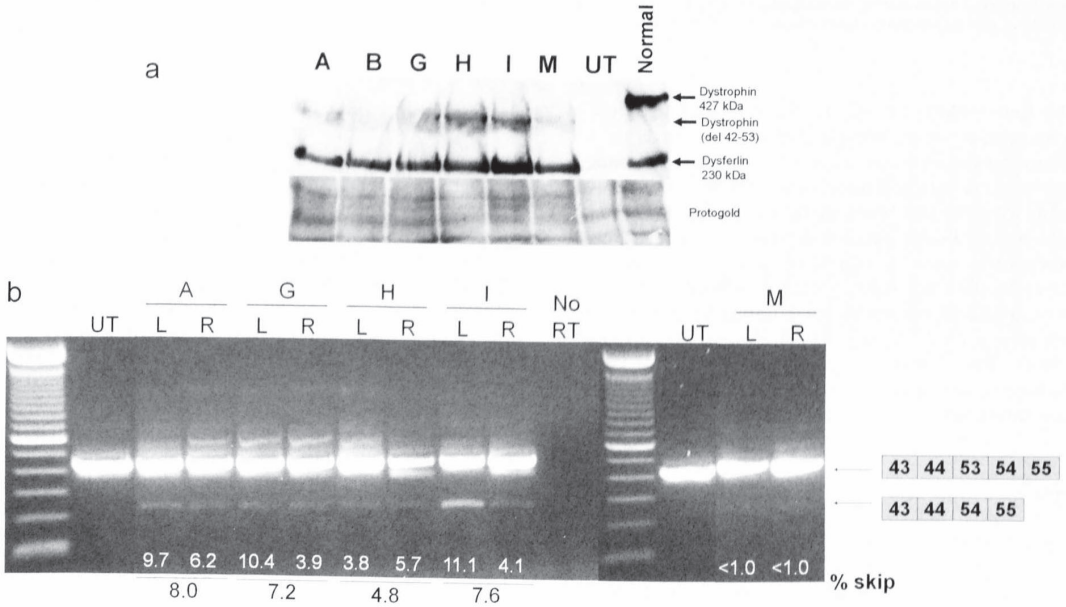


Fig. 5. Analysis of induced dystrophin protein expression in del DMD cells and in vivo efficacy of Type 1 PMOs in hDMD mice. (a) Western blot analysis was performed on total protein extracts from del 45–52 DMD cells 7 days after transfection with the six best PMOs (300 nM). Blots were probed with antibodies to dystrophin, to dysferlin as a muscle-specific loading control, and protogold for total protein loading control. CHQ5B myoblasts, after 7 days of differentiation were used as a positive control for dystrophin protein (normal). (b) PMOs (20 μg) were injected in a blind experiment into the gastrocnemius muscle of hDMD mice in duplo. RT-PCR analysis of RNA harvested from muscle isolated 1 week after injection was performed and products visualised by agarose gel electrophoresis. Quantification of PCR products was performed using a DNA LabChip.

group [28] suggest that the optimal 2′OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured control cells and 7% in the hDMD mouse. This 2′OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our PMOs produce higher levels of exon skipping could be a (combined) consequence of the different AO chemistries, length of AO used, type of cell used (patient vs. control) and the absolute target site of AO. No direct comparison was made here between PMO and 2′OMe AOs targeted to the same sequence of exon 53, since the purpose of this study was not to elucidate which chemistry was superior, but to ascertain the optimal target site for a PMO. A direct comparison would in fact be difficult as 2′OMe AOs are generally only 20 nucleotides long, whereas the PMOs used here were 25 and 30mers.

A PMO can be classified as an effective AO if it produces strong (over 50%), consistent and sustained exon skipping after administration to myogenic cells in vitro at low concentrations (25 nM). An AO reagent that produces sustained exon skipping at low concentrations would be preferable in the clinical setting as a gene therapy as this would lower the cost of treatment, extend the therapeutic action of exon skipping and reduce the possible toxicity of chronic AO administration. The levels of skipping produced in vitro by those PMOs targeting the sequence +30+65 are comparable, or indeed superior, to those reported pre-clinically for PRO051 [28] and AVI4658 [9], the 2′OMe and PMO AOs that are now both being used in Phase I/II clinical trials with encouraging results [14,15]. Indeed we directly demonstrate here the greater skipping efficacy of PMO-G relative to AVI4658 (H51A) over a range of concentrations (see Fig. 4b). However predicting the amount of skipping needed in vitro for an AO to be therapeutic in a patient is impossible; the efficiency of exon skipping is likely to differ from patient to patient and mutation to mutation, and the levels of dystrophin protein restoration will depend on the quality of the muscle itself when a clinical treatment is started.

When considering the data presented previously [24] and here as a whole, the superiority of the PMOs targeting the sequence +30+65 (i.e. PMOs -A, -B, -G and -H) is strongly indicated. The 30mers PMO-G and PMO-H produce higher levels of skipping relative to the 25mers PMO-A and PMO-B. In normal myoblasts, liposomal-carrier mediated transfection of leashed forms of these 30mer PMOs targeting produced over 50% skipping of exon 53, implying that they act extremely efficiently within the cell. This was confirmed in patient myoblasts using nucleofection as the entry method of naked PMOs into the cells. The different levels of exon skipping seen here in the patient cells relative to control cells is due to the different concentrations used (300 nM in patient cells versus 500 nM in normal cells), the different techniques used to introduce the PMOs into the cells, and the differences between the cells themselves. The dystrophin in patient cells is generally more readily skippable than normal cells [5,6,31]. The results seen in patient cells were reproducible, implying that sufficient PMO is getting into the cells to induce such skipping, and the order of efficiency of exon skipping induced in patient cells mirrored that seen in normal cells (Table 1, and Fig. 1). Further, these PMOs generate the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important for potential therapeutic application, exert their activity at concentrations as low as 25 nM. The exon skipping activity of these PMOs is also persistent, with over 60% exon skipping for 21 days in culture for PMO-G. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMOs targeting the sequence +30+65 of exon 53 of the *DMD* gene were also shown to skip exon 53 correctly in vivo in the hDMD mouse. It should be noted that the levels of exon skipping in the hDMD mouse by each particular PMO was variable. This has been reported previously [12], and is likely to be due to the poor uptake into the non-dystrophic muscle of the hDMD mouse. However this does not compromise the importance of the finding that the PMOs tested here are able to elicit the targeted skipping of exon 53 in vivo. A summary of the relative efficacy of the different Type 1 PMOs tested over the different assays used is presented in Fig. 4a. The recommendation of PMO-G as a potential clinical trial reagent of choice for the targeted skipping of exon 53 of the *DMD* gene relative to the other Type 1 PMOs, is based primarily on it's more persistent longevity of action. Repeated administration of PMO will be required for prolonged antisense therapeutic action, and the prolonged action of PMO-G makes it an attractive choice with PMOs-A and -H providing viable alternatives if required. These RNA results were further confirmed by the detection of dystrophin protein in extracts from patient cells treated with these PMOs.

Although efficiency of exon skipping is perhaps the most important quality an AO can possess, it is not the only one. The potential for the PMOs targeting sequence +30+65 of exon 53 to have off-target effects could be considered to be negligible, since no completely homologous sequences were found on BLAST analysis (results not shown). There is a common single nucleotide polymorphism (SNP) seen on exon 53 of the *DMD* gene. PMOs -J, -K, -L and -M had this SNP (c7728C>T) in the last, fourth to last, seventh to last and second to last base of their target sites, respectively. There is the potential that this allelic mismatch could influence the binding and bioactivity of these PMOs. However, the more active PMOs (-A, -B, -G, -H and -I) all had their target sites away from the SNP, thus removing the possible effect of a mismatch weakening binding and bioactivity, allowing definitive comparisons between these PMOs to be made. The DMD patient (del 45–52) carried the normal (T) allele, hence the SNP would not affect the binding of the PMOs that anneal at this site. Additionally, there was no evidence that the PMOs produced cellular cytotoxicity (results not shown). This, together with the predicted stability of the PMO–target complexes [24], suggests these PMOs have potential as a clinical therapy.

We would therefore recommend that PMOs targeting sequence +30+65 of exon 53 of the *DMD* gene worthy of consideration for any upcoming clinical trial. In this study, sequence +30+65 was effectively targeted by PMOs-A, -B, -G and -H, resulting in exon 53 skipping. Since repeated delivery would be required for therapeutic action, the more persistent action of PMO-G may suggest this to be the PMO of choice for the targeted skipping of exon 53, and PMOs-A and -H providing viable alternatives if required.

Acknowledgements

The authors wish to thank members of the UK Muscular Dystrophy Exon skipping (MDEX) Consortium for helpful discussions, and the Muscular Dystrophy Campaign, Action Duchenne (formerly Parent Project UK), Duchenne Family Support Group, Muscular Dystrophy Ireland, NIH, Muscular Dystrophy Association USA, Clinigene EC Network of Excellence (LSHB-CT-2006018933) and participating patients for supporting this study. All work was carried out with informed consent and with the approval of the institutional ethics committee. The authors would also like to thank Prof. Ryszard Kole for his advice and careful reading of the paper. The authors also thank Janet Rose Christensen and Peter O'Hanley for the support of AVI Biopharma in the supply of PMO AOs. The work was funded by the Department of Health (UK). JEM was supported by a MRC collaborative career development fellowship in stem cell research and is currently funded by a Wellcome Trust University Award. CA was funded by the Muscular Dystrophy Campaign, Association Francais Contre les Myopathies, EU MYOAMP and is currently funded by the Duchenne Parent Project. The MRC Neuro-

muscular Centre support to the Biobank is also gratefully acknowledged.

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Neuromuscular Disorders

Volume 20 Number 2 February 2010

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0960-8966(201002)20:2;1-H

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