Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study


Summary

Background We report clinical safety and biochemical efficacy from a dose-ranging study of intravenously administered AVI-4658 phosphorodiamidate morpholino oligomer (PMO) in patients with Duchenne muscular dystrophy.

Method We undertook an open-label, phase 2, dose-escalation study (0·5, 1·0, 2·0, 4·0, 10·0, and 20·0 mg/kg bodyweight) in ambulant patients with Duchenne muscular dystrophy aged 5–15 years with amenable deletions in DMD. Participants had a muscle biopsy before starting treatment and after 12 weekly intravenous infusions of AVI-4658. The primary study objective was to assess safety and tolerability of AVI-4658. The secondary objectives were pharmacokinetic properties and the ability of AVI-4658 to induce exon 51 skipping and dystrophin restoration by RT-PCR, immunohistochemistry, and immunoblotting. The study is registered, number NCT00844597.

Findings 19 patients took part in the study. AVI-4658 was well tolerated with no drug-related serious adverse events. AVI-4658 induced exon 51 skipping in all cohorts and new dystrophin protein expression in a significant dose-dependent (p=0·0203), but variable, manner in boys from cohort 3 (dose 2 mg/kg) onwards. Seven patients responded to treatment, in whom mean dystrophin fluorescence intensity increased from 8·9% (95% CI 7·1–10·6) to 16·4% (10·8–22·0) of normal control after treatment (p=0·0287). The three patients with the greatest responses to treatment, in whom mean dystrophin fluorescence intensity increased from 8·9% (95% CI 7·1–10·6) to 16·4% (10·8–22·0) of normal control after treatment (p=0·0287). The three patients with the greatest responses to treatment, in whom mean dystrophin fluorescence intensity increased from 8·9% (95% CI 7·1–10·6) to 16·4% (10·8–22·0) of normal control after treatment (p=0·0287) were cohort 1 (dose 0·5 mg/kg), cohort 2 (dose 1·0 mg/kg), and cohort 3 (dose 2 mg/kg), respectively.

Interpretation The safety and biochemical efficacy that we present show the potential of AVI-4658 to become a disease-modifying drug for Duchenne muscular dystrophy.

Funding UK Medical Research Council; AVI BioPharma.

Introduction Duchenne muscular dystrophy is a progressive, severely disabling neuromuscular disease that affects one in 3500 newborn boys and causes premature death.1 In Duchenne muscular dystrophy, the open reading frame of the X-linked dystrophin gene (DMD) is disrupted by deletions (roughly 65%), duplications (10%), point mutations (10%), or other smaller rearrangements. Dystrophin is located underneath the sarcolemma and assembles with sarcosomal proteins such as dystroglycan, α-sarcoglycan, and neuronal nitric oxide synthase (NOS) to form the dystrophin-associated glycoprotein complex. The essential function of dystrophin in muscle is to connect the subsarcolemmal cytoskeleton to the sarcolemma by binding N-terminally to F-actin and C-terminally to β-dystroglycan. Loss of dystrophin results in inflammation, muscle degeneration, and replacement of muscle with fibroadipose tissue.2

In the mdx mouse, administration of 2′O-methyl-ribooligonucleoside-phosphorothioate (2′OMe) and phosphorodiamidate
morpholino oligomers (PMOs) identified PMOs as more effective for induction of exon skipping and restoration of long-lasting dystrophin production after intramuscular or intravenous administration.7 In the X-linked muscular dystrophy dog, PMO administration was followed by dystrophin restoration and clinical benefit without adverse reactions.8

Two proof-of-principle clinical trials in patients with Duchenne muscular dystrophy, who received one intramuscular administration of either 2′OMe or PMO targeted to skip exon 51, showed efficient dystrophin restoration. More recently, in an open-label, dose-escalation study in 12 boys with Duchenne muscular dystrophy,6 weekly subcutaneous injections of PRO051, a 2′OMe splice switching oligomer, at 0·5, 2, 4, and 6 mg/kg bodyweight for 5 weeks induced skipping of exon 51 and increased dystrophin concentrations. A 12-week extension with a dose of 6 mg/kg bodyweight of PRO0519 was well tolerated and was followed by stabilisation of muscle function, but no significant improvement in a 6-min walk test. We report biochemical efficacy and clinical safety from a dose-ranging study of the first intravenous systemically administered PMO, AVI-4658, in patients with Duchenne muscular dystrophy.

Methods
Study design and participants
This open-label phase 2 study was approved by the UK Medicines and Healthcare Products Regulatory Agency. The UK Gene Therapy Advisory Committee provided ethics approval and site-specific approval under the number GTAC157 (EudraCT number 2007-004695-39) for both active trial sites in London and Newcastle, UK. Additionally, the study was adopted by the institutional review boards at both sites. Participants were aged 5–15 years and had genetically confirmed diagnosis of Duchenne muscular dystrophy with an out-of-frame deletion eligible for correction by skipping of exon 51. The absence of additional deletions and variants in the splice switching oligomer duplex formation region was confirmed in all participants by dystrophin exon multiplex ligation-dependent probe amplification and DNA sequencing. Participants were enrolled after written informed assent from the child and written informed consent of a parent or legal guardian was obtained. Further study details are shown in the webappendix pp 1–3; the protocol is available online. If no original diagnostic muscle biopsy sample was available, a sample was obtained from the biceps brachii muscle (16 participants) and a post-treatment sample was taken from the contralateral biceps 2 weeks after the last dose of the study drug.

Figure 1 shows the dose escalation and cohort assignment. The data safety monitoring board met with clinical investigators and the sponsor to review safety before dose escalations took place. Each cohort led with a single patient and, after three doses, a safety monitoring committee consisting of clinical investigators, the sponsor medical monitor, and the medical study monitor reviewed all safety data from that patient before enrolment of subsequent patients to expand that cohort. Dose escalation occurred after informed discussion of the data safety monitoring board when all patients in the previous cohort had received at least 3 weeks of treatment.

Procedures
AVI-4658 (sequence CTCCAACATCAAGGAAGATGGC-ATTTCTAGC),10 an exon 51-targeting PMO, was provided by AVI BioPharma (Bothell, WA, USA) at 100 mg/mL in phosphate-buffered saline (1 mL/vial). It was diluted in phosphate-buffered saline (1 mL/vial). It was diluted in...
up to 50 mL saline (NaCl 0·9% v/w) for intravenous infusion over 1 h.

Exon 51 skipping in muscle biopsy samples was assessed by RT-PCR.9 Dystrophin expression in pretreatment and post-treatment muscle was investigated first by immunohistochemical detection of dystrophin with MANDYS106 (MDA Monoclonal Antibody Resource, Glenn Morris, Oswestry, UK) and Dys2 (Novacastra, UK) antibodies, initially assessed by two masked investigators. To control for the presence of trace levels of dystrophin in the pretreatment muscle, as well as revertant fibres,13,14 we set a baseline using sections of pretreatment muscles from each patient when counting dystrophin-positive fibres in post-treatment muscles, so that only any revertant fibres were seen as positive. We then used this threshold to count the dystrophin-positive fibres in sections of post-treatment muscle of that patient.2

The investigator had to be unmasked to which section came from the pretreatment and post-treatment biopsies. The semi quantitative measurements of dystrophin, α-sarcoglycan, and neuronal NOS expression levels were done as previously described.13 Finally, western blotting was done with the antidystrophin antibody Dys1 15 and quantified as explained in the webappendix p 4.

Inflammatory infiltrates in muscle biopsy samples were examined by immunohistochemistry with antibodies against CD3 pan T cells, CD4 T-helper cells, and CD8 cytotoxic T cells (webappendix p 3). Antidystrophin antibody induction in patients' sera was tested at week 1 and week 12. Finally, pharmacokinetic parameters of AVI-4658 were established from plasma and urine taken over 24 h at the first, sixth, and 12th doses. Experimental details are

<table>
<thead>
<tr>
<th>Cohort 1 (0·5 mg/kg)</th>
<th>Mutation*</th>
<th>Age (years)†</th>
<th>Weight (kg)†</th>
<th>6-min walk test at baseline (m)‡</th>
<th>Cardiomyopathy at recruitment</th>
<th>Dose and regimen of corticosteroid treatment</th>
<th>Other regular treatments taken at study entry</th>
<th>Serious or severe adverse events and relation to AVI-4658</th>
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<tr>
<td>P1 Del 48–50</td>
<td>9</td>
<td>31</td>
<td>410</td>
<td>No</td>
<td>Prednisolone 25 mg, intermittent (0·81 mg/kg per day)</td>
<td>···</td>
<td>···</td>
<td>···</td>
</tr>
<tr>
<td>P2 Del 45–50</td>
<td>8</td>
<td>29</td>
<td>254</td>
<td>Yes</td>
<td>Prednisolone 12·5 mg, daily (0·43 mg/kg per day)</td>
<td>Perindopril, calcium, vitamin D</td>
<td>···</td>
<td>···</td>
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<td>38</td>
<td>437</td>
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<td>Omeprazole</td>
<td>···</td>
<td>···</td>
</tr>
<tr>
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<td>139</td>
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<td>···</td>
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<td>Cohort 2 (1 mg/kg)</td>
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<td>250</td>
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<td>Prednisolone 22·5 mg, intermittent (0·86 mg/kg per day)</td>
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<td>···</td>
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<td>371</td>
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<td>Ranitidine</td>
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<td>···</td>
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<tr>
<td>Cohort 3 (2 mg/kg)</td>
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<td>47</td>
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<td>Prednisolone 15 mg daily (0·32 mg/kg per day)</td>
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<td>P8 Del 49–50</td>
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<td>38</td>
<td>350</td>
<td>No</td>
<td>Prednisolone 15 mg daily (0·4 mg/kg per day)</td>
<td>Ranitidine</td>
<td>Post-anaesthesia hospitalisation for 1 day due to vomiting, unrelated</td>
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<td>Cohort 4 (4 mg/kg)</td>
<td>P9 DelS2</td>
<td>10</td>
<td>30</td>
<td>301</td>
<td>Regional wall hypokinesia, normal FS</td>
<td>Prednisolone 15 mg daily (0·49 mg/kg per day)</td>
<td>Risedronate, calcium, vitamin D</td>
<td>Cardiomyopathy, possible</td>
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<td>62</td>
<td>146</td>
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<td>Not on steroids because of side-effects</td>
<td>···</td>
<td>···</td>
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<td>477</td>
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<td>···</td>
<td>···</td>
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<td>25</td>
<td>317</td>
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<td>Calcium, vitamin D</td>
<td>···</td>
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<td>22</td>
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<tr>
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<td>52</td>
<td>138</td>
<td>Yes</td>
<td>Deflazacort 30 mg daily (0·58 mg/kg per day)</td>
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<td>···</td>
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<tr>
<td>P15 Del 49–50</td>
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<td>39</td>
<td>169</td>
<td>No</td>
<td>Prednisolone 20 mg daily (0·5 mg/kg per day)</td>
<td>···</td>
<td>···</td>
<td>···</td>
</tr>
<tr>
<td>Cohort 6 (20 mg/kg)</td>
<td>P16 Del 45–50</td>
<td>9</td>
<td>31</td>
<td>515</td>
<td>No</td>
<td>Prednisolone 20 mg daily (0·65 mg/kg per day)</td>
<td>···</td>
<td>···</td>
</tr>
<tr>
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<td>25</td>
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<td>No</td>
<td>Prednisolone 17·5 mg daily (0·6 mg/kg per day)</td>
<td>···</td>
<td>···</td>
<td>···</td>
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<td>P18 Del 49–50</td>
<td>10</td>
<td>45</td>
<td>265</td>
<td>Yes</td>
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<td>Lisinopril</td>
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<td>405</td>
<td>No</td>
<td>Prednisolone 15 mg daily (0·5 mg/kg per day)</td>
<td>Calcium, vitamin D</td>
<td>···</td>
<td>···</td>
</tr>
</tbody>
</table>

Intermittent=10 days on and 10 days off treatment. FS=fractional shortening. *Deleted exons in the dystrophin gene. †At the first dose of AVI-4658. ‡Assessed a week before the first dose.

Table 1: Clinical summary
Mean plasma concentrations of AVI-4658 versus nominal elapsed time averaged across weeks 1, 6, and 12. Area under the curve (AUC) over 24 h accounted for greater than 95% of AUC_0–∞, suggesting that most of the drug eliminated from the plasma was cleared within 24 h. AVI-4658 plasma exposure increased in a nearly proportional manner with dose for maximum concentration, AUC_0–24, and AUC_0–∞. Error bars show SDs.

Figure 2: Plasma pharmacokinetics of AVI-4658
Mean plasma concentrations of AVI-4658 versus nominal elapsed time averaged across weeks 1, 6, and 12. Area under the curve (AUC) over 24 h accounted for greater than 95% of AUC_0–∞, suggesting that most of the drug eliminated from the plasma was cleared within 24 h. AVI-4658 plasma exposure increased in a nearly proportional manner with dose for maximum concentration, AUC_0–24, and AUC_0–∞. Error bars show SDs.

provided in the webappendix p 3; pharmacokinetic parameters were calculated with WinNonlin Professional (version 5.2.1). Muscle function testing (North Star ambulatory assessment, myometry, step activity monitoring, and 6-min walk test) was undertaken to identify any dose-dependent changes.

The primary objective of this study was to assess the safety of systemic administration of escalating doses of AVI-4658. The secondary objectives were to investigate the pharmacokinetics of AVI-4658 and the biochemical efficacy as assessed by induction of dystrophin expression in muscle. The functionality of restored dystrophin was assessed by study of both the relocalisation of dystrophin-associated glycoprotein complex proteins to the sarcolemma and the effect of restored dystrophin on muscle inflammation.

Statistical analysis
No formal sample size calculations were done. All descriptive statistical analyses were done with Graph Pad Prism 4 statistical software. We calculated p values using a paired t test to compare pretreatment versus post-treatment samples. The exact Cochran-Armitage trend test was used to show the dose response to AVI-4658.

The study was registered at ClinicalTrials.gov, number NCT00844597.

Role of the funding source
The UK Medical Research Council funded the study teams, patients’ travel, and the acquisition and analysis of the clinical and biochemical outcome measures. AVI BioPharma funded the regulatory submission, the study sponsorship including clinical and medical monitoring, data collection, and provision of the study drug. FM, KB, SC, and SBS had complete access to the data and FM as corresponding author was responsible for submission for publication.

Results
19 patients were enrolled within the UK: 12 at Great Ormond Street Hospital for Sick Children (London) and seven at the Royal Victoria Infirmary (Newcastle). Participants fulfilled eligibility criteria, had a mean age of 8.7 years (range 6–13), and had the clinical phenotype of Duchenne muscular dystrophy (table 1). A pretreatment muscle biopsy confirmed less than 5% revertant fibres in all patients. Adverse events were generally mild (63%) to moderate (32%), consistent with complications related to the disorder and to the paediatric age range, and showed no dose-dependent increase in frequency or severity (webappendix p 7). Difficult venous access largely due to cushingoid features resulted in failure to administer four of the planned 228 doses. Five other doses were not administered because of an adverse event in participant P9 leading to discontinuation of treatment after the seventh dose. In total, 219 doses of AVI-4658 were administered.

Participant P11 was enrolled to ensure that at least two participants in each cohort (dose) completed the dosing period and had a post-treatment muscle biopsy.

The 219 intravenous PMO administrations, including cannulation, were generally well tolerated. Laboratory safety assessments did not show any effect of AVI-4658 on pulmonary, kidney, liver, or bone-marrow functions. We did not identify any widespread rash or other clinical signs indicating an adverse reaction; four infusions were associated with a local rash due to application of local anaesthetic cream before venepuncture. Two serious adverse events were reported, both unrelated to the study drug (table 1). Participant P9 had normal cardiac fractional shortening (34%) before study entry, although mild regional impairment of the left ventricular inferior segment was detected on Doppler imaging. Over the first few weeks of study treatment, mild intermittent sinus tachycardia (maximum 125 beats per min) was noted. After the seventh dose, an echocardiogram revealed that his fractional shortening had fallen to 22%, and treatment with ACE inhibitor and β blocker was started. The investigators and study sponsor agreed to discontinue PMO administration and that this patient should not undergo general anaesthesia for the post-treatment muscle biopsy. At the final visit at week 26, his heart function had stabilised with a fractional shortening of 26%. This complication was judged most likely to be part of the well described pattern of cardiomyopathy associated with Duchenne muscular dystrophy. Cardio investigations in the remaining participants were consistent with findings in the disorder. Forced vital
Creatine kinase concentrations did not show any dose-dependent trends. There was no induction of anti-dystrophin antibodies after treatment (data not shown). Muscle function testing did not reveal any dose-dependent changes (webappendix pp 12–21, 25–27). These assessments showed that most patients remained stable during the study period; four lost ambulation during follow-up, as was expected on the basis of their ambulatory stage at study entry (table 1).

The plasma half-life of AVI-4658 was short (1.62–3.60 h; figure 2), and no accumulation between doses was recorded. Clearance was 233–615 mL/h per kg, and volume of distribution was 450–981 mL/kg. As a result of the small sample size, a clear relation between dose and clearance and volume of distribution could not be established. Renal clearance of AVI-4658 ranged between 116 and 229 mL/h per kg and was between 32.3% and 46.2% of total (plasma) clearance at doses between 0.5 and 4 mg/kg. At 10 and 20 mg/kg doses, renal clearance accounted for 60·5% and 63·8% of total clearance from the plasma in the first 24 h after dosing.

Experiments on MyoD-converted patient fibroblasts treated with a 2′OMe congener of AVI-4658 were done in all participants to confirm before the trial that the patients were able to correctly skip exon 51. This preliminary step was done with a 2′OMe antisense oligonucleotide because PMO cannot be used to transfect cells in culture, whereas 2′OMe can be efficiently delivered by transfection. The results of this experiment were positive in all patients’ cells and no qualitative differences in exon skipping between patients were noted (data not shown). When post-treatment muscle biopsies were assayed by RT-PCR, all patients had variable AVI-4658 induced skipping of exon 51, confirmed by sequencing (webappendix p 22).

Seven patients had a post-treatment increase in dystrophin protein expression compared with their pretreatment biopsies, evidenced by at least two of the three methods of quantification: number of dystrophin-positive fibres, western blotting, and semiquantitative immunocytochemistry measurements (figure 3, A–C).
There was no difference in α-sarcoglycan intensity between dystrophin-positive and dystrophin-negative fibres in the post-treatment muscle biopsy sample and neuronal NOS showed only a small increase in dystrophin-positive fibres (paired two-tailed t test, p≤0·0001; α-sarcoglycan mean intensity as percentage of control: dystrophin-negative fibres 45% [SD 16], dystrophin-positive fibres 52% [SD 15], p=0·0007). For participant P18, neuronal NOS and α-sarcoglycan intensity was significantly increased in the dystrophin-positive fibres (neuronal NOS mean intensity as percentage of control: dystrophin-negative fibres 7% [SD 7], dystrophin-positive fibres 28% [SD 17], paired two-tailed test, p=0·0001; α-sarcoglycan mean intensity as percentage of control: dystrophin-negative fibres 4% [SD 16], dystrophin-positive fibres 75% [SD 31], p=0·0001).

(A) Expression of dystrophin, α-sarcoglycan, and neuronal NOS in post-treatment muscle biopsy samples from participants P18 and P19 was quantified relative to control muscle in 40 dystrophin-positive or dystrophin-negative muscle fibres and normalised to β-spectrin expression. To overcome high background seen with the neuronal NOS antibody, the average background intensity of neuronal NOS-negative membranes was subtracted from control and patient values. For participant P19, there was no difference in α-sarcoglycan intensity between dystrophin-positive and dystrophin-negative fibres in the post-treatment muscle biopsy sample and neuronal NOS showed only a small increase in dystrophin-positive fibres (paired two-tailed t test, p=0·0007). For participant P18, neuronal NOS and α-sarcoglycan intensity was significantly increased in the dystrophin-positive fibres (neuronal NOS mean intensity as percentage of control: dystrophin-negative fibres 7% [SD 7], dystrophin-positive fibres 28% [SD 17], paired two-tailed test, p=0·0001; α-sarcoglycan mean intensity as percentage of control: dystrophin-negative fibres 4% [SD 16], dystrophin-positive fibres 75% [SD 31], p=0·0001). (B) Sarcolemmal restoration of the dystrophin-associated glycoprotein complex by AVI-4658. Post-treatment muscle biopsy samples from participants P19 and P18 were stained with antibodies against dystrophin (exons 4–3), MAN DYS106, α-sarcoglycan, neuronal NOS, and β-spectrin. The arrows show the same dystrophin-positive fibre in each panel. In P19 (deletion of exons 45–50) the fibre shown by the arrow has increased α-sarcoglycan sarcolemmal expression, but not neuronal NOS because this patient is deleted for part of the dystrophin neuronal NOS binding site. (C) Inflammatory infiltrates quantification on pretreatment and post-treatment muscle samples. Muscle sections were incubated with antibodies (DAKO, UK) raised against human CD3 (pan T cell), CD4 (helper T cell), and CD8 (killer T cell). For each section, the number of CD3-positive cells was represented as a percentage of the total number of muscle fibres. Participants with pretreatment and post-treatment values of zero are not represented. NOS=nitric oxide synthase.

**Table 2: Summary of response to AVI-4658**

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Dystrophin-positive fibres (% of total muscle fibres)</th>
<th>Mean fluorescence intensity per fibre*</th>
<th>Dystrophin expression assessed by western blotting</th>
<th>Response to AVI-4658†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment Post-treatment</td>
<td>Pretreatment Post-treatment</td>
<td>p value‡</td>
<td>Increase (%)</td>
</tr>
<tr>
<td></td>
<td>(% of control; SD)</td>
<td>(% of control; SD)</td>
<td></td>
<td>(% of control; SD)</td>
</tr>
<tr>
<td><strong>Cohort 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>1%</td>
<td>1%</td>
<td>5% (2.2)</td>
<td>8% (4.5)</td>
</tr>
<tr>
<td>P2</td>
<td>3%</td>
<td>0%</td>
<td>5% (2.3)</td>
<td>5% (1.6)</td>
</tr>
<tr>
<td>P3</td>
<td>1%</td>
<td>7%</td>
<td>5% (5.4)</td>
<td>5% (2.1)</td>
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<td>5% (0.0)</td>
<td>6% (1.7)</td>
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<td></td>
<td></td>
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<td>0%</td>
<td>0%</td>
<td>4% (1.9)</td>
<td>4% (1.3)</td>
</tr>
<tr>
<td>P6</td>
<td>5%</td>
<td>1%</td>
<td>8% (4.2)</td>
<td>6% (2.7)</td>
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<tr>
<td>Mean (SD)</td>
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<td>6% (2.8)</td>
<td>5% (1.4)</td>
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<tr>
<td>P7</td>
<td>1%</td>
<td>21%</td>
<td>5% (1.5)</td>
<td>13% (28)</td>
</tr>
<tr>
<td>P8</td>
<td>1%</td>
<td>5%</td>
<td>7% (3.4)</td>
<td>5% (2.1)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.0% (0.0)</td>
<td>13.0% (11.3)</td>
<td>6.0% (5.4)</td>
<td>12.0% (9.9)</td>
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<td>5%</td>
<td>4%</td>
<td>9% (7.2)</td>
<td>10% (6.7)</td>
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<td>1%</td>
<td>1%</td>
<td>8% (4.3)</td>
<td>11% (13)</td>
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<tr>
<td>Mean (SD)</td>
<td>3.0% (2.8)</td>
<td>2.5% (2.1)</td>
<td>8.5% (0.7)</td>
<td>10.5% (0.7)</td>
</tr>
<tr>
<td><strong>Cohort 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P12</td>
<td>3%</td>
<td>6%</td>
<td>9% (9.8)</td>
<td>17% (27)</td>
</tr>
<tr>
<td>P13</td>
<td>2%</td>
<td>6%</td>
<td>11% (4.5)</td>
<td>10% (4.2)</td>
</tr>
<tr>
<td>P14</td>
<td>0%</td>
<td>7%</td>
<td>10% (13)</td>
<td>13% (12)</td>
</tr>
<tr>
<td>P15</td>
<td>1%</td>
<td>15%</td>
<td>9% (3)</td>
<td>27% (24)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.5% (1.3)</td>
<td>8.5% (4.4)</td>
<td>9.8% (1.0)</td>
<td>16.8% (7.4)</td>
</tr>
<tr>
<td><strong>Cohort 6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P16</td>
<td>3%</td>
<td>5%</td>
<td>11% (1.6)</td>
<td>13% (1.4)</td>
</tr>
<tr>
<td>P17</td>
<td>3%</td>
<td>8%</td>
<td>9% (6)</td>
<td>10% (7)</td>
</tr>
<tr>
<td>P18</td>
<td>3%</td>
<td>55%</td>
<td>9% (4.2)</td>
<td>19% (1.7)</td>
</tr>
<tr>
<td>P19</td>
<td>5%</td>
<td>7%</td>
<td>10% (5.5)</td>
<td>13% (10)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3.5% (1.0)</td>
<td>18.8% (24.2)</td>
<td>9.8% (1.0)</td>
<td>13.8% (3.8)</td>
</tr>
</tbody>
</table>

Pre-treatment muscle biopsy samples from participants P1, P3, and P6 were diagnostic quadriceps samples at the time of diagnosis, all other samples were obtained from the biceps muscle. Participant P4, who had missed two PMO doses because of cannulation challenges, declined the post-treatment muscle biopsy, but completed the rest of the study. Participant P9 did not undergo general anaesthesia for the post-treatment muscle biopsy because of an adverse event (cardiomyopathy) and was discontinued from treatment after week 7. Participant P11 was enrolled in cohort 4 to substitute for P9. Therefore, only 17 of 19 post-treatment muscle biopsy samples were obtained and participants P4 and P9 are not listed in this table. PMO=phosphorodiamidate morpholino oligomer. *Mean fluorescence signal intensity of MANDYS10612 as percentage of control muscle (details in webappendix p 23). ‡Two-tailed t test comparing the mean fluorescence intensity in the pretreatment versus post-treatment biopsy sample for each patient; we assessed the dose response to AVI-4658 across cohorts using the Cochran-Armitage method and confirmed a significant linear trend of dose response leading to increase in dystrophin expression (responders with ++ or +++ with increasing dose (p=0·0203).
A Dystrophin (MANDYS106)

- **Control**
- **P19 del 45-50**
- **P18 del 49-50**

B Low dose CD3

- **0.5 mg/kg**
- **1 mg/kg**

C Low dose CD4

- **2 mg/kg**
- **4 mg/kg**

D Low dose CD8

- **10 mg/kg**
- **20 mg/kg**

E High dose CD3

- **p = 0.0007**

F High dose CD4

- **p = 0.0115**

G High dose CD8

- **p = 0.0001**

H Neuronal NOS

- **p = 0.0001**

- **p = 0.0007**
Articles

In these seven patients, mean dystrophin fluorescence intensity increased from 8·9% (95% CI 7·1–10·6) to 16·4% (10·8–22·0) of normal control after treatment (p=0.0287). In the low-dose cohorts 1 to 4, there was no increase in dystrophin expression, with the exception of participant P7 in cohort 3. However, six of eight patients in the two high-dose cohorts 5 and 6 showed an increase in protein expression. Three patients, one in each of cohorts 3 (P7), 5 (P15), and 6 (P18), had a very substantial response to AVI-4658, having 21%, 15%, and 55% dystrophin-positive fibres, respectively. These three patients had also 314%, 198%, and 110% increases in dystrophin fluorescence intensity compared with pretreatment biopsy on semiquantitative immunocytochemistry. Western blot analysis of these patients also showed an increase after treatment of protein levels from 2% to 18%, from 0·9% to 17%, and from 0% to 7·7% of normal muscle, respectively (table 2).

A dose-dependent significant linear increase in dystrophin expression was noted (table 2; exact Cochran-Armitage trend test, p=0.0203). Additionally, AVI-4658 administration induced significant increase of dystrophin expression on immunocytochemistry pretreatment versus post-treatment in cohorts 5 and 6 (paired two-sided t test, p=0·04). Participant P19 had dystrophin levels on blot of 12·3% of control muscle, but a smaller increase in fluorescence intensity and percentage of dystrophin-positive fibres, probably reflecting variability in different blocks of the muscle biopsy sample studied with the different techniques (table 2). The functional properties of restored dystrophin were confirmed by quantification of α-sarcoglycan and neuronal NOS expression. Dystrophin-positive fibres had roughly a 30% average increase in α-sarcoglycan expression and neuronal NOS expression. Dystrophin-positive fibres had roughly a 30% average increase in α-sarcoglycan expression and neuronal NOS expression. Dystrophin-positive fibres had roughly a 30% average increase in α-sarcoglycan expression and neuronal NOS expression.

Finally, the inflammatory infiltrate was investigated to establish whether dystrophin restoration had any effect on the prominent inflammatory response seen in Duchenne muscular dystrophy (figure 4B). A reduction in inflammatory infiltrates was recorded in cohorts 5 and 6, apart from in participant P15 in whom the CD8 cell count was increased, but not around dystrophin-positive fibres. Furthermore, this patient did not have antidystrophin antibodies. Despite the small number of samples, the significant reduction in CD3 cell count (paired two-tailed t test, p=0.0115) shows that restored dystrophin is tolerated by the immune system. The quotient between CD3% and dystrophin intensity in the seven patients who responded to treatment showed a significant reduction in the post-treatment muscle biopsy samples (one-tailed paired t test, p=0.0078), confirming the correlation with the increase in dystrophin expression.

Discussion

We show for the first time that repeated systemic administration of a PMO splice switching oligomer (AVI-4658) induces targeted exon skipping in skeletal muscle in patients with Duchenne muscular dystrophy, restoring correctly localised dystrophin at the sarcolemma (panel). The administration of AVI-4658 was very well tolerated, without clear drug-induced adverse events with single doses of up to 900 mg and cumulative exposure exceeding 10000 mg. The absence of drug-related adverse events after 12 weeks is encouraging, but caution is still needed because any splice switching oligomer would need to be given lifelong.

A clear and significant dose response was recorded in terms of dystrophin protein expression, leading to seven patients who responded to treatment at higher doses. This finding was accompanied by a significant reduction of inflammatory infiltrates in patients in the two highest dose cohorts. Patients with the highest levels of dystrophin also had increased sarcolemmal expression of proteins of the dystrophin-associated glycoprotein complex. This outcome included restoration of neuronal NOS to the sarcolemma in patients with deletions that did not disrupt the NOS binding site localised in spectrin repeats 16/17 of the rod domain. The restoration of neuronal NOS is beneficial for patients in whom ongoing muscle damage is compounded by paradoxical exercise-induced vasoconstriction as a result of dysfunctional fine-tuning of blood flow.

The reduced inflammation in muscle could be related to reduced necrosis due to improved sarcolemmal function with better resistance to mechanical load, induced by the restored dystrophin. We noted variable levels of protein restoration in the seven patients who responded to treatment and considered possible reasons for this variability. Variability due to specific deletions and intronic breakpoints of individual patients could not be detected in vitro, because the response to splice switching oligomer in all patients’ myotubes was qualitatively similar. The stability of the resulting protein might be relevant since the patients with the three greatest responses to treatment all had 49–50 deletions and mildly affected patients with Becker muscular dystrophy or even asymptomatic individuals with deletion of exons 49–51 have been described, suggesting that this shortened protein is highly functional. However two patients who did not respond to treatment (cohort 1, P3, who had no increase in either dystrophin-positive fibres or dystrophin expression, and cohort 3, P8, who had an increase in dystrophin-positive fibres, but not in dystrophin expression) and one with a small response (cohort 5, P12) also have the deletion 49–50. We noted variable levels of protein restoration in the seven patients who responded to treatment and considered possible reasons for this variability. Variability due to specific deletions and intronic breakpoints of individual patients could not be detected in vitro, because the response to splice switching oligomer in all patients’ myotubes was qualitatively similar. The stability of the resulting protein might be relevant since the patients with the three greatest responses to treatment all had 49–50 deletions and mildly affected patients with Becker muscular dystrophy or even asymptomatic individuals with deletion of exons 49–51 have been described, suggesting that this shortened protein is highly functional. However two patients who did not respond to treatment (cohort 1, P3, who had no increase in either dystrophin-positive fibres or dystrophin expression, and cohort 3, P8, who had an increase in dystrophin-positive fibres, but not in dystrophin expression) and one with a small response (cohort 5, P12) also have the deletion 49–50; additionally, patients with exon 45–50 deletions did not have more
protein than did patients with other genotypes, although asymptomatic individuals with 45–51 deletions are on record.15–19 An aspect to consider is the genetic background, which in humans is variable, including the intronic deletions breakpoints. A further variable could be differences in pharmacodynamics of PMO, although our analysis suggests that there was not a clear correlation between response and maximum concentration and area under the curve for AVI-4658. When concomitant treatments, age, and extent of muscle pathological changes were taken into account, no obvious pattern emerged. Immune response to novel dystrophin epitopes induced by exon skipping could be another reason for the variability; however, this explanation seems unlikely because we did not detect humoral immunity in any of the patients and furthermore we documented a reduction in T cells in muscle biopsy samples in cohorts 5 and 6, who showed the highest dystrophin levels.

In a recent systemic exon skipping trial in Duchenne muscular dystrophy using the 2′OMe chemistry, high numbers of dystrophin-positive fibres but low dystrophin levels were described.19 However, the study had no pretreatment muscle biopsy in patients in whom a dystrophin response was reported, making establishment of a proper baseline to distinguish minimally positive from negative fibres difficult. Indeed, in Duchenne muscular dystrophy, sections of muscle often have discernible trace levels after immunostaining in any of the antibodies to dystrophin used,19 and these pretreatment levels need to be taken into account to provide an accurate measurement of both number of positive fibres and dystrophin intensity18,19. In our study, we first established the level of dystrophin in the pretreatment muscle biopsy sample, and regarded those levels as the baseline for that individual—i.e., judged fibres as positive only if they exceeded the intensity levels of the pretreatment biopsy.

With respect to the variable dystrophin restoration we have reported, the most plausible conclusion is that stochastic events affect muscle splice switching oligomer targeting and contribute to variability. Animal models treated with both 2′OMe and PMO also showed targeting and contribute to variability. Animal models stochastic events affect muscle splice switching oligomer have reported, the most plausible conclusion is that exceeded the intensity levels of the pretreatment biopsy.

Sample, and regarded those levels as the baseline for that level of dystrophin in the pretreatment muscle biopsy.5,29 Because we did not detect humoral immunity in any of the patients and furthermore we documented a reduction in T cells in muscle biopsy samples in cohorts 5 and 6, who showed the highest dystrophin levels.

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With respect to the variable dystrophin restoration we have reported, the most plausible conclusion is that stochastic events affect muscle splice switching oligomer targeting and contribute to variability. Animal models treated with both 2′OMe and PMO also showed substantial variability of dystrophin expression, even in contralateral muscles from the same animal.29 Because splice switching oligomers do not target skeletal muscle specifically, their uptake is partly dependent on local events such as muscle perfusion, damage, and inflammation. To obtain more uniform protein production, either high doses of PMO or prolonged frequent administration, or both, could be considered because PMOs seem to be well tolerated.14 The safety profile we noted with AVI-4658 at doses of 20 mg/kg, supported by animal testing at up to human equivalent doses of 100 mg/kg,16 is encouraging and bodes well for longer administration periods and higher clinical dose. Preclinical data suggest that repeated administration of even small doses over an extended time achieves more homogeneous restoration of dystrophin than does the same cumulative dose administered as a bolus injection of PMO.15 This finding suggests that a long period of administration will be necessary to achieve homogeneous dystrophin expression.

In terms of the clinical efficacy, the PRO051 study16 claims that eight of 11 boys who were ambulant at entry to the extension study showed improvement in the 6-min walking test of 35·2 m (SD 28·7) after 12 weeks’ treatment; however, this change was not significant. Moreover, several of these children were younger than 7 years and, according to longitudinal observation,16 boys younger than 7 years with Duchenne muscular dystrophy gain motor function. Additional confounding factors are the variability in the walking test (SD 36 m16) and the

Panel: Research in context

Systematic review

We searched PubMed in March, 2011, using the keywords “Duchenne”, “antisense”, “exon skipping”, and “clinical trial”. Splice-switching oligomers have been tested previously after intramuscular injection in animal models and in patients affected by Duchenne muscular dystrophy.42–46 An open-label, dose-escalation study47 in 12 boys with Duchenne muscular dystrophy receiving weekly subcutaneous injections of the 2′OMe PRO051 at 0·5, 2, 4, and 6 mg/kg bodyweight for 5 weeks induced skipping of exon 51. Low dystrophin levels were reported after treatment, although the absence of pretreatment samples makes precise quantification of the biochemical efficacy of PRO051 difficult. This study was followed by a 12-week extension study using a dose of 6 mg/kg bodyweight of PRO051, with stabilisation of muscle function, but no significant improvement in a 6-min walk test.

Interpretation

We report for the first time that the systemic administration of a splice-switching oligomer based on PMO chemistry (AVI-4658) induced restoration of dystrophin expression in skeletal muscle of boys with Duchenne muscular dystrophy. The clinical and laboratory safety data in our open-label, dose-escalation, repeated intravenous administration study showed that AVI-4658 was well tolerated. Seven patients had a significant dose response, six of whom were in the two high-dose cohorts, showing restoration of dystrophin protein expression. This finding was associated with increased expression of proteins associated with dystrophin, such as α-sarcoglycan and neuronal nitric oxide synthase, the sarcolemmal localisation of which is disrupted in Duchenne muscular dystrophy. Additionally, we showed a dose-dependent reduction in the inflammatory infiltrate in muscles of boys with Duchenne muscular dystrophy in whom dystrophin expression was restored. This finding is encouraging because it suggests that the restored dystrophin attenuates the inflammation that is a hallmark of the disease’s pathology; it also suggests that the newly produced dystrophin does not produce novel immunogenic epitopes.
powerful placebo effect of open-label studies. Despite these limitations, this observation is encouraging.

In our study, boys remained mostly stable during follow-up, but because the period during which AVI-4658 was administered was only 12 weeks, we did not observe any significant clinical improvement, and the lack of a study extension was a limitation of our study, since only extended exposure to the drug is able to affect progression of the disease. Nevertheless, our results are very encouraging because they prove that doses of 10 mg/kg and 20 mg/kg of AVI-4658, which were very well tolerated, consistently induced dystrophin expression in the seven patients who responded to treatment up to levels typically found in patients with Becker muscular dystrophy or disease of intermediate severity between Duchenne muscular dystrophy and Becker muscular dystrophy. The restoration of the dystrophin-associated glycoprotein complex suggests that the produced dystrophin is functional. Because of the variability of dystrophin restoration, MRI or spectroscopy of muscle are promising methods to assess the effect of systemic treatment in Duchenne muscular dystrophy. Recent studies have described the correlation between MRI and the degree of dystrophic muscle changes,23 the effect of exercise in Duchenne muscular dystrophy,24 and reduction of muscle inflammation after POM treatment in dogs.25

On the basis of our data and recent preclinical data,26 we expect that extended administration of AVI-4658 at doses of 10 mg/kg or higher will result in sufficient dystrophin expression to have a positive effect on the prevention of muscle degeneration in Duchenne muscular dystrophy. Indeed, chronic administration (1 year) of doses of POMO similar to the one used in our study produced significant improvement in muscle pathology and function in mdx mice.27 AVI-4658 has the potential to ameliorate the progressive natural history of Duchenne muscular dystrophy and now needs to be investigated in clinical efficacy trials.

Contributors
SC, FM, KB, MG, and SBS designed and wrote the clinical trial protocol together with the amendments. SC and MG identified patients and coordinated clinical teams, executed the trial procedures, collected data, and followed up the study participants, under the supervision of FM and KB. SC, VAG, and JEM coordinated the collaborative work between clinical and laboratory teams and the biochemical work-up of clinical trial samples under the supervision of FM. FM, SC, MG, and KB obtained patient consent. JB oversaw and interpreted cardiac tests for the Newcastle cohort. SA was responsible of the acquisition of the genetic data. FM and KB managed the study budget. SC, VAG, JEM, and FM drafted the first report and have seen and approved the final version. DW, GD, MJAW, SDW, and VS contributed to the interpretation of results and drafting of the report. VAG and KA did RT-PCR, image capture, and dystrophin expression analysis. RK contributed to the design of the RT-PCR assay and to interpretation of results. ST did the western blot analysis. LF and CS processed muscle biopsy specimens and immunofluorescence staining and their analysis. MEG devised and oversaw the psychiatric assessments of the subjects and families. SC did the statistical analysis. SBS was responsible as sponsor’s medical officer for overseeing safe conduct of the study and participated in all safety monitoring discussions and data reviews. All authors contributed to the interpretation of results and drafting of the report and have seen and approved the final version.

Conflicts of interest
FM serves on scientific advisory boards for Acceleron Pharma, Genzyme, AVI BioPharma, Debiopharma Group, Prosensa, and Santhera Pharmaceutical, receives research support from AVI BioPharma, and has received funding for trials from AVI, Trophos, and PTC. KB has served on scientific advisory boards for Acceleron, AMT, AVI Biopharma, Debiopharma, Genzyme, GlaxoSmithKline, Prosensa, PTC, and Santhera and has received funding for trials from AVI and PTC. SBS is employed full time as Chief Medical Officer and Senior Vice President by AVI BioPharma and owns AVI stock. RK is employed full time as Senior Vice President and Distinguished Scientist by AVI BioPharma and owns AVI stock. VS has served on scientific advisory boards for Acceleron and Genzyme and has received funding for a trial from GlaxoSmithKline. GD and SDW hold patents in the area of exon skipping. MJAW serves as a scientific adviser for AVI BioPharma and Novartis. All other authors declare that they have no conflicts of interest.

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References


