Persistent Dystrophin Protein Restoration 90 Days after a Course of Intraperitoneally Administered Naked 2′OMePS AON and ZM2 NP-AON Complexes in mdx Mice

In Duchenne muscular dystrophy, the exon-skipping approach has obtained proof of concept in animal models, myogenic cell cultures, and following local and systemic administration in Duchenne patients. Indeed, we have previously demonstrated that low doses (7.5 mg/Kg/week) of 2′-O-methyl-phosphorothioate antisense oligoribonucleotides (AONs) adsorbed onto ZM2 nanoparticles provoke widespread dystrophin restoration 7 days after intraperitoneal treatment in mdx mice. In this study, we went on to test whether this dystrophin restoration was still measurable 90 days from the end of the same treatment. Interestingly, we found that both western blot and immunohistochemical analysis (up to 7% positive fibres) were still able to detect dystrophin protein in the skeletal muscles of ZM2-AON-treated mice at this time, and the level of exon-23 skipping could still be assessed by RT real-time PCR (up to 10% of skipping percentage). In contrast, the protein was undetectable by western blot analysis in the skeletal muscles of mdx mice treated with an identical dose of naked AON, and the percentage of dystrophin-positive fibres and exon-23 skipping were reminiscent of those of untreated mdx mice. Our data therefore demonstrate the long-term residual efficacy of this systemic low-dose treatment and confirm the protective effect nanoparticles exert on AON molecules.

1. Introduction

Duchenne muscular dystrophy (DMD) is an inherited X-linked degenerative muscle disorder mainly caused by frame-disrupting mutations following large rearrangements in the dystrophin gene [1]. DMD boys are affected by severe skeletal muscle wasting and cardiomyopathy. However, therapeutic approaches for this debilitating disease are now a realistic hope.
0.9 mg of AVI-4658 AON (PMO backbone), inducing exon-51 skipping in all cases [5, 6]. In phase I/II systemic clinical trials, AVI-4658 and PRO051/GSK2402968 have been administered by intravenous (i.v.) and subcutaneous (s.c.) injection, using incremental doses from 0.5 to 20.0 mg/Kg and from 0.5 to 6.0 mg/Kg, respectively [7, 8]. Although for the most part studies are ongoing, they have already revealed the absence of severe adverse effects, at least at the doses tested, and confirmed the therapeutic potential of specific exon skipping to induce dystrophin restoration in DMD patients.

Preclinical studies on the mdx mouse (the most frequently studied animal model of dystrophy) are also underway, with a view to identifying the most appropriate and safe delivery system for the AON molecules, irrespective of their chemical formulation. The aims of these studies are to (i) ensure more efficient muscle targeting, and (ii) define the optimal effective therapeutic AON dose that will enable the chronic life-long treatment required by DMD patients.

Rather inconveniently, the different chemical properties of the two AON backbones preclude the use of a universal carrier for efficient delivery. Nevertheless, recent studies have described the use of PEG-PEI copolymers and nonionic polymersomes as efficient carriers for local delivery of 2′OMePS AONs in mdx mice [9, 10]. Furthermore, a new formulation of PEG-PEI copolymer associated with functionalized derivatives containing either the cell-penetrating peptide TAT, adsorbed colloidial gold, or both, have also yielded promising results [11]. Grounds for optimism have also been provided by an approach exploiting a set of lipid nanoparticles with different compositions of cationic lipids and polyethylene glycol (PEG) when tested for their ability to deliver a luciferase siRNA to the liver via systemic administration in mice [12, 13]. Moreover, chitosan-coated nanoparticles have been used in mice as carriers for the in vivo delivery of active siRNA to papillary thyroid carcinoma by systemic administration [14]. Unfortunately, however, activation of the complement system by conversion of C3 into C3b in serum incubated with chitosan-coated nanoparticles is known to cause significant side effects [15]. Hopes are therefore pinned on a clinical trial involving the systemic administration of siRNA using targeted nanoparticles as a delivery system in patients with solid cancers that is currently underway [16].

Regarding our efforts in the field, we have previously demonstrated that nonviral biocompatible nanoparticles (NPs) (named T1 and ZM2) bind and deliver 2′OMePS M23D AON in mdx mice by systemic intraperitoneal (I.P.) injections. These complexes showed a body-wide distribution and efficiently induced dystrophin restoration in the skeletal muscles of the quadriceps, gastrocnemius and diaphragm, the arrector pili smooth muscle, and the cardiac muscle of the heart, as assessed by a routine cohort of biochemical outcome measures (skipping quantification, immunostaining, and positive fibres counting, as well as western blotting). Our results also suggest that these nanoparticles could afford protection to antisense RNA molecules [17, 18]. Moreover, the use of ZM2 nanoparticles in particular has allowed us to employ very low doses of M23D AON (7.5 mg/Kg/week, 52.5 mg/Kg in total), and to observe the efficacy of this systemic treatment at 1 week after the last injection [18].

In this further study, we tested whether the protective effect of ZM2 nanoparticles on AON molecules noted was still measurable at 3 months from the end of the same low dosage treatment (52.5 mg AON/Kg in total) and whether the restored dystrophin protein was detectable by the same outcome measures employed in our previous work [18]. Encouragingly, we discovered the long-term persistence of reframed dystrophin protein expression in the skeletal muscles of mdx mice following treatment with the same low dosage of M23D AON, corresponding to 1/20 of the effective I.P. dosage described for naked AONs [19]. We show that the effect of ZM2–AON on dystrophin expression persists up to 3 months (western blot positive, skipping percentage up to 10%, and immunofluorescence positive fibres up to 7%) from the suspension of treatment, contrasting with results yielded by an identical naked AON dose regimen (western blot negative, skipping percentage, and immunofluorescence positive fibres reminiscent of untreated mdx mice).

2. Materials and Methods

2.1. Animals. All experiments were performed on male mdx mice (C57BL/10ScSn–Dmdmdx/J) and age-matched wild-type (WT) male mice (C57BL/10SnJ). All procedures were approved by the Animal Experimentation Ethics Committee. Mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in temperature-controlled rooms (22°C) at a humidity level of 50% and a 12:12 hour light–dark cycle.

2.2. ZM2-M23D AON-Loading Experiments. ZM2s are cationic core-shell nanoparticles made up of a PMMA core surrounded by a random copolymer shell consisting of units derived from N-isopropyl acrylamide+ (NIPAM) and reactive methacrylate-bearing cationic groups [18]. M23D AON, which contains a 2′-O-methyl RNA phosphorothioate backbone, was synthesized as previously described [17]. Loading experiments showed that ZM2 nanoparticles (1 mg/mL) adsorbed 2′OMePS M23D oligoribonucleotide onto their surface in the concentration range 10–100 μg/mL. M23D adsorption onto ZM2 nanoparticles is a highly reproducible process with a loading value of 90 μg/mg [18].

2.3. I.P. Injections of ZM2-M23D AON Complexes or Naked M23D AON in mdx Mice. Mdx male mice (6 weeks of age) were I.P. injected with either 200 μL of ZM2-M23D complex containing 225 μg of M23D AON and 2.5 mg of ZM2 nanoparticles dissolved in sterile, unpreserved saline solution (0.9% sodium chloride), or 200 μL containing 225 μg of M23D dissolved in unpreserved saline solution (naked AON) and monitored according to approved NIH and University guidelines. A summary of the injection schedule is shown in Table 1. Four additional mdx mice were injected with 200 μL containing 2.5 mg of ZM2 NPs dissolved in sterile unpreserved saline solution (0.9% sodium chloride). The
total amount of M23D AON received by each animal was 7.5 mg/Kg/week, 52.5 mg/Kg in total. One group of mdx mice (n = 4) received seven injections of ZM2-M23D complex once a week for seven weeks, the second group (n = 4) received seven injections of M23D–naked AON, and the third (n = 4) received seven injections of ZM2 NPs at the same times. Four noninjected (not treated) age-matched mdx mice were used as controls.

2.4. RNA Studies. Total RNA was extracted from frozen sections of muscle biopsies using TRIzol (Invitrogen, Milan, Italy), and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Italy), and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Frankfurt, Germany).

Exon-specific real time assays (ESRAs) [20] were used on exons 8, 23, and 25 to quantify the percentage of exon-23 skipping in treated, with respect to untreated mice (ΔΔCt method), using β-actin as endogenous control, as previously described [17].

2.5. Immunofluorescence Analysis. At 12 weeks after the last injection, all mdx mice were killed, and their diaphragm, quadriiceps and cardiac muscles isolated, blotted dry, trimmed of external tendon, snap-frozen in liquid N2-cooled isopentane, and stored at −80°C until further processing. Seven-micrometer-thick frozen transverse sections were cut from at least two-thirds of the length of the heart, diaphragm and quadriceps muscles, and for each muscle at least 5 slices were cut at 150 μm intervals.

To analyse dystrophin restoration, freshly cut muscle sections were labelled with a polyclonal antidystrophin antibody (H-300, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and revealed with anti-rabbit Cy3-conjugated secondary antibody (Jackson Immunoresearch, Suffolk, UK). For the semiquantitative analysis of dystrophin, all samples were double-labelled with a rat monoclonal antibody for laminin-2 (a2-chain, 4H8-2, diluted 1:500; Alexis Biochemicals, Farmingdale, NY, USA), followed by anti-rat Cy2-conjugated secondary antibody (Jackson Immunoresearch, Suffolk, UK). The percentage of fibres with a dystrophin labelling that covers at least 80% of the sarcolemma perimeter was then calculated in treated (ZM2-AON and naked AON) compared to untreated (NT) mdx mice [17]. At least 1000 muscle fibres obtained from five different levels of tissue blocks for each sample were studied for statistical evaluation. All images were observed with a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments, Florence, Italy) connected to a high-resolution CCD camera (Nikon Instruments, Florence, Italy) at 20x magnification. For statistical analysis, all the data were analysed by means of Student’s t-test.

2.6. Tissue Morphology Analysis. Sections obtained from several blocks of kidney of mdx mice treated with ZM2-AON and untreated mdx (control) were stained with haematoxylin and eosin (H&E) (Sigma-Aldrich, Milan, Italy) and anticollagen I antibody (diluted 1:80, Abcam, Cambridge, UK), and observed at 10x and 20x with a Nikon Eclipse 80i microscope. Images were recorded with a Nikon digital camera.

2.7. Western Blot Analysis. Western blot analysis was performed as previously described [17]. Briefly, twenty-micrometer-thick frozen muscle sections were homogenized with a lysis buffer (7 mol/L urea, 2 mol/L thiourea, 1% amidosulfobetaine-14, and 0.3% dithioerythritol). Aliquots of proteins from wild type mice (15 μg) and from the muscles of treated or untreated mdx mice (150 μg) were loaded onto a 6% sodium dodecyl sulphate–polyacrylamide gel and separated by electrophoresis. Samples were transferred to a nitrocellulose membrane at 75 V, and the membrane was incubated overnight at 4°C with the specific antibody DYS2 (NovoCastra, Newcastle, UK). To quantify the restoration of dystrophin protein in treated versus wild-type mice, a densitometric analysis of autoradiographic bands was performed with a Bio-Rad Densitometer GS 700 (Bio-Rad, Milan, Italy), followed by normalization with the quantity of total protein loaded onto the gels.

3. Results

3.1. I.P. Injections of ZM2–AON Complexes and Naked AON in mdx Mice. 12 mdx male mice were treated (from 6 weeks of age) via intraperitoneal injections once a week for 7 weeks: 4 mice received the ZM2–AON complex (7.5 mg/Kg/week of AON), 4 mice received naked AON (7.5 mg/Kg/week of AON), and 4 mice received the ZM2 NPs. Controls were 4 age-matched mdx mice that did not receive any treatment. All animals were sacrificed 12 weeks after the last injection (at 6 months of age). Table 1 summarizes the treatment and sacrifice of the mice analysed in the present study.

Intraperitoneal injection was adopted as the best administration route after preliminary studies showed that i.v. injections of nanoparticles via caudal vein induced venous lesions and subsequent necrotic areas in the tail in a significant number of mice, probably due to obstructing nanoparticle aggregates; furthermore, subcutaneous injection resulted in local accumulation with no circulation of nanoparticles and no AON release (Ferlini A. and Rimessi P., unpublished observations).

3.2. Dystrophin RNA Analysis: Evaluation of Exon-23 Skipping in mdx Mice. In order to test the efficacy of the two treatments, we conducted three different experiments to assess exon-23 skipping levels, using a Real-Time PCR exon-specific assay (ESRA) of our own devising as previously reported [17, 21]. Skipping percentages were calculated in treated with respect to untreated mice.

Figure 1 summarizes the results obtained in the quadriceps, diaphragm and heart. Small percentages of exon-23 skipping were detected in all muscles from ZM2–AON- and naked AON-treated mice: 9.5% in the diaphragm, 7.8% in cardiac muscle and 6.3% in the quadriceps of mice receiving ZM2-AON treatment, and 6.5% in the diaphragm, 3.3% in cardiac muscle and 2.6% in the quadriceps of those treated with naked AON. Only in the diaphragm and quadriceps
TABLE 1: Injection schedule.

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<thead>
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<th>Group (n° of mice)</th>
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<td>225 μg/injection (7.5 mg/Kg/week)</td>
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<td>ZM2-AON (n = 4)</td>
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<td>ZM2 (n = 4)</td>
<td>ZM2 2.5 mg/injection</td>
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were the differences between the treatment with or without ZM2 nanoparticles statistically significant. In fact, in the cardiac muscle from naked AON- and ZM2–AON-treated mdx mice, the values of exon-23 skipping (ranging from 3.3% to 7.8%) were similar to the physiological skipping present in untreated mdx mice (Figure 1). In muscles from mice receiving ZM2 uncomplexed NP, the skipping percentages were similar to those measured in untreated mdx mice (data not shown).

3.4. Tissue Morphology Analysis. Tissue morphology analysis of mdx mice treated with ZM2–AON versus untreated mdx (control) showed no apparent tissue damage, an absence of morphological alterations, no cell architecture modifications, and an absence of inflammatory infiltrates in the kidney in either group (Figure 4).

3.5. Western Blotting. Western blot analysis, performed as previously described [17], confirmed the presence of high-molecular-weight dystrophin protein only in the quadriceps and diaphragms of ZM2–AON-treated mdx mice (Figure 5). Interestingly, no protein was detected in the muscles from mice treated with naked AON.

4. Discussion

These findings demonstrate the persistence of dystrophin protein in mdx mice systemically treated with 2′OMePS AON adsorbed onto ZM2 nanoparticles, 3 months after the
Figure 2: Immunofluorescence analysis. (a) Restored dystrophin is detectable and correctly expressed at the membrane of muscle fibres (quadriceps and diaphragm) from ZM2–AON-treated mdx mice. Traces are also visible in skeletal muscles from naked AON-treated mice, although the distribution at the sarcolemma is not continuous and very faint. Cardiac muscles were found to be negative for restored dystrophin. (Scale bar = 100 μm in quadriceps, 50 μm in diaphragms, 25 μm in heart). (b) Here are represented some enlarged details of immunofluorescence images that highlight the different expression patterns of rescued dystrophin in the quadriceps and diaphragm of ZM2–AON- and naked AON-treated mdx mice. These images show both a brighter dystrophin labelling and a more continuous distribution along the fibre membranes in muscles from ZM2–AON-treated mice (NT, untreated; WT, wild type).
Figure 3: Dystrophin-positive fibre counting. Dystrophin-positive fibre counting was performed on fibres with a labelling that covers at least 80% of the sarcolemma perimeter. Images were recorded and processed using identical exposure parameters. The graph shows the percentage of dystrophin-positive fibres detected in the muscles and heart from ZM2–AON- (black columns) and naked AON-treated (grey columns) mdx mice, calculated with respect to untreated mice, on sections representative of the total muscle. Quadriceps and diaphragm from ZM2–AON-treated mice show significantly higher percentages of dystrophin-positive fibres (7.2% in quadriceps and 4.5% in diaphragm) than the same muscles from mice treated with naked AON (reminiscent of untreated mdx mice). Negligible levels of dystrophin-positive fibres were detected in the heart of both treatment groups. Percentages of positive fibres in muscles from treated mice (ZM2-AON and naked AON) were calculated by subtracting the percentages detected in untreated mdx mice (up to 3%), considered as baseline (% positive fibres in treated mice − % positive fibres in untreated mice). The significance was calculated with respect to naked AON-treated mice (error bars represent mean ± S.E.M., n = 10, t-test, and *P < 0.05, **P < 0.001).

more difficult disease target to be reached, at least when using inefficient delivery systems or low doses of AONs [19, 24, 25]. Indeed the use of peptide-conjugated PMO molecules, in particular CPP-PMO and Pip5e-PMO, have been shown as very efficient at dealing with dystrophin restoration in the heart, due to the presence of stretches of arginines and a central hydrophobic core, which enhanced nuclear PMO delivery in cardiomyocytes [26–28].

5. Conclusions

In conclusion, this is the first study on phosphorothioate oligoribonucleotides, both naked and loaded onto NPs, documenting a long-lasting effect at such a low dose (7.5 mg/Kg/week, 52.5 mg AON/Kg in total). Indeed, this corresponds to 1/20 of the doses used for the mdx mouse in previous systemic studies [19, 29].

At this point in time, although encouraging enough to warrant clinical trials, the results obtained in mice with naked 2′OMePS AON seem to indicate that the doses required for a therapeutic effect might be too high for long-term treatment in humans. In fact, systemic naked AON delivery in animal models only yielded satisfactory levels of dystrophin rescue in both skeletal and cardiac muscles at amounts of AON ranging from 300 mg/Kg to 1250 mg/Kg [19, 30]. Nevertheless, better results have been reached with more moderate doses of PMO AONs, that is, roughly 600 mg/Kg [31].

Until now, the long-term persistence of restored dystrophin protein after low-dosage systemic treatment has only been achieved using naked morpholino or, more promisingly, molecules conjugated with a cell-penetrating peptide (CPP) containing a stretch of arginines [19, 32–34]. All these different AON formulations still raise safety implications, regarding their nondegradable nature (PMO, [9]), their possible toxicity (PPMO, [35]) and the renal overload (2′OMePS, [19, 36, 37]). This is especially true for long-life treatments as those required for DMD.

In this paper, we reemphasize our previous results regarding the use of ZM2 nanoparticles as a nontoxic vehicle for RNA molecules [18], and we show that the protective effect of the ZM2 remains at 3 months from the interruption of treatment. In fact, the dystrophin restoration
Nanoantisense oligonucleotides (AONs) interfere with the expression of dystrophin, which is a protein that is critical for muscle function. In this study, ZM2-AON complexes were used to treat mdx mice, a model for Duchenne muscular dystrophy, by inducing exon-23 skipping. The effects of these treatments were studied using various methods, including western blot analysis, immunofluorescence, and morphological assessment.

**Figure 5:** Western blot analysis. Immunoblotting for dystrophin using DY2 antibody showed restored expression of the protein in the quadriceps and diaphragm of ZM2–AON-treated mice (lane 4), while no protein was detected in either untreated control (NT, lane 2) or naked AON-treated mdx mice (lane 3). Dystrophin protein was undetectable in the hearts of both untreated and treated mdx mice. Quadriceps, diaphragms, and hearts from WT mice were used as positive controls (lane 1). For WT samples, the total protein loaded was 1/10 (15 μg) of the quantity in the other lanes (150 μg). Quantification performed by densitometric analysis of autoradiographic bands followed by normalization with the quantity of total protein loaded onto the gel showed a dystrophin recovery of 8 ± 0.6% in quadriceps and 7 ± 0.17% in diaphragm (P = 0.0002) of ZM2–AON-treated mice with respect to WT control mice. The results are representative of three separate experiments. Data are given as means ± S.E.M. and the statistical significance has been calculated with the Student’s t-test for unpaired data.

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**References**


A. Ferlini, P. Sabatelli, M. Fabris et al., "Dystrophin restoration

H. Yin, A. F. Saleh, C. Betts et al., "Pip5 transduction peptides
direct high efficiency oligonucleotide-mediated dystrophin


