

highly nuclease-resistant and thermodynamically stable, we reported that an antisense RNA/ENA chimera is 40 times as effective as corresponding conventional phosphorothioate oligonucleotides in inducing exon 19 skipping. To develop the broad therapeutic applicability of this exon skipping therapy, it is necessary to identify the specific antisense oligonucleotides that can skip exons located in the deletion hot spots in the *dystrophin* gene. We focus to induce skipping of exon 45 that is located in one of the deletion hot spots. We designed many antisense RNA/ENA chimeras against exon 45 and examined their ability to induce exon skipping. Here, we identified the best antisense RNA/ENA chimera could induce exon 45 skipping in cultured myotubes from DMD patients with deletion mutations neighboring exon 45. The production of functional dystrophin in the treated myotubes was also confirmed. It is proposed that antisense RNA/ENA chimera can be applied to treat a broader spectrum of DMD cases.

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P3.02

AAV-U7snRNA mediated multi exon-skipping for Duchenne muscular dystrophy

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Most cases of Duchenne muscular dystrophy (DMD) are caused by mutations that disrupt the dystrophin mRNA reading frame. In many cases, skipping of a single exon could restore the reading frame, giving rise to a shorter but still functional quasi-dystrophin protein. It has previously been proposed to use small nuclear RNAs, especially U7snRNA, to shuttle antisense sequences designed to mask key elements involved in the splicing of targeted exons.

Our present project focuses on the optimisation of U7snRNA constructs to complete rescue of dystrophin by exon-skipping in DMD patients. In particular, we are investigating the multi exon-skipping of exons 45–55, which could rescue up to 63% of DMD patients with a deletion.

In order to achieve this multi-skipping, we have first developed U7snRNA constructs targeting every exon between 45 and 55. Each construct has been inserted into lentiviral vectors for *in vitro* analysis in myoblasts from DMD patients. After transduction of these cells with lentiviral vectors encoding the various U7 constructs, specific skipping of the targeted exon was confirmed by RT-PCR. In parallel, we demonstrated the efficacy of these constructs *in vivo* in transgenic mice carrying the entire human DMD locus (*hDMD mice*) after intramuscular injection of AAV vectors encoding the U7snRNAs. Based on *in vitro* and *in vivo* results, the U7snRNA constructs inducing the most efficient skipping for each targeted exon were selected and combined into multi-skipping vectors. These AAV vectors are currently being tested in DMD myoblasts and in *hDMD mice* and efficient skipping of up to four exons has been demonstrated thus far.

These very encouraging results provide evidence that efficient multi exon-skipping can be achieved *in vitro* and *in vivo* using AAV vectors encoding multiple U7snRNAs. These new constructs offer therefore very promising tools for clinical treatment of DMD.

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P3.03

Nanoparticles are effective vehicles for systemic delivery of 2'OMePS antisense oligonucleotides in exon skipping-mediated dystrophin restoration

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We used a novel PMMA/N-isopropyl-acrylamide+ (NIPAM) cationic nanoparticle (ZM2-NPs) for systemic delivery of 2'OMePS AONs in the mdx animal model. Six weeks-old mdx mice were intraperitoneally treated: group 1 received 225 µg of naked M23D AON and group 2 received of 225 µg of M23D AON conjugated with 2.5 mg of ZM2 NPs. Four non-injected mdx mice were used as controls. The mice were injected weekly for 7 weeks (7.5 mg/kg/injection) and sacrificed 1 week (4) and 12 weeks (4) after the final administration. In treated animals sacrificed 1 week and 12 weeks after final administration we observed restored dystrophin protein synthesis in arrector pili skeletal and cardiac muscles (only for 1 week sacrificed animals), allowing protein localization in up to 40% of muscle fibers. The mdx exon 23 skipping level was up to 20% and 8% respectively. We measured the dystrophin transcript amount in all animal groups by four exon specific real time PCR assays (ESRAs). In non-treated mice, we found that the dystrophin transcript amount ranged 20–30% in all muscles of younger mice (7 weeks of age), whereas it was lower in skeletal muscles (10–20%) and higher in the heart (up to 30%) in older mice. In ZM2-AON or naked AON treated animals sacrificed 1 week after final administration, the dystrophin transcript amount did not vary significantly. Differently, in 12 weeks/sacrificed ZM2-AON treated animals, the transcript level was significantly higher in skeletal muscles but reduced in the heart. In mice treated with naked AONs the amount of transcript was higher also in the heart. In conclusions, ZM2-AON complexes are able to induce dystrophin restoration, which still persists in skeletal muscles, though at low level, 12 weeks after final administration. The basal dystrophin transcription amount in the heart seems to be age-related, having a different behavior during AON therapy.

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P3.04

Skipping of exons 6 and 8 of the DMD gene has been achieved in myogenic cells from an exon-7 deleted DMD patient: direct application of antisense sequences found in study with canine muscular dystrophy

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Exon skipping approach using anti-sense oligonucleotide (AO) is a promising therapeutic approach for Duchenne muscular dystrophy (DMD). The multi-exon skipping could theoretically rescue up to 63% of DMD patients. We have previously tried a systemic delivery of phosphorodiamidate morpholino oligomer (PMO) AO targeting exons 6 and 8 of the DMD gene in canine X-linked muscular dystrophy in Japan (CXMDJ), which harbors a point mutation within the 3' splice site of intron 6. We successfully achieved recovery of dystrophin

expression throughout the body with improvement of muscle function and pathology (Ann Neurol 2009;65:667–76). To date, AO used for exon skipping in DMD animal models have not been directly applied to DMD patients having the same exon deletions. We recently experienced a DMD patient with exon 7 deletion, therefore, we evaluated the multi-exon skipping in cultured cells derived from the patient and compared the efficiency with cells from CXMDJ. We isolated fibroblasts from skin biopsies of CXMDJ and exon 7 deleted DMD patient, and converted them to myogenic cells by MyoD transduction. PMO cocktails with antisense sequences targeting exons 6 and 8 were administered to canine or human myogenic cells, respectively. Skipping of exons 6 and 8 were achieved with similar efficiency between canine and human cells, but skipping of exon 9 was different between these cells, which did not alter the reading frame. The similarity of exon sequences of targeted region, and the antisense design methodology targeting identical sequence, may assure similar skipping efficiency between dogs and humans. This study shows directing an AO to the same motif in both humans and dogs induce comparable exon skipping, provides a proof of concept for systemic multi-exon skipping therapy for the DMD patient with exon 7 deletion using the AO sequences optimized in CXMDJ.

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P3.05

Checking exon-skipping events in candidates for clinical trials of morpholino

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Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are caused by abnormalities in the dystrophin gene. DMD, which manifests as a severe muscle weakness phenotype, results from an out-of-frame deletion(s) in the dystrophin gene. In contrast, BMD, which results from an in-frame deletion(s) in the dystrophin gene, causes a milder muscle weakness. Antisense-mediated exon-skipping, which changes out-of-frame deletions to in-frame deletions, is a promising therapeutic approach for DMD. Morpholino, a type of antisense molecule, is a powerful tool because it has higher affinity for the target nucleic acid sequences and greater resistance to degradation than conventional nucleic acids. It is important to confirm whether the morpholino, which has been tested in animal models, is effective in cells from clinical trial candidates. Fibroblasts from DMD patients with deletions of exons 45–50, 48–50, 49–50 and 50 of the dystrophin gene were isolated, induced to differentiate into the myogenic lineage by MyoD expression, and transfected with antisense morpholino; then, the exon-skipping event was checked by RT-PCR and sequence analysis. One of the two types of antisense molecules did not induce exon-skipping, which was the result that we anticipated. We also evaluated the quantity of dystrophin in morpholino treated cells by Western blotting analysis. Additionally, the contractile response of morpholino treated cells and non-morpholino treated cells were compared in order to analyze dystrophin function after inducing by KCL depolarization. The result shows the morpholino treated cells were more contractile than non-morpholino treated cells.

In this study, we developed an in vitro system that can easily screen the effectiveness of antisense sequences. This system can be used to identify patients who are good candidates for the therapy.

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P3.06

Evaluation of the truncated products of exon and multiple exon skipping in DMD therapy

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Duchenne's muscular dystrophy (DMD) is a severe muscle wasting disorder affecting 1/3500 male births. Lack of dystrophin in skeletal muscle compromises the integrity of the muscle membrane and thus muscle fibres are prone to contraction induced injury. Further rounds of muscle degeneration and regeneration leads to the replacement of muscle fibres with non contractile fatty/fibrotic tissue. These alterations lead to progressive muscle wasting, weakness and death in late adolescence.

Gene therapy for DMD have been hampered by a number of factors (e.g. gene transfer efficiency, immune responses). A promising therapeutic approach for DMD is antisense-mediated exon skipping; antisense oligonucleotides (AONs) targeting specific exons to restore the DMD reading frame. The resultant truncated forms of dystrophin, restore the integrity of the muscle cell membrane and improve muscle pathology. An ideal therapy could target multiple exons, thereby treating many more patients whilst still producing a partially functional truncated dystrophin protein product. Some of these AONs are currently in clinical trial for single exon skipping.

To evaluate the therapeutic value of these therapies, different inframe truncated human dystrophins have been generated; these represent the dystrophins created by skipping different single exons or skipping multiple exons currently being investigated. The truncated dystrophins were electro-transferred into mdx mice muscle with a subset of constructs have been used to generate transgenic mice. Truncated dystrophins, akin to multiple exon skipping products, localise to the sarcolemma and re-establish the dystrophin associated protein complex. Further histological and functional data will be presented.

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P3.07

Preclinical safety of AVI-4658, a phosphorodiamidate morpholino oligomer (PMO) being developed to skip exon 51 in Duchenne muscular dystrophy

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Background: AVI-4658 is a PMO that skips dystrophin exon 51, restores the reading frame and enable dystrophin expression in selected DMD patients, proven by a single IM dose study in the UK. To enable clinical trials in the US, three 12-week GLP studies in animals were performed. Published data suggests the older phosphorothioate antisense oligonucleotides have dose limiting toxicities. **Objective:** The current studies were designed to assess the safety of AVI-4658 (and PMO in general). **Method:** (1)mdx mice were dosed IV with 0, 12, 120 or 960 mg/kg (the maximum feasible dose (MFD)), or subcutaneously at 960 mg/kg; wild type C57 mice at 0 and 960 mg/kg (i.e., 7 groups) with AVI-4658. (2) A second identical study with AVI-4225, the PMO to skip exon 23 of in the dystrophic mouse and restore dystrophin (looking for mechanistic toxicity), was also performed. (3) Cynomolgus monkeys were dosed IV with 0, 5, 40 or 320 mg/kg (MFD) and 320 mg/kg subcutaneously. A 28 day recovery period was included in all studies. **Results:**