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Identification of Potent, Muscle-Targeting Investigational Stereopure Oligonucleotides for Exon 53 DMD Therapy

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Summary

- Wave is developing investigational exon 53 skipping oligonucleotides as a potential disease-modifying therapy for the treatment of patients with Duchenne muscular dystrophy (DMD) amenable to skipping of exon 53 of the *dystrophin (DMD)* gene.
- Stereopure oligonucleotides, which are intended to target exon 53 *DMD* pre-mRNA, induced dose-dependent skipping in up to 50% of transcripts and dystrophin protein restoration up to 70% *in vitro* in DMD patient-derived Δ45–52 myoblasts compared with healthy human myoblasts.
- Wave's optimized stereopure oligonucleotides demonstrated entry into muscle cell nuclei *in vivo*
- In *mdx23* mice, exon skipping and dystrophin protein restoration were observed at 7 days and for at least 28 days following a single intravenous (IV) dose of a stereopure oligonucleotide. Four weekly IV doses achieved dystrophin restoration of 70%–90%, as quantified by western blot, compared with wild-type control. Creatine kinase levels were reduced by 87% compared with untreated mice. The same principles were used to optimize the chemical configuration and backbone stereochemistry of both the exon 53 targeting and exon 23 targeting sequences.
- These studies support further development of Wave's stereopure oligonucleotide targeting exon 53 as a potential treatment for DMD patients amenable to exon 53 skipping.

Introduction

- In DMD, a fatal, X-linked neuromuscular disorder, mutations in the *DMD* gene result in absent or defective dystrophin protein.[1]
- Antisense oligonucleotides that promote exon skipping in *DMD* may enable partial restoration of dystrophin protein, which is expected to result in therapeutic benefits for patients with DMD.[1,2]
- Wave has developed a proprietary technology platform that is a targeted approach to designing stereopure nucleic acid compounds based on the interplay among sequence composition, chemical design, and backbone stereochemistry.
- Wave's technology platform enables the production of oligonucleotides that are stereopure, defined as precisely controlled chirality of each phosphorothioate internucleotide backbone linkage during chemical synthesis; control of stereochemistry enables more fine-tuned pharmacologic profiles for each target based on the desired therapeutic profile.[3]
- WVE-210201, an investigational antisense oligonucleotide that is designed to target exon 51 in the *DMD* gene, is currently being studied in a Phase 1 clinical trial in patients with DMD amenable to exon 51 skipping (ClinicalTrials.gov Identifier: NCT03508947).
- Wave has also designed stereopure oligonucleotides as a potential treatment for patients with *DMD* gene mutations amenable to exon 53 skipping. Here we present preclinical data on the potency of stereopure oligonucleotides targeting exon 53.

Methods

In Vitro Skipping and Dystrophin Protein Expression

- Cultured DMD patient-derived myoblasts or murine *mdx23*-derived myoblasts were treated with oligonucleotide under free-uptake conditions at 0.1 μM–10 μM in differentiation media in the absence of transfection reagents.
- After 4 days of oligonucleotide treatment, RNA was extracted with Trizol and skipping efficiency was evaluated by TaqMan® assays (Applied Biosystems) using custom primer-probe sets specific for the exon junction of skipped *DMD* transcripts or total *DMD* transcripts, normalized to an internal control (hSFSR9) for human myoblasts or to gene-block standard curves for murine myoblasts.
- After 6 days of oligonucleotide treatment, protein lysate was analyzed by western blot. Dystrophin signal was normalized to vinculin loading control and quantified according to a standard curve of primary human myoblast lysate (pooled from 4 donors) titrated into DMD myoblast lysate.

Immunogenicity Assessment

- HEK-Blue-hTLR9® cells (InvivoGen) were treated with oligonucleotides under free-uptake conditions for 16 hours and analyzed for TLR9 reporter activity.
- Human peripheral blood mononuclear cells (PBMCs) from 3 healthy donors were incubated for 24 hours with oligonucleotides; supernatant was analyzed by Luminex® multiplex assay (EMD Millipore) for quantification of 29 cytokines.

Nuclear Localization

- Dystrophin-deficient *mdx23* mice received a single intravenous (IV) dose of 30 mg/kg of oligonucleotide and were necropsied at 24 hours postdose. Oligonucleotide was visualized by ViewRNA® technology. Nuclei were stained with Hoechst33342 or hematoxylin.

In Vivo Exon 23 Skipping, Dystrophin Protein Expression, and Serum Enzyme Profiles in *mdx23* Mice

Treatment of *mdx23* mice was conducted as follows:

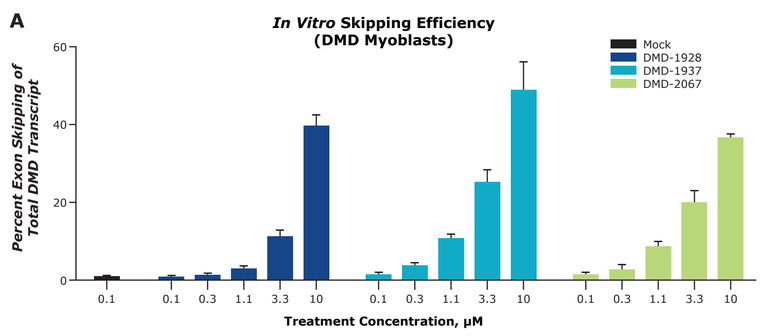
- Four weekly IV doses of 75 mg/kg or 150 mg/kg of oligonucleotide, necropsy at 4 days after the last dose
- Single IV dose 150 mg/kg of oligonucleotide, necropsies at 1, 3, 7, 14, and 28 days postdose
- Tissues were fresh frozen for RNA skipping analysis (as above) or western blot analysis (as above) or microscopy. Western blot standard curve was composed of CD1 wild-type mouse lysate titrated into untreated *mdx23* lysate. Immunofluorescent microscopy was performed with a polyclonal rabbit anti-dystrophin and DAPI nuclear stain.
- Serum and plasma clinical chemistry were measured with an Olympus AU640 (Olympus America) and the manufacturer's reagents and procedures, at Charles River Laboratory.

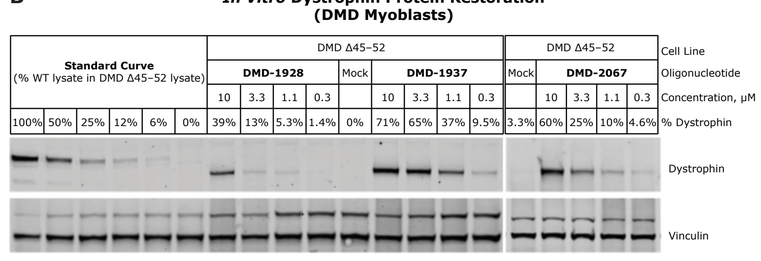
Results

In Vitro Potency

- Stereopure oligonucleotides induced dose-dependent exon 53 skipping *in vitro* in DMD Δ45–52 myoblasts after 4 days of treatment under free-uptake conditions (**Figure 1A**).
- Stereopure oligonucleotides also induced dose-dependent dystrophin protein restoration in DMD Δ45–52 myoblasts following 6 days of treatment under free-uptake conditions (**Figure 1B**).

Figure 1. Potency of stereopure oligonucleotides targeting exon 53 *in vitro*





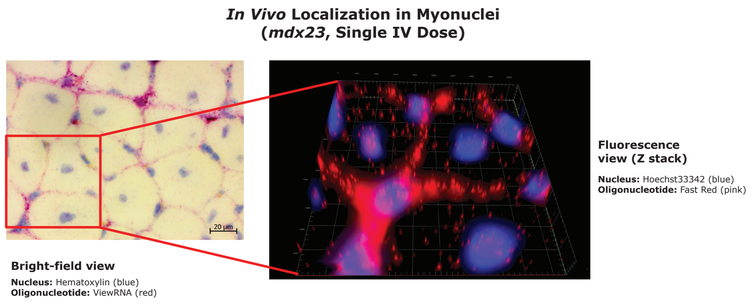
WT=wild-type human myotubes.

- Stereopure oligonucleotides targeting exon 53 did not activate a TLR9 innate immune response at doses of up to 30 μM; in addition, the oligonucleotides stimulated minimal to no activation of cytokines when incubated with human PBMCs at doses up to 30 μM (data not shown).

Nuclear Localization of Stereopure Oligonucleotides *In Vivo*

- To engage the target DMD transcripts to induce exon skipping, oligonucleotide therapeutics must reach the nuclei of muscle cells.
- A stereopure oligonucleotide targeting exon 53 reached the myonuclei in gastrocnemius tissue of treated *mdx23* mice within 24 hours following a single IV dose of 30 mg/kg (**Figure 2**).

Figure 2. Visualization of nuclear entry of stereopure oligonucleotide targeting exon 53 *in vivo*



IV=intravenous.
mdx23 mice received a single IV dose of 30 mg/kg of oligonucleotide; gastrocnemius was analyzed by ViewRNA at 24 hours postdose and imaged with bright field (left) or confocal fluorescence imaging (right).

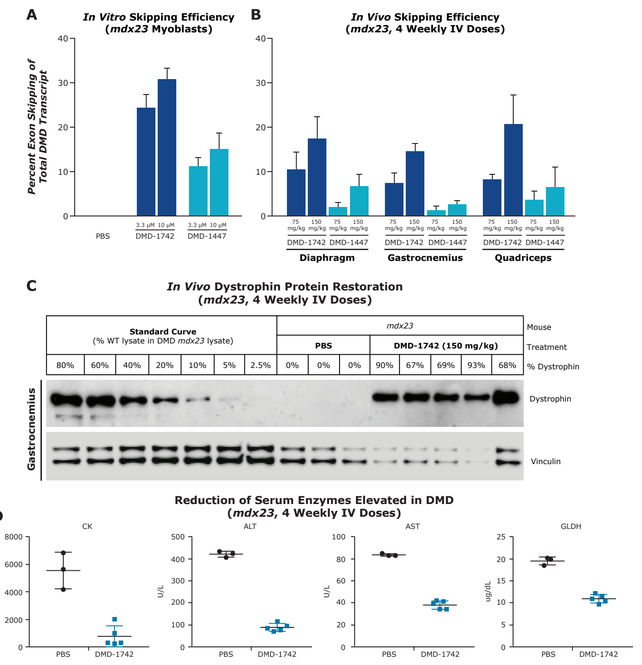
- Stereopure oligonucleotides targeting exon 53 were detectable by hybridization ELISA in the diaphragm, heart, gastrocnemius, and quadriceps of *mdx23* mice 24 hours, 7 days, and 14 days after receiving a single 30-mg/kg IV dose (data not shown).

Duration of Effect *In Vivo*

- Similar chemical design principles were applied to generate stereopure oligonucleotides targeting murine exon 23, to explore the potency and duration of effect of stereopure oligonucleotides for *in vivo* exon skipping and dystrophin protein restoration in the *mdx23* mouse model of DMD, and to explore the relationship between *in vitro* and *in vivo* models.
- Stereopure oligonucleotides targeting exon 23 induced dose-dependent exon 23 skipping *in vitro* in *mdx23*-derived myoblasts (**Figure 3A**) and in target muscle tissues *in vivo* in *mdx23* mice, including gastrocnemius, diaphragm, and quadriceps following 4 weekly IV doses (**Figure 3B**).

- Stereopure oligonucleotides targeting exon 23 induced dystrophin protein restoration in target muscle tissues, including gastrocnemius (**Figure 3C**), diaphragm, quadriceps, and heart (data not shown) following 4 weekly IV doses.
- Stereopure oligonucleotides targeting exon 23 reduced serum enzymes elevated in DMD. Creatine kinase (CK) levels were reduced by 87%.

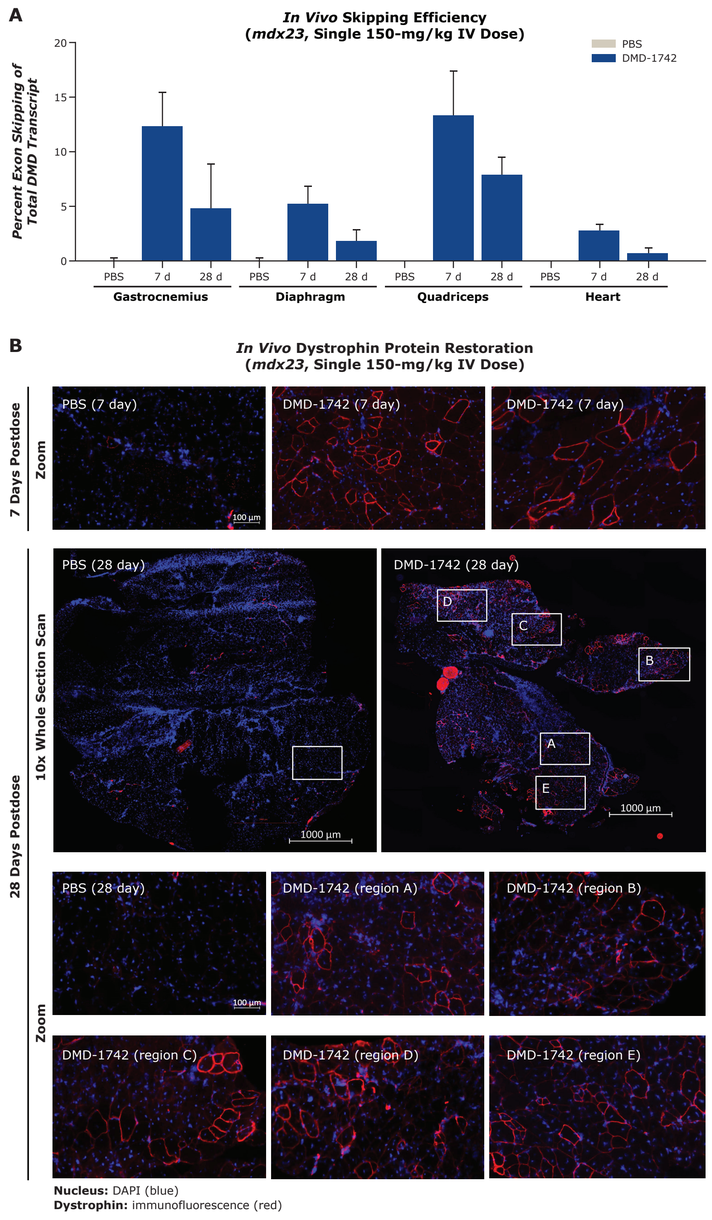
Figure 3. Potency of stereopure oligonucleotides targeting exon 23 *in vitro* and *in vivo*



ALT=alanine aminotransferase; AST=aspartate aminotransferase; CK=creatine kinase GLDH=glutamate dehydrogenase IV=intravenous; PBS=phosphate-buffered saline; WT=wild type.

- Single IV doses of stereopure oligonucleotides induced efficient exon 23 skipping in diaphragm, gastrocnemius, quadriceps, and heart that was observed for at least 28 days after administration (**Figure 4A**).
- Single IV doses of stereopure oligonucleotides induced dystrophin restoration for at least 28 days postdose in gastrocnemius of *mdx23* mice, as measured by western blot (data not shown).
- Single IV doses of stereopure oligonucleotides resulted in myofiber membrane localization of restored dystrophin for at least 28 days postdose in gastrocnemius tissue, as would be expected for functionally restored dystrophin (**Figure 4B**).

Figure 4. Durability of effect of stereopure oligonucleotide targeting exon 23 *in vivo* following single IV dose



IV=intravenous; PBS=phosphate-buffered saline.
(A) Exon skipping in indicated tissues analyzed by TaqMan.
(B) Microscopy of dystrophin expression and membrane localization in gastrocnemius cross-section. Blue=nuclei (DAPI stain); red=dystrophin (rabbit anti-dystrophin); 10× magnification.

References: 1. Mah JK. *Neuropsychiatr Dis Treat*. 2016;12:1795-1807. 2. Nakamura A. *J Hum Genet*. 2017;62(10):871-876. 3. Iwamoto N, et al. *Nat Biotechnol*. 2017;35(9):845-851.
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