Electronic Supplementary Material (ESI) for ChemComm.

Evaluation of Anhydrohexitol nucleic acid, Cyclohexenyl nucleic acid and D-Altritol nucleic acid-modified 2'-*O*-methyl RNA mixmer antisense oligonucleotides for exon skipping *in vitro*

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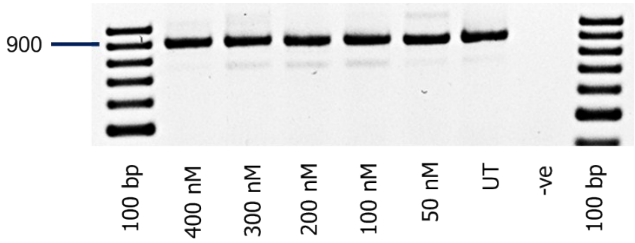


Fig. S1 RT-PCR analysis of exon-23 skipping in mdx mouse myotubes by 2'-OMePS mismatch AO showed no skipping.

Experimental Procedures

1. Design and synthesis of chemically modified antisense oligonucleotides (AOs)

All AOs (Table 1) were prepared in-house on an Expedite 8909 DNA synthesizer via standard phosphoramidite chemistry in 1 µmol scale. Synthesized oligonucleotides were deprotected and cleaved from the solid support by treatment with NH_4OH at 55°C overnight. The crude oligonucleotides were then purified, desalted and verified by MALDI-ToF MS analysis.

2. Cell culture and transfection

Immortalised *mdx* myoblasts (H2K cells, provided by Prof. Sue Fletcher and Prof. Steve Wilton's laboratory, Murdoch University, Australia) were cultured as described previously [1,2]. Briefly, when 60–80% confluent, primary *mdx* myoblast cultures were treated with trypsin (Life Technologies) and seeded at a density of 2×10^4 cells/well into 24 well plates. The plate was pre-treated with 50 μg/ml poly-D-lysine (Sigma) and 100 μg/ml Matrigel (Corning). Cultures were induced to differentiate into myotubes in Dulbecco's Modified Eagle Medium (DMEM) containing 5% horse serum by incubation at 37°C, 5% CO_2 for 48 hours. Antisense oligonucleotides were complexed with Lipofectin (Life Technologies) at the ratio of 2:1 (lipofectin:AO) and used in a final transfection volume of 500 μl/well in a 24-well plate as per the manufacturer's instructions, except that the solution was not removed after 3 hours.

3. RNA extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from transfected cells using Direct-zol™ RNA MiniPrep Plus with TRI Reagent® (Zymo Research) as per the manufacturer's instructions. The dystrophin transcripts were then analysed by nested RT-PCR across exons 20–26 as described previously [2]. PCR products were separated on 2% agarose gels in Tris–acetate–EDTA buffer and the images were captured on a Fusion Fx gel documentation system (Vilber Lourmat, Marne-la-Vallee, France). Densitometry was performed by Image J software.

4. Cell toxicity assay

Cells were seeded and transfected with AOs as described previously. After 24 hrs, cell viability was measured by WST-1 assay kit (Sigma). Briefly, WST-1 solution was added at ratio 1:10 (v/v) per well and incubated for 2 hrs at 37°C, 5% CO_2. The absorbance was measured with a microplate reader (FLUOstar Omega, BMG Labtech, Germany) at 450 nm.

5. *In vitro* stability study of antisense oligonucleotides

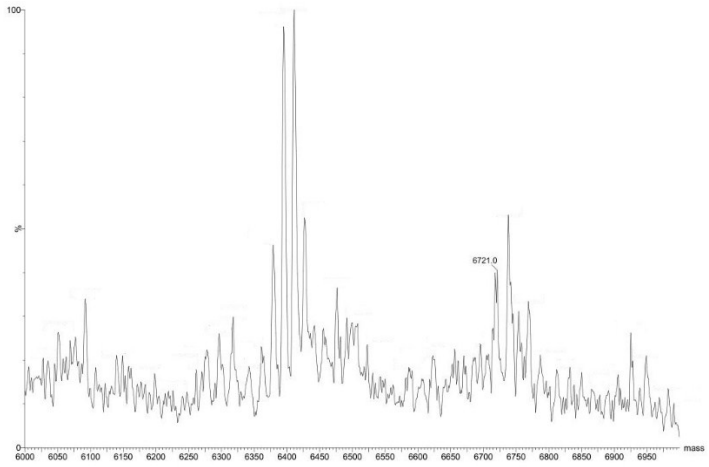
Nuclease stability of the oligonucleotides was assayed at 5 uM oligonucleotide using 0.8 units/ml snake venom phosphodiesterase (Sigma) in a buffer of 10 mM Tris-HCl, pH 8.9, 100 mM NaCl, and 15 mM $MgCl_2$ in a final volume of 60 ul. Briefly, 8 ul reaction aliquots were removed at the indicated times, added to an equal volume of 80% formamide containing bromphenol blue and xylene cyanol gel tracking dyes, and then heated for 5 min at 95 °C. Aliquots were then stored at -20 °C until analysis by 20% denaturing polyacrylamide electrophoresis. Quantitation was performed on a Biorad Chemidoc XRS imaging system.

7. MALDI-ToF analysis of antisense oligonucleotides

Spectra were acquire on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3uL/min and spectra were obtained in positive or: negative ionization mode with a resolution of 15000 (FWHM) using leucine enkephalin as lock mass.

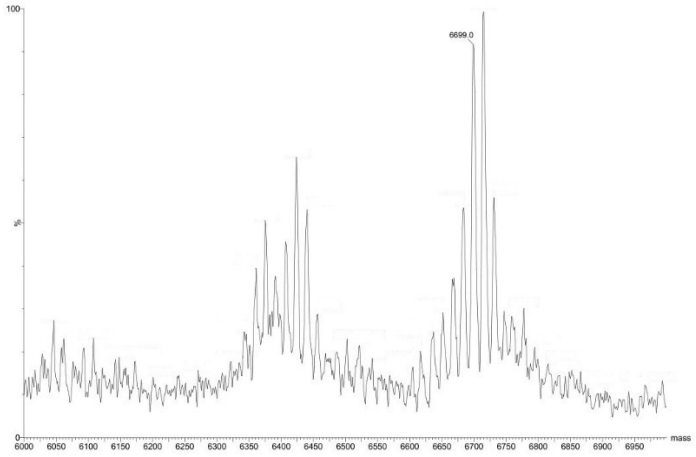
HNA/2'-OMePS:5'-hG mG mC mC hA mA mA mC hC mU mC mG mG hC mU hT mA mC mC hT-3'

Calcd 6720,5; found 6721,0



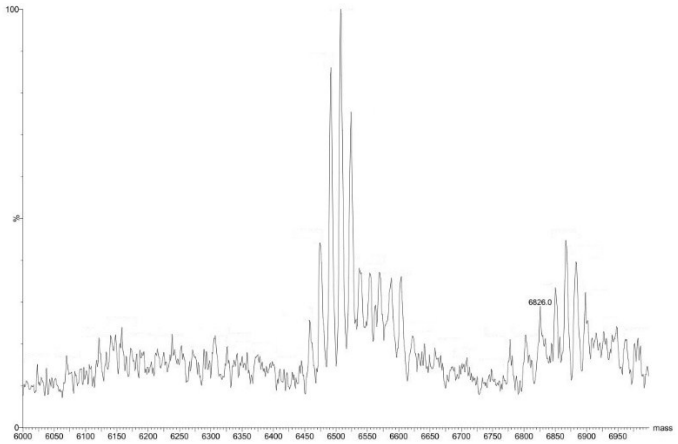
CeNA/2'-OMePS: 5'-cG mG mC mC cA mA mA mC cC mU mC mG mG cC mU cT mA mC mC cT-3'

Calcd 6696,6; found 6699,0



ANA/2'-OMePS: 5'-aG mG mC mC aA mA mA mC aC mU mC mG mG aC mU aT mA mC mC aT-3'

Calcd 6820,0; found 6826,0



6. Melting temperature study of the antisense oligonucleotides.

Five antisense oligonucleotides: control 2'-OMePS, mismatch 2'-OMePS and three mixmers HNA/2'-OMePS, CeNA/2'-OMePS and ANA/2'-OMePS were prepared at 2 uM concentration in melting buffer containing 10 mM NaCl, 0.01 mM EDTA adjusted to pH 7.0 by 10 mM sodium phosphate buffer. The AOs were then mixed with the complementary RNA sequence (2 uM) at equal volume and denatured at 95 °C for 10 minutes, cooled down to room temperature and loaded onto a 1-mm path-length quartz cuvettes. The melting process was monitored by Shimadzu UV-

1800 with the temperature controller over the range of 20-90 °C with ramp rate at 1.0 °C/ min. Tm values were calculated by the first derivative.

References

1. T. A. Rando, H. M. Blau, *J Cell Biol*., 1994, **125**, 1275–1287.
2. S. Forrest, P. L. Meloni, F. Muntoni, J. Kim, S. Fletcher, S. D. Wilton, *Neuromuscul Disord*., 2010, **20**, 810–816.