**SUPPLEMENTARY INFORMATION**

# Enhancement of exon skipping in *mdx*52 mice by 2′-*O*-methyl-2-thioribothymidine incorporation into phosphorothioate oligonucleotides

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## Contents

## Experimental Section

**General procedure for oligonucleotide synthesis.** Oligonucleotides were synthesized on an ABI 392 DNA/RNA synthesizer using standard phosphoramidite chemistry with 5′-*O*-(4,4′-dimethoxytrityl) 3′-(2-cyanoethyl*N*,*N*-diisopropylphosphoramidite) monomers of 2′-*O*-methylated nucleobase modified uridine derivatives and commercially available 2′-*O*-methylated uridine, 4-*N*-acetylcytidine, 6-*N*-phenoxyacetyladenosine, and 2-*N*-(4-isopropylphenoxy)acetylguanosine (Glen Research). The 5′-*O*-(4,4′-dimethoxytrityl) 3′-(2-cyanoethyl*N*,*N*-diisopropylphosphoramidite) monomers of 2′-*O*-methyl-ribothymidine[1], 2′-*O*-methyl-2-thiouridine[2], and 2′-*O*-methyl-2-thioribothymidine[3] were synthesized by following previous reports. Each synthesized phosphoramidite derivative was coevaporated with dry pyridine, toluene, and $CH_2Cl_2$ three times for each solvent in this order. Then each derivative was dissolved in dry MeCN to give 0.1 M solution under argon atomosphere. Benzylthiotetrazole (Glen Research) was used as the coupling reagent. The coupling time (10 min) was applied at steps incorporating phosphoramidite derivatives. 3-((*N,N*-dimethyl-aminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT, Glen Research) in MeCN was used as the sulfurizing reagent. After completion of the synthesis, the cleavage of modified oligonucleotides from the support and deprotection of all protecting groups were carried out in 28% $NH_3$ aq (1 mL) at room temperature for 3 h, and then reaction mixture was filtered. The filtrate was concentrated under reduced pressure and then subjected to C-18 cartridge purification. Each product was analyzed by using 4% agarose gel-electrophoresis. Finally, all synthesized oligonucleotides were identified by MALDI-TOF Mass.

**MALDI-TOF-Mass data**

mA20 sequence: 5′-GCAAAGAAGAXGGCAXXXCX

| | |
|---|---|
| U20 (X = 2′-*O*-methyl uridine) | calcd for $C_{212}H_{277}N_{79}O_{117}P_{19}S_{19}$ $[M+H]^+$ 7001.6 found 6998.6 |
| sU20 (X = 2′-*O*-methyl 2-thiouridine) | calcd for $C_{212}H_{277}N_{79}O_{112}P_{19}S_{24}$ $[M+H]^+$ 7081.9, found 7078.7 |
| sT20 (X = 2′-*O*-methyl 2-thiothymidine) | calcd for $C_{217}H_{286}N_{79}NaO_{112}P_{19}S_{24}$ $[M+Na]^+$ 7174.0, found 7177.07 |

mB30 sequence: 5′-CXCCAACAGCAAAGAAGAXGGCAXXXCXAG

| | |
|---|---|
| U30 (X = 2′-*O*-methyl uridine) | calcd for $C_{317}H_{416}N_{118}O_{174}P_{29}S_{29}$ $[M+H]^+$ 10491.4, found 10486.2 |
| T30 (X = 2′-*O*-methyl thymidine) | calcd for $C_{323}H_{428}N_{118}O_{174}P_{29}S_{29}$ $[M+H]^+$ 10575.6, found 10574.6 |
| sU30 (X = 2′-*O*-methyl 2-thiouridine) | calcd for $C_{317}H_{416}N_{118}O_{168}P_{29}S_{35}$ $[M+H]^+$ 10587.8, found 10583.7 |
| sT30 (X = 2′-*O*-methyl 2-thiothymidine) | calcd for $C_{323}H_{428}N_{118}O_{168}P_{29}S_{35}$ $[M+H]^+$ 10672.0, found 10574.6 |

**Exon skipping experiments.** Exon 52-deficient X chromosome-linked muscular dystrophy mice (*mdx52* mice, 5weeks old) were used. As antisense oligonucleotides against exon 51 of dystrophin gene, U20, sU20, sT20, U30, T30, sU30, and sT30 were used. Each antisense oligonucleotide (2 nmol) was dissolved in saline. One nmol of antisense oligonucleotide was injected into each tibialis anterior muscle. Two weeks after injection, mice were sacrificed and muscles were dissected. Total RNA was extracted from frozen tissue and 50 ng of total RNA was used for one-step RT-PCR according to the manufacturer's instructions. The primer sequences were Ex50F 5′-TTTACTTCGGGAGCTGAGGA and Ex53R 5′-ACCTGTTCGGCTTCTTCCTT for amplification of cDNA from exons 50-53. The PCR conditions were 95 °C for 5 min, then 35 cycles of 94°C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 7 min. The intensity of PCR bands was analyzed by using ImageJ software (http://rsbweb.nih.gov/ij/). All experimental protocols in this study were approved by The Experimental Animal Care and Use Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan.

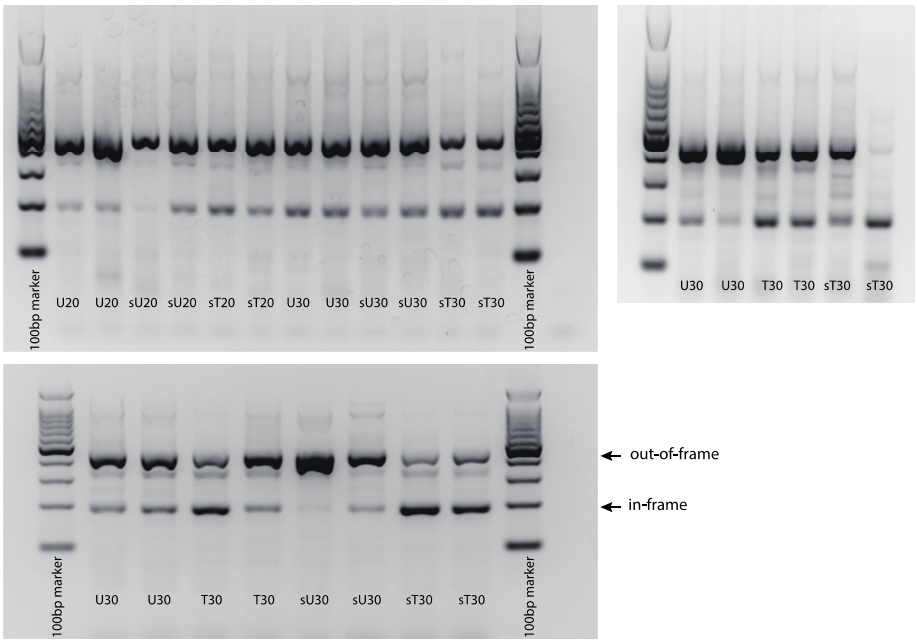


**Figure S1**. Gel-images for exon skipping experiments.

**UV-melting Experiments.** Duplex (1.2 nmol) was dissolved in 10 mM sodium phosphate buffer (pH 7.0, containing 100 mM NaCl and 0.1 mM EDTA) (600 μL) to arrange the final concentration of each oligonucleotide to be 2 μM. The solution was separated into quartz cells (10 mm) and incubated at 95 °C. After 10 min, the solution was cooled to 5 °C at a rate of annealing and melting procedure (0.5 °C·min$^{-1}$), the absorption at 260 nm was recorded and used to draw UV-melting curves. $T_m$ values were calculated as the temperature that gave the maximum of the first derivative of the UV−melting curve.

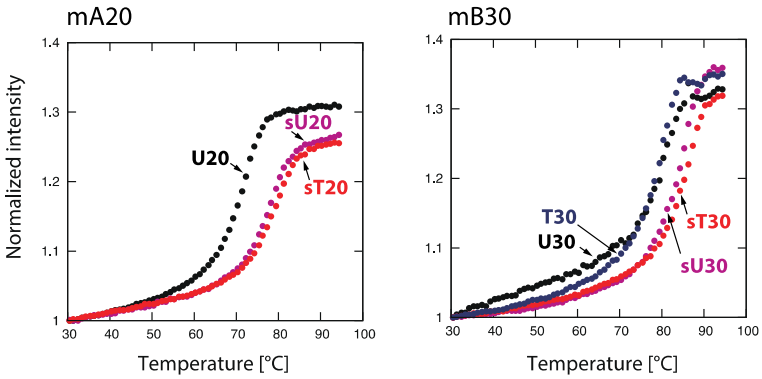


**Figure S2**. UV-melting curves for each duplex.

**Human Serum Albumin Binding Assay.** Each oligonucleotide (10 pmol) was dissolved in phosphate buffered saline (pH 7.4, 2.68 mM KCl, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 8.10 mM $Na_2HPO_4$ at 25 °C). To the solution was added human serum albumin (HSA) to arrange the final concentration of each oligonucleotide to be 500 nM, and human serum albumin (HSA) to be 0 to 100 μM. 20 μL of each sample was loaded on 4% agarose gel (E-Gel® EX, life technologies), and electrophoresis was conducted for 15 min at room temperature. The amount of oligonucleotides that did not bind to the HSA was quantified by using ImageJ software (http://rsbweb.nih.gov/ij/).

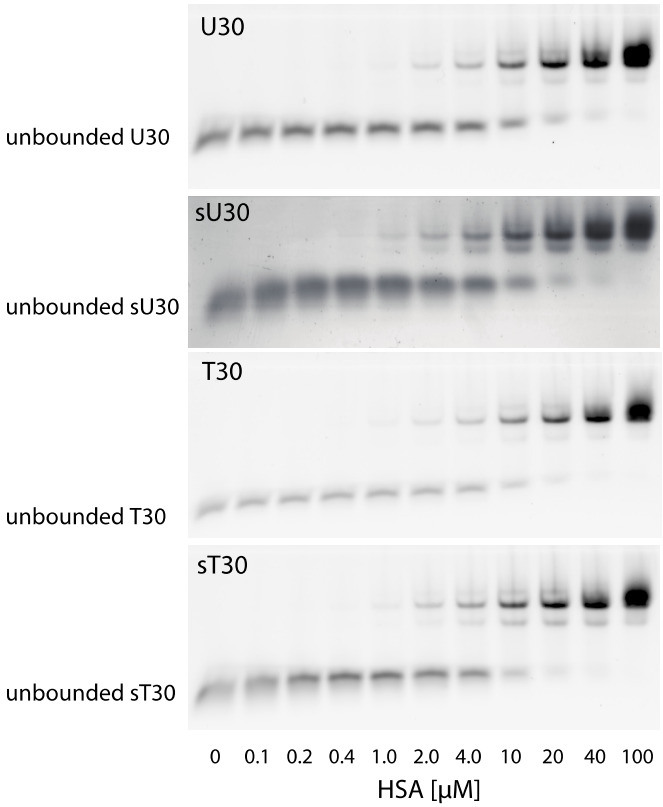


**Figure S3**. Gel-images for gel-shift assay of HSA binding.

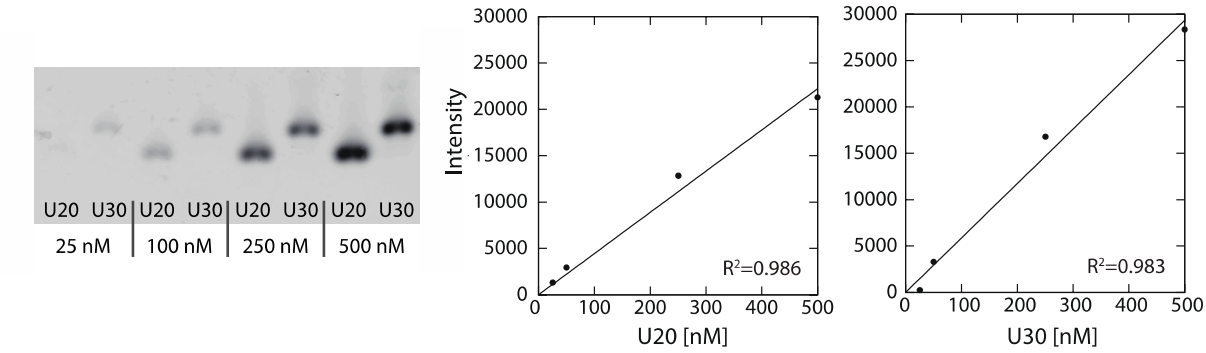


**Figure S4**. Linearity of gel-intensities and AON concentrations under HSA binding assay condition.

## References


1. T. Ostrowski, J. C. Maurizot, M. T. Adeline, J. L. Fourrey and P. Clovio, *J. Org. Chem*., **2003**, *68*, 6502-6510.
2. I. Okamoto, K. Shohda, K. Seio and M. Sekine, *J. Org. Chem.*, **2003**, *68*, 9971-9982.
3. A. Ohkubo, Y. Nishino, A. Yokouchi, Y. Ito, Y. Noma, Y. Kakishima, Y. Masaki, H. Tsunoda, K. Seio and M. Sekine, *Chem. Commun.*, **2011**, *47*, 12556-12558.