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

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Incorporation of 2′-*O*-Methyl-2-thioribothymidine (s^2T_m) into antisense oligoribonucleotides significantly enhanced the exon skipping activity of Duchenne Muscular dystrophy model mice.

Duchenne Muscular Dystrophy (DMD) is a lethal X-linked progressive muscular deterioration disease.[1] This disease affects one in every 3500 newborn males. The affected males gradually lose the ability to walk in their early teens and die in their twenties due to refractory cardiac or respiratory failure. In DMD treatment, exon skipping therapy mediated by antisense oligonucleotides (AONs), such as phosphorodiamidate morpholino oligomers (PMOs)[2] and phosphorothioate antisense 2′-*O*-methyloligonucleotides (PS-2′OMe-RNAs)[3] is expected to be the closest to clinical application.

One of the major problems in exon skipping therapy for DMD is that the relatively high dose of AON is required. For example, the dose of PMO in a phase II clinical study (NCT01540409) was 0.9 or 1.5 g/week PMO for a 30-kg child.[4] Because present nucleic acid drugs require high doses to maintain efficacy, it is crucial that more efficient AONs will be developed. To improve the efficacy of AONs, various kinds of sugar and backbone-modified AONs[5], such as 2′-*O*-methylcarbamoylethyl-modified AON[6], 2′-*O*-methoxyethyl-modified AON[7], LNA/BNA[8], ENA[9] and tricycloDNA have been reported.[10] However, to the best of our knowledge, there has been no report on the effect of nucleobase-modified AONs on exon skipping. It is expected that nucleobase modification could have an additional effect on those resulting from the modification of sugar and phosphate backbones previously reported.

To find efficient nucleobase modifications for exon skipping, we synthesised oligoribonucleotides containing thymine (T), 2-thiouracil (s^2U) and 2-thioribothymine (s^2T) bases, which are non-cannonical nucleobases found in RNAs. These nucleosides are known to not only play an important role in stabilising tRNA structures but also affect aminoacylation kinetics because of their precise recognition of codon-anticodon base pairs.[11] Many research groups have reported the synthesis and properties of s^2U and s^2T derivatives, showing that 2-thiocarbonyl modifications considerably stabilised the A–U Watson–Crick base pair. This is a result of their strong stacking interaction with 3′-downstream nucleobases and the steric preference of the C3′-*endo* conformation of ribofuranose rings.[12] Although these modified nucleobases could stabilise duplexes, the G-s^2U (or s^2T) wobble base pair is destabilised, as suggested by the *ab initio* calculation of the base pair between the modified base and G.[13] As the result, s^2U/s^2T can replace U/T to enforce binding to the target mRNA and reduce the off-target effect. For example, incorporation of s^2U into short double stranded RNAs produced 5–10 times more effective gene silencing.[14] Despite the promising properties of s^2U_m and s^2T_m, no papers have reported their incorporation into phosphorothioate oligonucleotides or evaluated their effect on exon skipping. Here we report the synthesis of phosphorothioate oligonucleotides containing 2′-*O*-methyl-2-

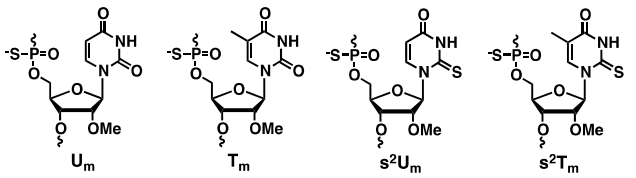

Figure 1. Chemical structures of 2′-*O*-methyl uridine (U_m), 2′-*O*-methyl ribothymidine (T_m), 2′-*O*-methyl 2-thioudirine (s^2U_m), and 2′-*O*-methyl 2-thioribothymidine (s^2T_m) residues in a phosphorothioate oligonucleotide

thioudirine (s^2U_m) or 2′-*O*-methyl-2-thioribothymidine (s^2T_m) residue and their exon skipping efficacy in *mdx*52 mice.

To evaluate the efficacy of nucleobase-modified AONs in exon skipping using DMD model mice, we selected the 20- and 30-nt sequences, A20 and B30, respectively, which were previously used for designing AONs to skip exon 51 of human dystrophin gene.[15] The A20 sequence incorporates part of the B30 sequence (30mer). The A20 and B30 sequences have also been previously used in clinical studies of PS-2′OMe RNAs and PMOs, respectively. To perform animal experiments using mice, the mA20 and mB30 sequences, which are adjusted to the mouse DMD pre-mRNA sequence, were used.[16] In this study, we synthesised five types of base-modified phosphorothioate 2′-*O*-methyl AONs. Among them, two AONs (sU20 and sT20) with the mA20 sequence, where U was replaced by s^2U_m and s^2T_m, respectively, were synthesised. The other AONs (sU30, sT30 and T30) were synthesised with the mB30 sequence containing s^2U_m, s^2T_m and T_m, respectively. In addition, unmodified AONs, U20 and U30, were synthesised as reference compounds. The phosphoramidite derivatives of 2-thiolated ribonucleosides were synthesised according to previously reported methods.[12b, c] These AONs were successfully synthesised without any detectable thiocarbonyl-associated side reactions by standard RNA synthesis protocol involving sulfurisation using DDTT for 6 min (Supplementary Information).

The exon skipping efficacy of the modified AONs in experiments using exon 52-deleted *mdx* mice (*mdx52*) was evaluated by local intramuscular injection (1 nmol each (10 μg for 30mer AONs and 7 μg for 20mer AONs), Figure 2). After two weeks, the efficacy of exon skipping was evaluated by one-step RT-PCR of total RNAs, as previously described (Figure S1).[6] The exon skipping efficiency of U20 was 7.1%, which is the relative percentage of the spliced product in RT-PCR products derived from the skipped mature mRNA. Similarly, efficiencies of sU20 and sT20 were 8.0% and 17.4%, respectively, with sT20 showing higher exon skipping efficacy than U20. The exon skipping efficiencies of U30, T30, sU30 and sT30 were 17.1%, 32.0%, 10.2% and 51.3%, respectively. Efficiency of s^2T30 showed statistically significant improvement from that of U30 ($p = 0.03$, Student's *t*-test). The use of sT30 roughly resulted in a 3-fold improvement at the mRNA level compared with U30. These results clearly indicated that the AONs having s^2T_m residues increased the exon skipping efficacy.

To understand the effect of nucleobase modification on the improvement of exon skipping efficacy, the binding affinities of the target pre-mRNA and human serum albumin (HSA) were studied. As summarised in Figure 3, the melting temperatures (T_m) of the AONs duplexes U20, sU20 and sT20 with their complementary RNA strands were determined to be 71.3 °C ± 0.6 °C, 77.8 °C ± 0.4 °C and 78.9 °C ± 0.8 °C, respectively. It was found that the 2-thiocarbonylation enhanced the T_m value by +6.5 °C and 5-methylation slightly enhanced the T_m value,

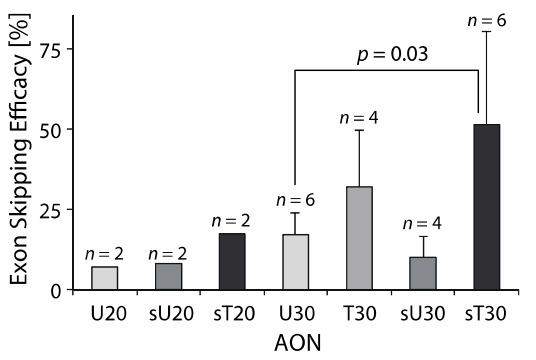

Figure 2. Exon skipping efficiency of AONs in *mdx52* mice. AON of 1 nmol each (10 μg for 30 mer AONs and 7 μg for 20 mer AONs) was injected into an 5-week-old *mdx52* mouse at the tibialis anterior muscle sites of its right and left legs. Two weeks after injection, mRNA levels were analysed. Exon skipping efficiency was calculated from the relative intensity of un-skipped and skipped mRNA bands.

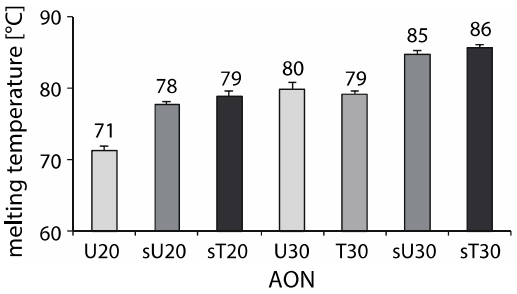

Figure 3. Melting temperatures (T_m) of AON duplexes with their complementary RNA strands. Each T_m measurement was conducted in a buffer containing 10 mM sodium phosphate (pH 7.0), 0.1 M NaCl, 0.1 mM EDTA and 2 μM duplex. Error bars indicate standard deviation of three independent measurements.

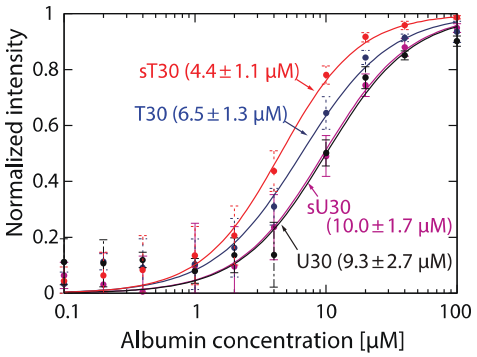

Figure 4. The apparent dissociation constants (K_d) of AONs with human serum albumin. The normalised intensity denotes the relative ratio of the gel intensity of unbound AONs. The values in brackets indicate the apparent dissociation constants calculated using Hill equation. See the details in the Supplementary Information.

with an increase of +1.1 °C. These results were consistent with those obtained when oligonucleotides containing s^2U_m and s^2T_m residues were used.[12b-d] Similarly, in AONs having the mB30 sequence, T_m values of the duplexes of AONs U30, T30, sU30 and sT30 with complementary RNA strands were 79.9 °C ± 0.9 °C, 79.2 °C ± 0.4 °C, 84.8 °C ± 0.5 °C and 85.7 °C ± 0.4 °C, respectively. In addition, 2-thiocarbonylation improved the T_m value, with an increase of +4.9 °C, while 5-methylation had

almost no effect on the T_m value (−0.7 °C–+0.6 °C). Melting temperature analysis suggested that the order of relative binding ability of the pre-mRNA was sT30 ≈ sU30 > U30 ≈ T30 ≈ sT20 ≈ sU20 > U20. Except for AONs containing s^2U_m residues, the T_m values showed positive correlation with exon skipping efficacies (R^2 = 0.77). Although the results of AONs sU20 and sU30 could not be explained, this tendency agreed well with those of a previous study.[17]

The dissociation constants of AONs with human serum albumin (HSA) were evaluated by gel shift assay. Previous studies have shown that affinity of the AONs for HSA plays an important role in *in vivo* pharmacokinetics.[18] The apparent K_d values of AONs U30, T30, sU30 and sT30 were 9.3 ± 2.7, 6.5 ± 1.3, 10.0 ± 1.7 and 4.4 ± 1.1 μM, respectively (Figure 4). U30 and sU30 only differed negligibly. On the other hand, sT30 exhibited a 2-fold greater affinity for HSA than U30. These results were consistent with those of the exon skipping experiments. It is said that the binding affinity of AONs for HSA has affected their distribution to all target tissues.[18] In addition, in acidic molecules, it is reported that their ability to bind to HSA was closely related to their lipophilicity,[19] which suggested sT30 was more lipophilic than the other AONs. Lipophilic AONs were reported to be preferentially incorporated into cells.[20] Furthermore, Yang *et al.* noted that preferential cellular uptake could lead to better exon skipping.[7] Together, these papers imply that the improved affinity of our AONs for HSA may reflect efficient cell permeability; thus, enhancing exon-skipping efficacy. However, further evaluation will be required.

In summary, we demonstrated the significant enhancement of exon skipping efficacy of 5-methylation and 2-thiolation of U. The phosphorothioate oligonucleotide (sT30) containing six s^2T_m residues showed a 3-fold enhancement of pre-mRNA exon skipping compared with unmodified AONs (U30). The data obtained by thermal melting analysis and gel shift assay of AONs with HSA indicated that these properties could be considerably improved by the introduction of s^2T_m residues into AONs. It should be noted that the use of nucleobase modification could provide a practical improvement of the properties of chemically modified AONs, previously reported in the antisense strategy, using exon skipping for treatment of muscular dystrophy.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details. See DOI: 10.1039/c000000x/

1. F. Muntoni and M. J. Wood, *Nat. Rev. Drug. Discov.*, 2011, **10**, 621.
2. (a) M. Kinali, V. Arechavala-Gomeza, L. Feng, S. Cirak, D. Hunt, C. Adkin, M. Guglieri, E. Ashton, S. Abbs, P. Nihoyannopoulos, M. E. Garralda, M. Rutherford, C. McCulley, L. Popplewell, I. R. Graham, G. Dickson, M. J. Wood, D. J. Wells, S. D. Wilton, R. Kole, V. Straub, K. Bushby, C. Sewry, J. E. Morgan and F. Muntoni, *Lancet Neurol.*, 2009, **8**, 918; (b) S. Cirak, V. Arechavala-Gomeza, M. Guglieri, L. Feng, S. Torelli, K. Anthony, S. Abbs, M. E. Garralda, J. Bourke, D. J. Wells, G. Dickson, M. J. Wood, S. D. Wilton, V. Straub, R. Kole, S. B. Shrewsbury, C. Sewry, J. E. Morgan, K. Bushby and F. Muntoni, *Lancet*, 2011, **378**, 595.
3. N. M. Goemans, M. Tulinius, J. T. van den Akker, B. E. Burm, P. F. Ekhart, N. Heuvelmans, T. Holling, A. A. Janson, G. J. Platenburg, J. A. Sipkens, J. M. Sitsen, A. Aartsma-Rus, G. J. van Ommen, G. Buyse, N. Darin, J. J. Verschuuren, G. V. Campion, S. J. de Kimpe and J. C. van Deutekom, *N. Engl. J. Med.*, 2011, **364**, 1513.
4. J. R. Mendell, L. R. Rodino-Klapac, Z. Sahenk, K. Roush, L. Bird, L. P. Lowes, L. Alfano, A. M. Gomez, S. Lewis, J. Kota, V. Malik, K. Shontz, C. M. Walker, K. M. Flanigan, M. Corridore, J. R. Kean, H. D. Allen, C. Shilling, K. R. Melia, P. Sazani, J. B. Saoud, E. M. Kaye and G. Eteplirsen Study, *Ann. Neurol.*, 2013, **74**, 637.
5. P. Jarver, L. O'Donovan and M. J. Gait, *Nucleic Acid Ther*, 2014, **24**, 37.
6. T. Yamada, N. Okaniwa, H. Saneyoshi, A. Ohkubo, K. Seio, T. Nagata, Y. Aoki, S. Takeda and M. Sekine, *J. Org. Chem.*, 2011, **76**, 3042.
7. L. Yang, H. Niu, X. Gao, Q. Wang, G. Han, L. Cao, C. Cai, J. Weiler and H. Yin, *PLoS ONE*, 2013, **8**, e61584.
8. A. Aartsma-Rus, W. E. Kaman, M. Bremmer-Bout, A. A. Janson, J. T. den Dunnen, G. J. van Ommen and J. C. van Deutekom, *Gene Ther*, 2004, **11**, 1391.
9. M. Yagi, Y. Takeshima, A. Surono, M. Takagi, M. Koizumi and M. Matsuo, *Oligonucleotides*, 2004, **14**, 33.
10. A. Goyenvalle, A. Babbs, A. Avril, G. Griffiths, B. Dugovic, K. E. Davies, C. Leumann and L. Garcia, *Neuromus. Disord.*, 2012, **22**, 907.
11. (a) A. Rodriguez-Hernandez, J. L. Spears, K. W. Gaston, P. A. Limbach, H. Gamper, Y. M. Hou, R. Kaiser, P. F. Agris and J. J. Perona, *J. Mol. Biol.*, 2013, **425**, 3888; (b) Y. Motorin and M. Helm, *Biochemistry*, 2010, **49**, 4934.
12. (a) R. K. Kumar and D. R. Davis, *Nucleic Acids Res.*, 1997, **25**, 1272; (b) K. Shohda, I. Okamoto, T. Wada, K. Seio and M. Sekine, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 1795; (c) I. Okamoto, K. Shohda, K. Seio and M. Sekine, *J. Org. Chem.*, 2003, **68**, 9971; (d) K. G. Rajeev, T. P. Prakash and M. Manoharan, *Org. Lett.*, 2003, **5**, 3005; (e) B.

Diop-Frimpong, T. P. Prakash, K. G. Rajeev, M. Manoharan and M. Egli, *Nucleic Acids Res.*, 2005, **33**, 5297; (f) 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; (g) Y. Masaki, R. Miyasaka, K. Hirai, H. Tsunoda, A. Ohkubo, K. Seio and M. Sekine, *Chem. Commun.*, 2012, **48**, 7313.

13. (a) J. Sponer, P. Jurecka and P. Hobza, *J. Am. Chem. Soc.*, 2004, **126**, 10142; (b) S. Kawahara, T. Wada, S. Kawauchi, T. Uchimaru and M. Sekine, *J. Phys. Chem. A*, 1999, **103**, 8516.
14. K. Sipa, E. Sochacka, J. Kazmierczak-Baranska, M. Maszewska, M. Janicka, G. Nowak and B. Nawrot, *RNA*, 2007, **13**, 1301.
15. V. Arechavala-Gomeza, I. R. Graham, L. J. Popplewell, A. M. Adams, A. Aartsma-Rus, M. Kinali, J. E. Morgan, J. C. van Deutekom, S. D. Wilton, G. Dickson and F. Muntoni, *Human gene therapy*, 2007, **18**, 798.
16. Y. Aoki, A. Nakamura, T. Yokota, T. Saito, H. Okazawa, T. Nagata and S. Takeda, *Mol. Ther.*, 2010, **18**, 1995.
17. L. J. Popplewell, C. Trollet, G. Dickson and I. R. Graham, *Mol. Ther.*, 2009, **17**, 554.
18. T. P. Prakash, M. Manoharan, A. M. Kawasaki, A. S. Fraser, E. A. Lesnik, N. Sioufi, J. M. Leeds, M. Teplova and M. Egli, *Biochemistry*, 2002, **41**, 11642.
19. N. A. Kratochwil, W. Huber, F. Muller, M. Kansy and P. R. Gerber, *Biochem. Pharmacol.*, 2002, **64**, 1355.
20. E. Vives, C. Dell'Aquila, J. C. Bologna, F. Morvan, B. Rayner and J. L. Imbach, *Nucleic Acids Res.*, 1999, **27**, 4071.