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Phosphorothioate Modification of Chimeric 2′-O-Methyl RNA/Ethylene-Bridged Nucleic Acid Oligonucleotides Increases *Dystrophin* Exon 45 Skipping Capability and Reduces Cytotoxicity

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ABSTRACT

Backgrounds, Antisense oligonucleotide (AO)-mediated exon skipping is the most promising way to express internally deleted dystrophin in Duchenne muscular dystrophy (DMD), by correcting the reading frame of *dystrophin* mRNA. An antisense chimeric oligonucleotide consisting of 2′-O-methyl RNA and ethylene-bridged nucleic acid (ENA), targeting exon 45 of the *dystrophin* gene, AO85, has been shown to induce exon 45 skipping efficiently. Since phosphorothioate (PS)-modification of AO85 has never been explored, we produced a PS-modified AO85 (AO88) and examined its exon skipping capability and cytotoxicity. Methods, Exon 45 skipping activity was examined in primary muscle cells established from a DMD patient carrying a deletion of *dystrophin* exon 44. Cytotoxicity was assessed by MTT assay. Results, AO88 induced *dystrophin* exon 45 skipping from 50 nM. More than 90% of products lacked exon 45 at 400 nM. AO88 showed significantly higher exon skipping activity than AO85. The EC50 of AO88 was 94.8 nM, while EC50 of AO85 was 66.7 nM. Cytotoxicity was lower for AO88 than for AO85. Conclusion, the PS-modified RNA/ENA chimera displayed stronger exon skipping activity and lower cytotoxicity than the phosphodiester-RNA/ENA chimera. AO88 has better potential for clinical use than AO85.

INTRODUCTION

Duchenne muscular dystrophy (DMD), a rapidly progressive muscle wasting disease, is caused by dystrophin deficiency. DMD is usually caused by out-of-frame exon deletion

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mutations in the *dystrophin* gene that create a premature stop codon in the *dystrophin* mRNA. Antisense oligonucleotide (AO)-mediated exon skipping was proposed as a form of DMD treatment, in which semi-functional, internally deleted dystrophin would be produced from in-frame *dystrophin* mRNA, derived by the splicing of out-of-frame pre-mRNA [1, 2]. Since then, studies on AO-mediated exon skipping for the treatment of DMD have focused on inducing exon 51 skipping; this is potentially applicable as a treatment for 13% of DMD patients [3]. Currently, two AOs are in clinical trials: 2′-O-methyl (2′-OMe) phosphorothioate (PS)-RNA and neutrally charged phosphorodiamidate morpholino oligomers to induce *dystrophin* exon 51 skipping [4-6]. Development of AO that induces exon 45 skipping is a next subject in the field of AO-mediated exon skipping therapy for DMD.

Several chemically modified nucleic acids have been developed, since oligonucleotides with phosphodiester (PO) backbones are very labile toward intracellular and extracellular nucleases [7].Various chemical modifications of nucleic acids have been developed to enhance nuclease resistance, prolong tissue half-life, increase affinity and potency, and reduce non-sequence-specific toxicity [8, 9]. PS-modification of DNA, in which a non-bridging oxygen atom is replaced with a sulfur atom, was developed as an artificial means to stabilize oligonucleotides against nuclease degradation and prolong their intact survival time [10, 11]. However, PS-modified AOs have been reported to produce non-specific effects by interactions with cell surface and intracellular proteins [8].

On the other hand 2′-O, 4′-C-ethylene-bridged nucleic acid (ENA) has been shown to have high binding affinity for the complementary RNA strand and more nuclease resistance than unmodified nucleic acid [12, 13]. A chimera of ENA and 2′-OMe RNA (RNA/ENA chimera) was shown to be 40 times more effective than conventional PS-backbone DNA oligonucleotides in inducing skipping of *dystrophin* exon 19 [14]. Considering this remarkable efficiency, we previously designed an 18-mer RNA/ENA chimera, AO85, targeting *dystrophin* exon 45. This has the theoretical capability to transform the *dystrophin* pre-mRNA of nearly 9% of DMD patients into in-frame mRNA [3, 15]. AO85 induced exon 45 skipping successfully in cultured human myocytes [16]. Subsequently, AO85 was shown to induce *dystrophin* exon 45 skipping efficiently even in a cell-free splicing system, and to have a low EC50 [17].

Structurally, AO85 has PO-backbones that are supposedly nuclease sensitive. Here, we examined the exon skipping capability and cytotoxicity of PS-modified AO85, namely AO88, and showed that AO88 has stronger exon skipping activity and lower cytotoxicity than AO85.

MATERIALS AND METHODS

RNA/ENA chimeric oligonucleotides

Oligonucleotides consisting of 2′-OMe RNA and ENA were designed to span 18 bp of the 5′ end of *dystrophin* exon 45. AO85, a PO-type RNA/ENA chimera (5′-CgCTgcCCaaTgCCatCC-3′; upper and lower case letters represent ENA and 2′-OMe RNA, respectively) was synthesized as described before [17]. The PS-type RNA/ENA chimera (AO88) was synthesized using PS-2′-OMeRNA and PS-ENA monomers (Figure 1) by KNC Laboratories Co. (Kobe, Japan). Both AOs were produced simultaneously to guarantee their identical quality.

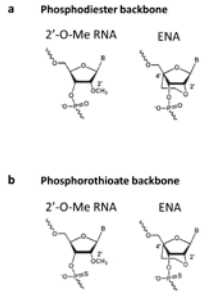

Figure 1. Structure of 2′-O-methyl RNA and ethylene-bridged nucleic acid (ENA) monomers.
a. PO-type structure of 2′-O-methyl RNA and ENA with phosphodiester backbone.
b. PS-type structure of 2′-O-methyl RNA and ENA with phosphorothioate backbone.

Muscle cell line

A primary muscle cell line was established from a muscle biopsy of a DMD patient (KUCG763) who carries a deletion in *dystrophin* exon 44. The muscle tissue was obtained from his quadriceps after obtaining informed consent, at Kobe University Hospital, Kobe, Japan. The study protocol was approved by the ethics committees of Kobe Kobegakuin Universities. The muscle tissue was minced and dissociated in 5% trypsin for 30 minutes at 37°C, and isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA) and 4% Ultroser-G (Ciphergen Biosystems, Fremont, CA). Myoblasts divided without differentiation in this medium. To induce muscle cell differentiation, the medium was changed to differentiation medium, which comprised DMEM supplemented with 2% horse serum (Sigma-Aldrich, St Louis, MO).

Transfection of chimeric oligonucleotides

One week after the induction of differentiation, muscle cells were transfected with RNA/ENA chimeras. Each of AO85 and AO88 was dissolved in 200 µl OptiMEM (Invitrogen) and incubated for 5 minutes at ambient temperature. The incubated solution was mixed with 8 µl Lipofectamine (Invitrogen) dissolved in 200 µl OptiMEM and incubated for 20 minutes. Then, the mixture was added to culture medium (1600 µl OPTIMEM) to a final RNA/ENA chimera concentration of 0–400 nM. After 3 hours of incubation, the medium was changed to DMEM +2% horse serum, and the incubation was continued for 2 days, at which point the myocytes were harvested and RNA was extracted.

mRNA analysis

RNA was isolated from the cultured myocytes, and cDNA was prepared from 60 ng purified RNA as described previously [14]. PCR amplification of cDNA spanning exons 43–48 was performed using the following primers: forward, 5′-CGCCTGTGGAAAGGGTGAAG-3′; reverse, 5′-CTTCAAGGTCTTCAAGCTTT-3′. The amplified products were then separated by high-resolution microcapillary electrophoresis with an Agilent 2100 Bioanalyzer on a DNA 1000 Labchip R (Agilent Technologies Inc., Santa Clara, CA). Electropherograms, PCR product size determination, and quantification were automatically performed using 2100 Expert software (Agilent Technologies, Inc.). The sequence of all detected bands was confirmed by DNA sequencing as described before [18]. As an internal standard, the *glyceraldehyde 3-phosphate dehydrogenase* gene mRNA was amplified by reverse transcription (RT)-PCR, as described before [17]. The percentage of exon 45-skipped product among the total product was calculated as [exon 45-skipped product / (normally spliced + exon 45-skipped products)] × 100.

The EC50 (concentration required to obtain a 50% exon skipping effect) was estimated using a comparative approach based on a regression model implemented in GraphPad Prism® version 5 (GraphPad Software Inc., San Diego, CA).

Cytotoxicity assay

The cytotoxicity of AO85 and AO88 was assessed by a colorimetric assay using an MTT Cell Proliferation Kit I (Roche Applied Science, Mannheim, Germany). Fibroblast cells were obtained from control having no neuromuscular disorder and seeded at a density of 20 000 cells/ well in 96-well plates and treated with AO85 or AO88 at a concentration of 0–400 nM as described above. After a 24-hour incubation, the medium was changed and MTT was added at a final concentration of 0.5 mg/ml. After a 4-hour incubation, formazan products were solubilized overnight with solubilization solution containing 10% SDS in 0.01 M HCl. The optical density was measured at 570 nm using an ARVO X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA). All experiments were performed in triplicate. Data were expressed as the mean percentage density compared with that of the untreated control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc.) The mean ± S.D. was derived from three or more independent experiments.

RESULTS

To characterize the effect of PS-modification, a PS-modified version of AO85, AO88, was synthesized using PS-2′-OMe RNA and PS-ENA monomers. The ability of AO88 to induce *dystrophin* exon 45 skipping was examined using muscle cells with a *dystrophin* exon 44 deletion mutation. AO88 was added to the culture medium at the indicated concentration (0-400 nM). The resulting *dystrophin* mRNA was analyzed by RT-PCR amplification of the region spanning exons 43 to 48. At 0 and 10 nM AO88, RT-PCR revealed a single band with the exon 44 deletion (Δ44dys) on microcapillary electrophoresis (Figure 2a). One additional small band was detected at a concentration of 50 nM and above. Sequencing of this small product revealed that it lacked exon 45 (Δ44•45dys), indicating AO88-mediated exon 45 skipping. The percentage of Δ44•45dys was 0% at 0 and 10 nM AO88; around 30% at 50 nM; and around 80% at 200 nM (Figure 2b). Thereafter, it increased slowly until Δ44•45dys accounted for more than 90% of the product at 400 nM AO88. This indicated that AO88 induced exon 45 skipping in a dose-dependent manner.

Next, we compared the ability of AO88 to induce exon 45 skipping with that of AO85. At 0 and 10 nM AO85, the percentage of Δ44•45dys was 0%; at 50 nM AO85 it was around 10%; and it reached a plateau at 100 nM (Figure 2b). At 400nM it was around 35%. In contrast, for AO88, the percentage of Δ44•45dys increased until 400 nM (Figure 2b). The percentage of Δ44•45dys was significantly higher in AO88-treated cells than in AO85-treated cells at 50 nM concentration ($p = 0.03$) and the difference was even greater at 400 nM ($p < 0.001$) (Figure 2b). This showed that AO88 has a higher capability than AO85 to induce exon 45 skipping.

The production of Δ44•45dys followed a non-linear pattern for both AO88 and AO85 (Figure 2c). The EC50 of AO88 was higher than that of AO85, at 94.8 nM and 66.7 nM, respectively (Figure 2c). This is expected result, as the amount of exon skipping induced by AO88 is much higher that the skipping induced by AO85.

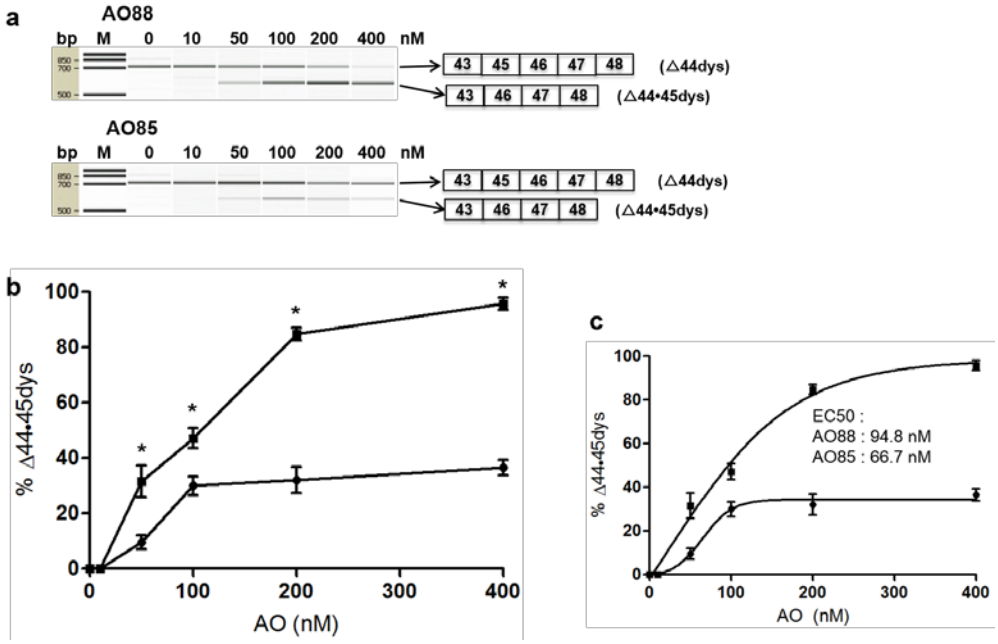

Figure 2. Splicing assay using DMD muscle cells.

Muscle cells obtained from a DMD patient were transfected with AO and resulting *dystrophin* mRNA was analyzed by RT-PCR. a. Microcapillary electrophoresis patterns of RT-PCR products. RT-PCR products spanning a region from *dystrophin* exons 43 to 48 are shown. Without the addition of AO, a single band was produced, lacking *dystrophin* exon 44 (∆44dys). Increasing AO85 or AO88 concentration resulted in the production of an additional smaller band that corresponded to an exon 45-skipped product (∆44·45dys). M, marker.

b. Percentage of exon 45-skipped product (∆44·45dys) in cells treated with different concentrations of AO85 (circles) or AO88 (squares). More ∆44·45dys was produced in AO88-treated cells than in AO85-treated cells. Asterisks indicate a significant difference (p = 0.03 at 50 nM and $p < 0.001$ at 400 *nM).* The data show the mean ± S.D. of three or more independent experiments.

c. Regression analysis of exon 45 skipping revealed a non-linear pattern of dose-dependency. . The EC_{50} of AO88 and AO85 was 94.8 nM and 66.7 nM, respectively. The data show the mean ± S.D. of three or more independent experiments.

We examined whether the more effective AO88 is more toxic than AO85 using an MTT assay. The number of viable fibroblasts was measured in the absence of AO, and its value set as 100%. AO85 reduced cell viability in a dose-dependent manner. In contrast, AO88 decreased cell viability only slightly (Figure 3). At 400 nM, AO85 caused a 30% decrease in cell viability, while AO88 caused a 5% decrease ($p < 0.05$). We concluded that AO88 is less cytotoxic than AO85.

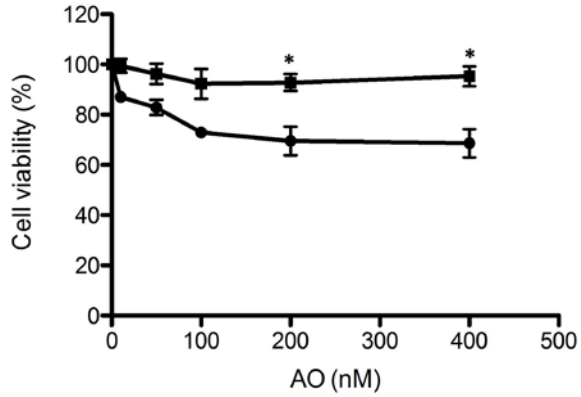

Figure 3. Cytotoxicity of AOs.

Fibroblasts were treated with the indicated concentrations of AO85 (circles) and AO88 (squares). Cell viability was assessed using an MTT assay and is expressed as the percentage of the viability in non-treated cells. Asterisks indicate a significant difference. AO85 was significantly more cytotoxic than AO88 ($p = 0.02$ at 400 nM). The data show the mean ± S.D. of three independent experiments.

DISCUSSION

The characteristics of PS-modified RNA/ENA chimeras have not been studied, although the PO-RNA/ENA chimera AO85 has been shown to induce exon 45 skipping in not only cultured myocytes but also cell-free splicing systems [16, 17, 19]. Our results showed that a PS-modified RNA/ENA chimera (AO88) is better at inducing exon 45 skipping than the equivalent PO-backbone chimera (AO85) in cultured muscle cells from a DMD patient (>90% vs. 35% skipped products, respectively, at the highest concentration of AO; Figure 2). AO88 had a higher EC50 than AO85. EC50 is the concentration needed to achieve half of the maximum effect achievable. Therefore it is expected that AO88 will have higher EC50 than AO85 since the maximum exon skipping achieved by AO88 is much higher than that achieved by AO85.

In this experiment, AO85 induced exon 45 skipping until only about 35 % at maximum concentration tested. This is much lower than our previous experiment which shows exon 45 skipping level of nearly 90 % by AO85. However, that experiment was performed in a cell free splicing system, while this experiment was performed using muscle cells. Some factors related to the use of living cells, like the need for cellular uptake and the more complex nature of natural pre-mRNA, probably contribute to this difference.

AOs are continually being chemically modified to improve their physiological properties. One of the early modifications was to incorporate a PS-modified backbone. PS-modification gives the AOs better resistance against nuclease degradation, leading to higher bioavailability [8]. However, PS-modified AOs exhibit several disadvantages, including relatively poor binding to complementary nucleic acids and significant nonspecific binding to proteins [20, 21], causing toxic side effects [21]. In spite of this, PS-oligonucleotides have shown excellent antisense activity both in laboratory settings and in clinical trials [22].

The increased exon skipping capability of AO88 compared with AO85 could be because of two factors. First, PS-modification would increase its nuclease resistance, thus increasing bioavailability in cells. Second, PS-modification would promote adsorption of the AOs to cell surface proteins, increasing internalization (cellular uptake) [8]. When AO88 was transfected without mixing it with a carrier, there was no visible production of ∆44•45dys

(data not shown), suggesting that PS-modification probably does not increase cellular uptake. However further experiment is still needed to confirm this.

One of the well-known disadvantages of PS-modification is an increase in toxicity. However, even at high concentrations, we found that AO88 did not significantly decrease cell viability (Figure 3); it was remarkable that AO88 had lower toxicity than AO85. The reduced cytotoxicity might be because of the increased nuclease resistance. Previous studies have shown that the cytotoxicity of PO- and PS-oligomers is, in part, caused by nucleoside released by mononucleotide dephosphorylation [22-24]. The released nucleoside inhibits thymidine kinase, resulting in inhibition of cell growth. It was supposed that PS-modification inhibits nucleoside production, thereby reducing cytotoxicity. However, further experiments to compare degradation of both AOs are needed to confirm this theory.

The exon skipping approach is mutation specific because different deletion mutations require skipping of different exons neighboring the deletion. It has been reported that exon 51 skipping is applicable to the largest group of deletion patients, occupying 11.0 and 13.0 % of all DMD patients in the DMD mutation data base of Kobe, Japan and Leiden, Netherland, respectively [3, 15]. Skipping of exon 45 has been reported applicable to 9.0 and 8.1 % in Kobe and Leiden, respectively [3, 15]. Since AOs that are able to induce exon 51 skipping are now under clinical trials [4-6], development of AO that induces exon 45 skipping is a next subject in the field of AO-mediated exon skipping therapy for DMD. A 2′-OMe AO, PRO45, is in a Phase I/IIa dose-escalating safety study to assess the safety and efficacy (www.clinicaltrials.gov). Although we have reported that PO-type AO85 is effective in inducing exon 45 skipping [19], our current results indicated that PS-type AO88 is more suitable for clinical use.

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