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ORIGINAL ARTICLE

# Effects of combinations of gapmer antisense oligonucleotides on the target reduction

**Mitsugu Yanagidaira[1,2] · Kotaro Yoshioka[1,2,3] · Tetsuya Nagata[1,2,3] · Shoichi Nakao[4] · Kenichi Miyata[4] · Takanori Yokota[1,2,3]**

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## Abstract

**Background** The co-administration of several therapeutic oligonucleotides targeting the same transcript is a beneficial approach. It broadens the target sites for diseases associated with various mutations or splice variants. However, little is known how a combination of antisense oligonucleotides (ASOs), which is one of the major modalities of therapeutic oligonucleotides, affects the potency. In this study, we aimed to elucidate the combination-effects of ASOs and the relationship between the target sites and potency of different combinations.

**Method and Results** We designed 113 ASOs targeting human superoxide dismutase 1 pre-mRNA and found 13 ASOs that had comparable silencing activity in vitro. An analysis of combination-effects on the silencing potency of 37 pairs of two ASOs on HeLa cells revealed that 29 pairs had comparable potency to that of two ASOs; on the other hand, eight pairs had reduced potency, indicating a negative impact on the activity. A reduced potency was seen in pairs targeting the same intron, exon-intron combination, or two different introns. The sequence distance of target sites was not the major determinant factor of combination-effects. In addition, a combination of three ASOs preserving the potency could be designed by avoiding two-ASO pairs, which had a reduced potency.

**Conclusions** This study revealed that more than half of the combinations retain their potency by paring two ASOs; in contrast, some pairs had a reduced potency. This could not be predicted only by the distance between the target sites.

**Keywords** Antisense oligonucleotide · Combination · Superoxide dismutase 1 · Oligonucleotide therapeutics

## Introduction

Antisense oligonucleotides (ASOs) are extensively investigated as a promising platform to treat several diseases, including infections [1], cancer [2], and neurodegenerative diseases [3]. ASOs are divided into two major categories: gapmer ASO and non-gapmer ASO [4]. Gapmers have the center portion composed of DNAs, while non-gapmers do not. Both ASOs bind to their target RNA to create a DNA/RNA duplex, but only gapmers recruit RNase H, followed by target cleavage [5]. Gapmers, which can downregulate disease-related gene expression, have been approved for clinical use [6, 7].

A combination of oligonucleotide therapeutics targeting the same transcript has been investigated and has shown clinical benefits. For instance, a combination of small interfering RNAs (siRNAs) against viral infections, such as SARS-CoV-2, is reasonable to reduce the risk of resistant strains by targeting multiple sites [8]. Moreover, a combination of

✉ Kotaro Yoshioka
kotanuro@tmd.ac.jp

✉ Takanori Yokota
tak-yokota.nuro@tmd.ac.jp

[1] Department of Neurology and Neurological Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), 1-5-45 Yushima, Bunkyo-Ku, Tokyo 113-0034, Japan

[2] Center for Brain Integration Research, Tokyo Medical and Dental University (TMDU), 1-5-45 Yushima, Bunkyo-Ku, Tokyo 113-0034, Japan

[3] Nucleotide and Peptide drug develop center, Tokyo Medical and Dental University (TMDU), 1-5-45 Yushima, Bunkyo-Ku, Tokyo 113-0034, Japan

[4] Research, Takeda Pharmaceutical Company Limited, Fujisawa, Japan

non-gapmer ASOs, which skips multiple exons, could treat a wider range of patients with Duchenne muscular dystrophy, while a single ASO could treat at most 8–13% of them [9]. Thus, the combination approach has clinical benefits for the treatment of specific diseases. However, another study implied that a certain siRNA combinations had a reduced potency [10]. This finding indicated that specific combinations of oligonucleotides would have negative effects on the potency of each component. For example, if two oligonucleotides have neighboring target sites, they likely hinder each other from binding to the target sequence, resulting in a reduced potency when co-administered. A fundamental knowledge of such combination-effect can help us verify and adopt the combination strategy. Although several studies have reported the potency of combinations composed of non-gapmers or siRNAs [10–12], there have been no systematic evaluation of gapmer combinations. Therefore, we evaluated combination-effect on the potency with 37 gapmer pairs targeting the same pre-mRNA. Furthermore, we also created three- or four-gapmer combinations and evaluated their potency.

## Materials & methods

### Antisense oligonucleotide

In this study, a series of gapmer ASOs were synthesized by Gene Design (Osaka, Japan). These ASOs were 3-10-3 gapmers using fully phosphorothioate-modified linkages with a central segment of 10-mer DNA flanked by the 3-mer locked nucleic acids (LNAs) on both wings. Individual gapmer ASOs were dissolved in PBS. To generate combinations, equimolar amounts of individual gapmer ASOs were mixed. The potency of a single gapmer ASO and the combination was compared at the same molarity. For example, a 10 nM combination of #6361/#6362 with 5 nM each was compared to 10 nM of each single gapmer.

## Cell culture and transfection

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C. Individual gapmer ASO or the combination was mixed with 50 µL Opti-MEM (Life Technologies) containing 0.5 µL Lipofectamine 2000 (Life Technologies) at 25 °C for 20 min; then, this was added to a 48-well plate. Then, the HeLa cells in DMEM were seeded to the plate at $6 \times 10^4$ cells/well and incubated for 6 h. Then, a transfection medium was replaced with fresh DMEM with FBS and PS. The cells were harvested for PCR analysis 24 h after transfection.

## Quantitative real-time PCR assay

The total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), following the manufacture's protocol. After a reverse-transcription with PrimeScript RT Master Mix (Takara Bio, Kusatsu, Japan), quantitative real-time PCR was performed using LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Delta Ct (ΔCt) values were calculated by subtracting the Ct of GAPDH from that of SOD1. Then, ΔΔCt was acquired by subtracting the ΔCt of vehicle group from the ΔCt of the treatment groups. The relative SOD1 expression was calculated as 2^(−ΔΔCt). The primers and probes (Applied Biosystems, Life Technologies) used in this study were as follows: SOD1 (forward: 5'- CGACGGCCCAGTGCA-3'; reverse: 5'-CCACACCTTCACTGGTCCATTA-3'; probe: 5'-FAM–TTCCTTCTGCTCGAAATTGATGATGCCC–MGB-3'), GAPDH (forward: 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse: 5'-GAAGATGGTGATGGGATTTC-3'; probe: 5'-FAM–CAAGCTTCCCGTTCTCAGCC–TAMRA-3').

## Statical significance

All experiments were performed at least three times, and data were presented as mean ± SEM. To assess the combination-effect, the mean of SOD1 expression level in the combination-treated group was compared to that in single gapmer groups. For the comparison of means between more than three groups, one-way ANOVA was performed, followed by Dunn's multiple comparisons test. The difference was considered statically significant if the p-value was $< 0.05$. All statistical analyses were performed using Prism version 8.4.3 (GraphPad Software).

## Result

### Most two-gapmer combinations showed a comparable potency compared to its component gapmer ASOs

The combination-effects were divided into three groups based on their potency. A pair was considered to be attenuating or synergic if the combination was significantly less or more active than both of each individual gapmer ASOs (Fig. 1a, b). When the combination was less active than single ASOs, we confirmed that single ASOs showed silencing activity at the concentrations equal to or lower

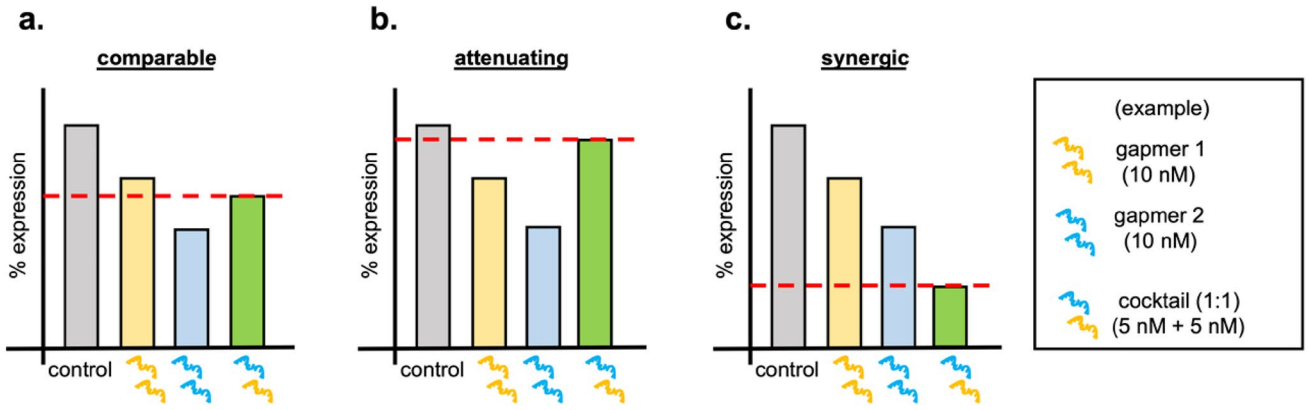



**Fig. 1** Possible knockdown potency of two-gapmer combination compared to individual gapmers. A two-ASO combination was generated by mixing two equimolar ASOs. The potency of the combination was compared to those of individual ASOs at the same molarity. For instance, a combination of gapmer 1 and 2 composed of 5 nM each (10 nM combined) was compared to a single ASO of 10 nM. The combination had a comparable potency if the potency was equal to or intermediate between those of its components (**a**). If the combination showed more or less active than both of its components, then the combination was attenuating (**b**) or synergic (**c**), respectively

than that used in the mixture (Supplementary Fig. 1). If the potency of the combination was comparable to or intermediate between those of its component gapmer ASOs, the pair was regarded as comparable (Fig. 1c).

Before creating combinations, 113 gapmer ASOs for exons and introns of human superoxide dismutase 1 (SOD1) pre-mRNA were designed. After screening them in vitro, the most active 13 gapmer ASOs were selected; these ASOs reduced SOD1 transcript by 40 ~ 60% at 1 nM (Fig. 2). Four-gapmer ASOs were targeted exon sequence and nine to intron; their target sequences had no overlap with each other.

Then, 37 two-gapmer combinations were created by mixing two ASOs and were co-transfected to HeLa cells. Most pairs (29/37 pairs) showed a comparable potency to single ASOs used to create a combination (Fig. 3a). For instance, the combination of #6360/#6361, both targeting intron 1, had a potency equal to that of single gapmers


**b.**

| oligo | target | ASO sequence (5' to 3') |
|---|---|---|
| #6360 | intron 1 | taaGTGCCAATTCggt |
| #6361 | intron 1 | cgtCTTAAGAATAgca |
| #6362 | intron 1 | ttcTCAGGTACAAcgg |
| #6364 | intron 1 | gtaTAGCCATTCAggt |
| #6367 | intron 2 | cctTACACAACTCgag |
| #6368 | intron 2 | cgtAGTATACTTTaca |
| #6369 | intron 3 | ccaGAAAGCTATCgcc |
| #6370 | intron 4 | cgtAATTGTCCAAtaa |
| #6371 | intron 4 | taaCCTTAGTTCCgca |
| #5400 | exon 1 | cgtCGCCCTTCAGcac |
| #7503 | exon 5 | gtcTTTGTACTTTctt |
| #7505 | exon 5 | ctgCTTTTTCATGgac |
| #5402 | exon 5 | cacCTTTGCCCAAgtc |

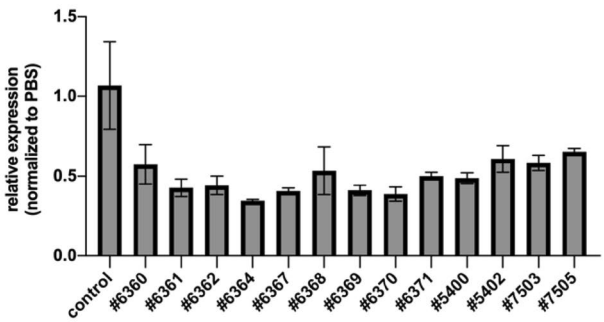
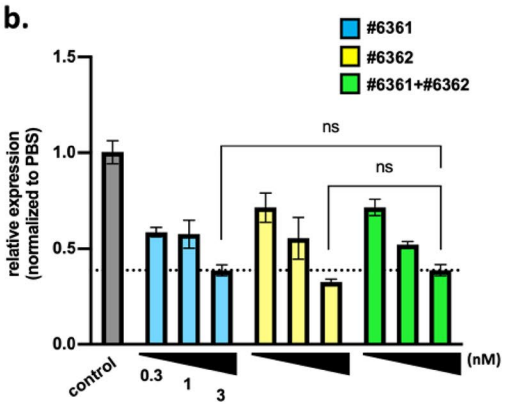



**Fig. 2** Relative positions, sequences, and knockdown effects of single 16-mer LNA gapmers against SOD1 pre-mRNA. **a** Relative target sites of gapmer ASOs. **b** List of ASO sequences. The ASOs used in this study was 3-10-3 LNA gapmers. Small letter, LNA; capital letter, DNA. **c** Single ASOs were transfected to HeLa cell at 1 nM. Thirteen ASOs showed comparable potency to each other. (n = 3; mean ± SEM.)

**a.**

| ASOs | | target sites | potency | distance (base) |
|---|---|---|---|---|
| #6360 | #6361 | intron 1 & intron 1 | comparable | 829 |
| #6360 | #6362 | intron 1 & intron 1 | comparable | 967 |
| #6361 | #6362 | intron 1 & intron 1 | comparable | 138 |
| #6362 | #6364 | intron 1 & intron 1 | comparable | 753 |
| #6367 | #6368 | intron 2 & intron 2 | attenuating | 124 |
| #6370 | #6371 | intron 4 & intron 4 | attenuating | 641 |
| #6360 | #6367 | intron 1 & intron 2 | attenuating | 4841 |
| #6360 | #6368 | intron 1 & intron 2 | comparable | 4965 |
| #6360 | #6369 | intron 1 & intron 3 | comparable | 5710 |
| #6360 | #6370 | intron 1 & intron 4 | attenuating | 6672 |
| #6367 | #6369 | intron 2 & intron 3 | attenuating | 869 |
| #6367 | #6370 | intron 2 & intron 4 | comparable | 1831 |
| #6369 | #6370 | intron 2 & intron 4 | comparable | 962 |
| #7505 | #7503 | exon 5 & exon 5 | comparable | 40 |
| #5402 | #7503 | exon 5 & exon 5 | comparable | 22 |
| #5402 | #7505 | exon 5 & exon 5 | comparable | 18 |
| #5400 | #5402 | exon 1& exon 5 | comparable | 8698 |
| #5400 | #6360 | exon 1 & intron 1 | comparable | 1047 |
| #5400 | #6362 | exon 1 & intron 1 | attenuating | 2014 |
| #5400 | #6364 | exon 1 & intron 1 | comparable | 2767 |
| #5400 | #6367 | exon 1 & intron 2 | comparable | 5888 |
| #5400 | #6368 | exon 1 & intron 2 | comparable | 6012 |
| #5400 | #6369 | exon 1 & intron 3 | comparable | 6757 |
| #5400 | #6370 | exon 1 & intron 4 | comparable | 7719 |
| #5402 | #6360 | exon 5 & intron 1 | comparable | 7651 |
| #5402 | #6361 | exon 5 & intron 1 | comparable | 6822 |
| #5402 | #6367 | exon 5 & intron 2 | comparable | 2810 |
| #5402 | #6368 | exon 5 & intron 2 | comparable | 2686 |
| #5402 | #6369 | exon 5 & intron 3 | attenuating | 1941 |
| #5402 | #6370 | exon 5 & intron 4 | comparable | 979 |
| #5402 | #6371 | exon 5 & intron 4 | attenuating | 338 |
| #7505 | #6360 | exon 5 & intron 1 | comparable | 7633 |
| #7505 | #6370 | exon 5 & intron 4 | comparable | 961 |
| #7505 | #6371 | exon 5 & intron 4 | comparable | 320 |
| #7503 | #6360 | exon 5 & intron 1 | comparable | 7673 |
| #7503 | #6370 | exon 5 & intron 4 | comparable | 1001 |
| #7503 | #6371 | exon 5 & intron 4 | comparable | 360 |



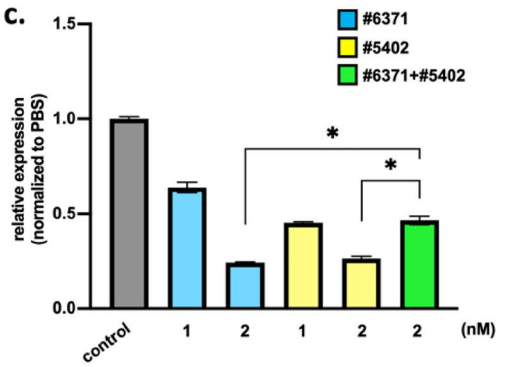




**Fig. 3** The potency of two-gapmer combinations. **a** List of combination-effects on the potency and distance between two target sites. We created 37 pairs from 13 gapmer ASOs and evaluated relative potency to the individual gapmer ASOs. **b** Representative pairs showing comparable potency compared to individual gapmers. ASO #6361, #6362 or combination of them were transfected to HeLa cell at 0.3, 1, and 3 nM. Knockdown potency by combination was comparable to #6361 and #6362. For instance, combination at 3 nM (1.5 nM each) showed equal potency to #6361 or #6362 at 3 nM. **c** Representative pairs with negative combination-effect. The combination at 2 nM (1 nM each) attenuated potency compared to #6371 or #5402 at 2 nM. (*$p < 0.05$; **$p < 0.01$; ns, not significant; $n = 3$; mean ± SEM.)

used in the combination at three different concentrations (Fig. 3b). There were no synergic pairs among the candidates we tested.

## Some combinations appeared to attenuate the potency when co-administered

Eight out of 37 pairs appeared to attenuate the potency. Before conducting the study, we speculated that two gapmers targeting neighboring sites, such as two sequences in the same intron, would likely attenuate the potency by sterically blocking each other from binding to RNA and/or recruiting RNase H. Indeed, two gapmers sharing the same target intron, such as #6367/#6368 (intron 2, 124 base apart) and #6370/#6371 (intron 4, 641 base apart), were attenuating. However, every combinations targeting two sequences in intron 1 showed a comparable potency. For example, the combination of #6361/#6362 demonstrated a comparable potency as mentioned above (Fig. 3b). The target sites of these two gapmers were 138 bases apart from each other, indicating that the distance of their targets was not the determinant factor of combination-effect.

The combination of #6371/#5402 at 10 nM, targeting intron 3 and exon 5, was significantly less potent than single gapmers (5 nM each) (Fig. 3c). Interestingly, other combinations targeting intron 3 and exon 5 with different sequences, such as the pair of #6370/#5402 or #6371/#7503, showed a comparable potency.

We also assumed that a gepmer-gapmer dimerization might lower the activity because this process could prevent each gapmer from binding to the target site. Therefore, the potential risk of dimerization of attenuating and comparable pairs was determined using Amplify4. None of the eight attenuating pairs had more than two complementary sequences with each other, whereas three comparable pairs, such as #6370/#7505, #5402/#6367, or #5402/#7505 had seven, five, or three complementary bases, respectively. This result suggested that the low potency of combinations was not simply explained by dimer formation.

## Three- or four-gapmer combination

A combination approach against the SARS-CoV-2 genome was investigated to minimize the risk of the emergence of escape mutants during treatment [8]. To meet this end, a mixture of more than two oligonucleotides seems to be one of suitable approaches. Moreover, a five-ASO combination was designed to induce multiple exon skipping in dystrophin transcript and treat a broader range of patients with Duchenne muscular dystrophy [9]. Thus, a combination with more than two components could be a reasonable strategy to enhance its clinical benefits in specific diseases.

To test the utility of the multiple-gapmer combination approach with preserved potency, a three-gapmer combination was generated. To avoid unfavorable combination-effects on the potency when mixing three gapmers, we presumed that it would be reasonable to combine two-gapmer combinations with comparable potencies. For instance, #7503, #5402, and #7503 were exon-targeting gapmers; each had equal potencies at 2, 10, 50 nM. Two-gapmer combinations showed a comparable potency to individual gapmers at the same molarity combined (1 nM each, 5 nM each, and 25 nM each) We mixed an equimolar amount of these three gapmers (0.67 nM each, 3.3 nM each, and 17 nM each), and the three-gapmer combination preserved the potency compared to single or two-gapmer combinations at the same molarity combined (Fig. 4a). We also hypothesized that if pairs attenuating the potency were mixed, the mixture would attenuate the potency. We chose #6360/#6367, #6367/#6369, and #6360/#6370 as attenuating two-gapmer pairs. The potency of these four-gapmer combination at 8 nM combined (2 nM each) was less than that of three individual gapmers at 8 nM (Fig. 4b). These results suggested that a judicious design of multiple-gapmer combinations can maintain the potency of single gapmers.

## Discussion

In this study, we evaluated the combination-effect of 37 pairs with two-ASO gapmers. We found that 29 pairs and eight pairs showed comparable and attenuating potencies, respectively, and no pairs showed a synergic effect (Fig. 2a). These combination-effects were not associated only with distance between the targets sites of ASOs. Although the mechanisms behind the combination-effects should be complex and need further investigation, we speculated at least two possible molecular mechanisms that attenuate the potency of combinations.

The first possible mechanism is the steric hindrance caused by conformational changes in the target RNA after the binding of ASO. For instance, #6367/#6368 (124 base apart) had target sites that were close to each other in intron 2, showing a reduced potency. Previous reports demonstrated that a gapmer ASO can recruit RNA-binding proteins (RBPs) competing with RNase H to the ASO/RNA duplex [13]. RBPs are associated with the structure of RNA, [14] which can influence the accessibility of ASOs [15, 16]. Therefore, one gapmer could change the local structure of RNA and attenuate the potency of other gapmers. We assumed that these interactions were more likely to occur between gapmers targeting close sites; however, all combinations targeting tandem sequences in exon 5, such

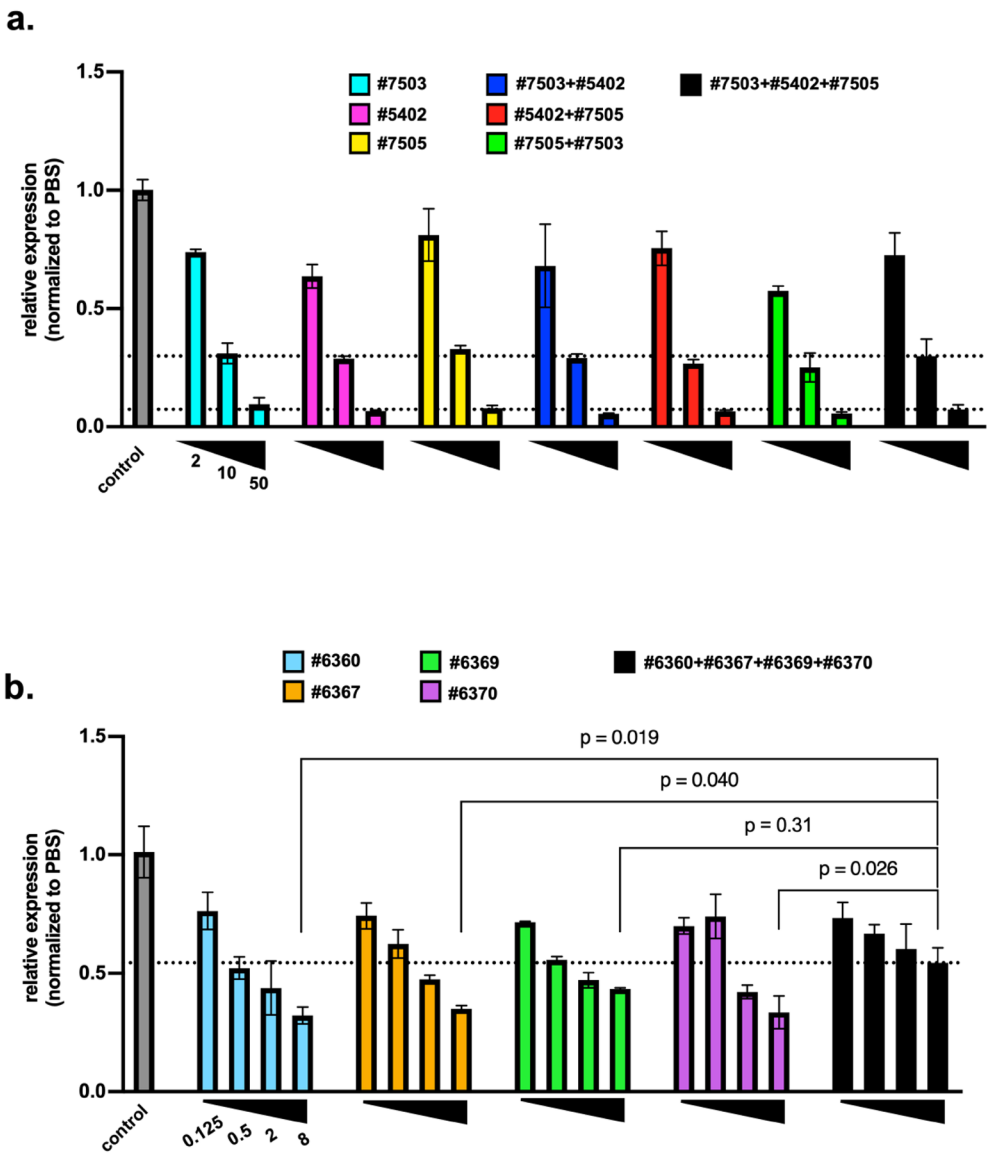



**Fig. 4** The potency of three- or four-gapmer combinations. **a** An example of comparable three–gapmer combination. #7503 (cyan), #5402 (magenta), and #7503 (yellow) had an equal potency to each other at 2, 10, and 50 nM. A two-gapmer combinations showed comparable potency to individual gapmers at the same molarity combined (1 nM each, 5 nM each, and 25 nM each) (#7503/#5402; blue, #5402/#7505; red, #7505/#7503, green). A three-gapmer combination (black) also preserved the potency at the same molarity (0.67 nM each, 3.3 nM each, and 17 nM each). **b** An example of attenuating four-gapmer combination. #6360/6367, #6367/#6369, and #6360/#6370 were attenuating pairs as demonstrated in Fig. 3. The combination (black) at 8 nM combined (2 nM each) appeared significantly less active compared to #6360, #6367, and #6370 at 8 nM. (n = 3; mean ± SEM)

as #5402/#7505, showed a comparable potency (Fig. 4a), indicating that the close distance between two target sites in some regions such as exon 5 might not cause a negative the combination-effect.

The second potential mechanism of attenuation is by the modulation of pre-mRNA processing. For instance, a reduced potency was observed in some pairs, including intron- and exon-targeting ASOs, such as #6371/5402 (Fig. 3c). Attenuation was also observed in combinations targeting different introns. Since these target sites were apart, other mechanisms than steric hindrance might be involved. Previous papers showed that exon-targeting ASOs can compete with splicing factors [17] and increase pre-mRNA levels [18]. Another study demonstrated that the splicing of different introns can cooperate with each other [19]. These modulation of pre-mRNA processing by one ASO may have a negative impact on the silencing activity of other gapmers.

It was also demonstrated that a three-gapmer combination composed of pairs with comparable potencies had a preserved potency. In contrast, a mixture of attenuating

pairs had a reduced potency (Fig. 4b). These findings suggested that a judicious combination of pairs could generate multiple-gepmer combinations with maintained potency by avoiding the negative effects of combinations.

In this study, there are several limitations related with the design or selection of ASOs. While all ASOs were designed as gapmer-type using LNA-modification in the wing portion, chemical modifications of ASOs have an effect on their interaction with RBPs recruited into the ASO-RNA duplex [20]. Since RBPs have crucial roles in the local structure of RNA and splicing process [21], gapmers with different chemical modification may have different effects on the silencing potency of the combinations. Although more than 10 ASOs targeting various exons or introns were utilized to evaluate the combination-effects of ASOs, the target sites of exon-targeting ASOs were limited within exon 1 or 5. This was because we could not find ASOs against exon 2–4 with a comparable knockdown activity. This was necessary to compare the activity of the combination and its components accurately.

## Conclusion

This study revealed that most two-gapmer combinations had a comparable potency, but some showed a reduced activity compared to single gapmers. The attenuation effect could not be predicted only by the distance between the target sites of ASOs. It was also demonstrated that a three-gapmer combination could be designed; this maintained the potency by combining two-gapmer combinations with comparable potencies. These findings showed that a judicious selection of ASOs for combinations, which is independent of the distance between their target sites, could allow combinations to maintain their potency, although the mechanism of negative interactions should be further investigated.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-08224-0.

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**Author contributions** All authors contributed to the study conception and design. MY and SN performed the experiments and analyzed data. TY, TN and KY conceived and supervised the study. MY and KY wrote the manuscript. TY and TN reviewed the manuscript.

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## Declarations

**Competing interests** T.Y. collaborates with Takeda Pharmaceutical Company, Ltd., Daiichi Sankyo Company, Ltd., Rena Therapeutics, Inc., and serves as the academic adviser for Rena Therapeutics, Inc., and Braizon Therapeutics, Inc. K. M. is and S.N. was paid employees of Takeda Pharmaceutical Company Limited. All other authors declare no competing financial interests. All authors read and approved the final manuscript.

**Ethical approval** This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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