Synergistic Effect of an Antisense Oligonucleotide and Small Molecule on Splicing Correction of the Spinal Muscular Atrophy Gene

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ABSTRACT: Spinal muscular atrophy (SMA) is treated by increasing the level of Survival Motor Neuron (SMN) protein through correction of *SMN2* exon 7 skipping or exogenous expression of SMN through gene therapy. Currently available therapies have multiple shortcomings, including poor body-wide distribution, invasive delivery, and potential negative consequences due to high doses needed for clinical efficacy. Here we test the effects of a combination treatment of a splice-correcting antisense oligonucleotide (ASO) Anti-N1 with the small compounds risdiplam and branaplam. We show that a low-dose treatment of Anti-N1 with either compound produces a synergistic effect on the inclusion of *SMN2* exon 7 in SMA patient fibroblasts. Using RNA-Seq, we characterize the transcriptomes of cells treated with each compound as well as in combination. Although high doses of each individual treatment trigger widespread perturbations of the transcriptome, combination treatment of Anti-N1 with risdiplam and branaplam results in minimal disruption of gene expression. For individual genes targeted by the 3 compounds, we observe little to no additive effects of combination treatment. Overall, we conclude that the combination treatment of a splice-correcting ASO with small compounds represents a promising strategy for achieving a high level of SMN expression while minimizing the risk of off-target effects.

KEYWORDS: Spinal muscular atrophy, SMA, survival motor neuron, SMN, antisense oligonucleotide, ASO, ISS-N1, risdiplam, branaplam, nusinersen, anti-N1

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Introduction

Spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality caused by low levels of Survival Motor Neuron (SMN) protein due to deletion of or mutations in the *SMN1* gene.[1] *SMN2*, a near identical copy of *SMN1*, cannot fully compensate for the loss of *SMN1* due to the predominant skipping of exon 7, leading to a truncated, unstable protein.[2-4] Skipping of *SMN2* exon 7 is facilitated by Intronic Splice Silencer N1 (ISS-N1) located close to the 5′ splice site (5′ss).[5] Nusinersen, the first FDA-approved therapy for SMA, is an antisense oligonucleotide (ASO) that restores *SMN2* exon 7 inclusion by blocking ISS-N1.[6] Nusinersen is highly effective at ameliorating the worst symptoms of SMA and preventing death when treatment is initiated early.[7] However, a sizable portion of patients treated presymptomatically with nusinersen still fail to achieve several motor milestones.[7] The obvious drawbacks of nusinersen treatment are invasive intrathecal administration, poor body-wide distribution, and off-target effects at concentrations used for clinical applications. Indeed, high concentrations of an ISS-N1 targeting ASO (Anti-N1) triggers massive perturbations of the transcriptome.[8] Risdiplam, a small molecule approved for the treatment of SMA, has the advantages of oral administration and body-wide delivery.[9] However, high concentrations of splicing-modulating small molecules such as risdiplam and branaplam also massively perturb the transcriptome.[10] In contrast, low concentrations of Anti-N1, risdiplam and branaplam display substantially less off-target effects while retaining the ability to significantly promote *SMN2* exon 7 inclusion.[8,10] It has been also found that the combined treatment with low concentrations of risdiplam and branaplam has a synergistic effect on *SMN2* exon 7 inclusion.[10] However, it is not known if the combined treatment with low concentrations of an ISS-N1-targeting ASO and a small molecule would have a synergistic effect on *SMN2* exon 7 inclusion while maintaining minimal off-target effects.

Here we reanalyze our recently reported high throughput sequencing data to capture the nature of overlapping off-target effects among Anti-N1, risdiplam and branaplam at both high and low concentrations of compounds. While our analysis captured several genes impacted by all 3 compounds, there were more overlaps between Anti-N1 and risdiplam than Anti-N1 and branaplam. We also observed many impacted genes unique to Anti-N1. Here we perform new experiments to determine the effect of combined treatment with low concentrations of Anti-N1 and risdiplam or branaplam on *SMN2* exon 7 splicing. Our results showed synergistic effects of combined treatments while retaining minimal off-target effects. Our findings reveal yet another avenue for improving the therapeutic strategy for an effective therapy for SMA using already approved compounds.

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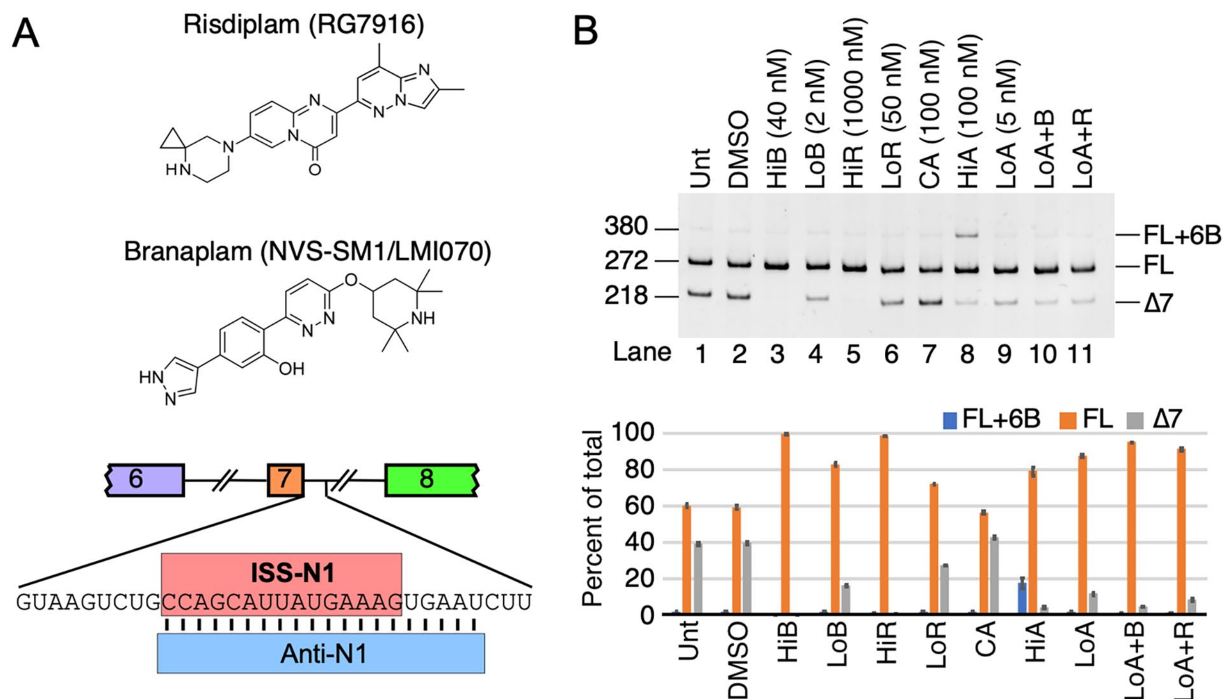


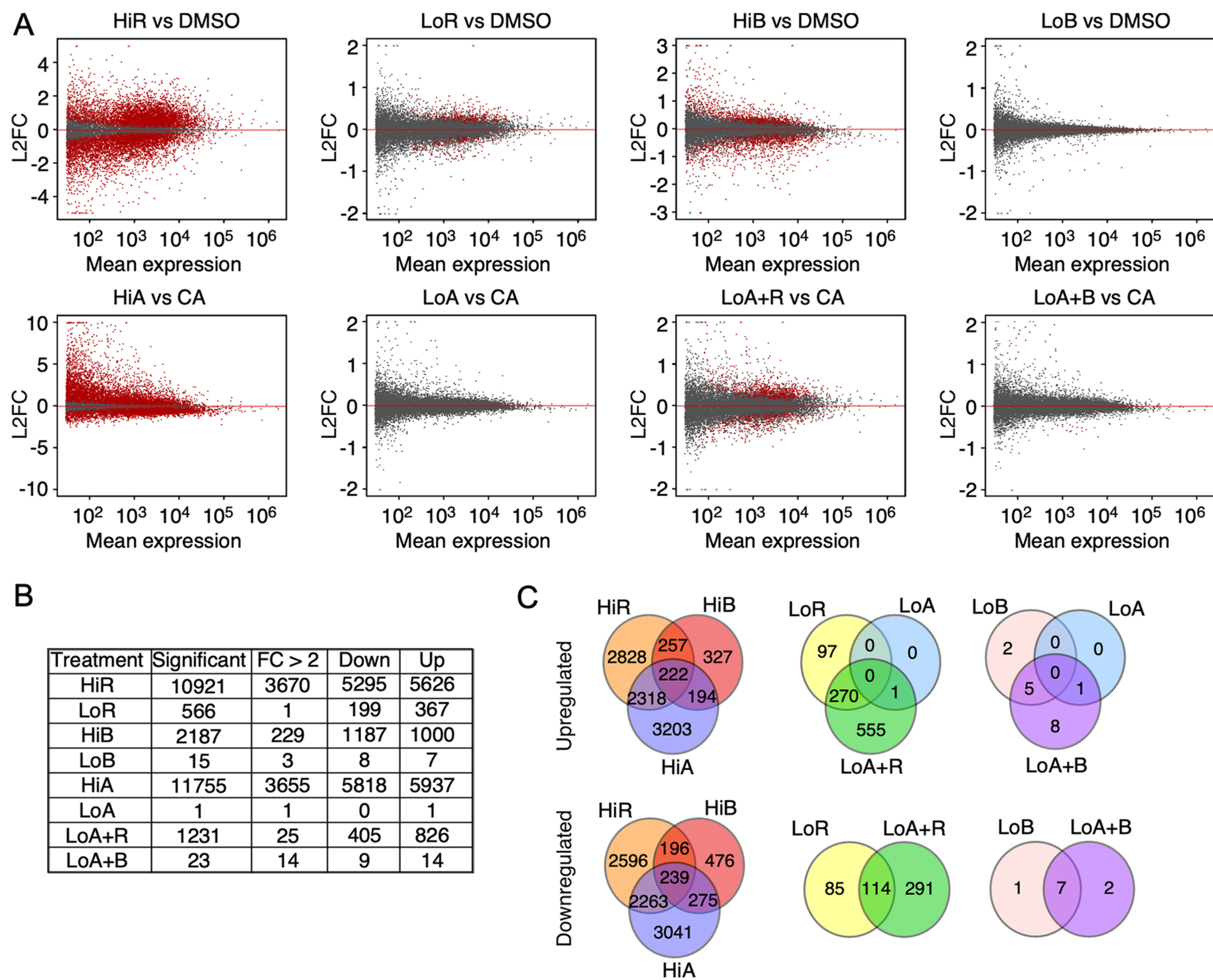
Figure 1. Combination treatment of Anti-N1 and small compounds has a synergistic effect on *SMN2* exon 7 inclusion: (A) Chemical structures of risdiplam and branaplam and graphical overview of the annealing location of Anti-N1. Exons are shown as colored boxes, introns as broken lines. ISS-N1 and its surrounding sequences are shown in the magnified area, with the annealing position of Anti-N1 indicated. (B) Treatment of GM03813 SMA patient fibroblasts with compounds. Upper panel: representative gel image of semi-quantitative PCR depicting splicing of *SMN2* exon 7. Treatments are indicated at the top of the gel and are labeled as follows: Unt, untreated; DMSO, 0.1% DMSO; HiB, 40 nM branaplam; LoB, 2 nM branaplam; HiR, 1000 nM risdiplam; LoR, 50 nM risdiplam; CA, 100 nM control ASO; HiA, 100 nM Anti-N1; LoA, 5 nM Anti-N1 + 95 nM control ASO; LoA + B, combined LoA and LoB treatment; LoA + R, combined LoA and LoR treatment. Calculated band sizes are labeled at the left side. Splice isoforms are labeled on the right side. Abbreviations: Δ7, exon 7 skipped product; FL, full-length *SMN2*; FL + 6B, full-length *SMN2* with inclusion of cryptic exon 6B. Lower panel: quantification of relative splice isoform abundance. Error bars represent the standard error of the mean (n = 3).

Results

To compare the effects of different concentrations of branaplam, risdiplam, and an ISS-N1 targeting ASO (Anti-N1) on splicing of *SMN2* exon 7, we treated GM03813 SMA patient fibroblasts with high and low concentrations of branaplam (HiB, 40 nM and LoB, 2 nM), risdiplam (HiR, 1000 nM and LoR, 50 nM), or Anti-N1 (HiA, 100 nM and LoA, 5 nM) (Figure 1). As previously observed, HiB and HiR completely prevented the skipping of *SMN2* exon 7, while LoB and LoR resulted in partial correction (Figure 1B).[10] Consistent with earlier results, HiA resulted in the predominant inclusion of *SMN2* exon 7 and partial inclusion of exon 6B, a cryptic exon located in intron 6 (Figure 1B).[8,11] LoA treatment resulted in partial restoration of *SMN2* exon 7 inclusion without triggering the inclusion of exon 6B. We also examined the effect of combination treatment of low concentrations of branaplam or risdiplam with Anti-N1 treatment together (LoA + B or LoA + R). Combination treatment resulted in almost complete inclusion of *SMN2* exon 7, more than any of the single low-dose treatments (Figure 1B).

We have previously shown that HiR, HiB, and HiA trigger widespread perturbations of the transcriptome of GM03813 SMA patient cells.[8,10] To determine the overlap between targets of HiA and the other 2 compounds, we reanalyzed our RNA-Seq data (Figure 2). Of the 5626 genes upregulated by HiR, 2318 were similarly impacted in HiA and 222 were affected by all 3 treatments, for a total overlap of 45.1% of affected genes (Figure 2C). Of 5295 downregulated genes in HiR, 2263 were downregulated by HiA as well and 239 by HiR and HiB, for a total 47.3% overlap (Figure 2C). 1000 genes were upregulated by HiB; 194 were shared by HiA alone (Figure 2C). Combined with the genes upregulated in all 3 treatments, 41.6% of all genes upregulated by HiB were similarly affected in HiA. 1187 genes were downregulated by HiB; 275 were also downregulated by HiA but not HiR (Figure 2C). With the genes affected in all three, 43.3% of the downregulated targets of HiB were shared with HiA. LoA only affected the expression of one gene, which was shared with LoR (Figure 2C).

To determine whether combination treatment exacerbates the off-target effects of the individual compounds, we performed RNA-Seq on transcripts isolated from cells treated with LoA + R and LoA + B (Figure 2A and B). LoA + R had a greater impact on the transcriptome than either of the individual treatments, altering the expression of 1231 genes, of which 25 were affected more than two-fold (Figure 2B). However, these changes are minimal compared to the widespread impact of HiA and HiR which changed 11755 and



Treatment	Significant	FC > 2	Down	Up
HiR	10921	3670	5295	5626
LoR	566	1	199	367
HiB	2187	229	1187	1000
LoB	15	3	8	7
HiA	11755	3655	5818	5937
LoA	1	1	0	1
LoA+R	1231	25	405	826
LoA+B	23	14	9	14

Figure 2. Transcriptomic changes after combination treatment with Anti-N1 and small compounds: (A) MA plots depicting gene expression changes upon individual and combination treatment of small compounds and/or Anti-N1. Comparisons between treatments are indicated above each plot. Each dot represents one gene, gray dots are unaffected while red dots are significantly affected by treatment (adjusted *P*-value < .05). *Y*-axis represents a $\log_2$ fold change of expression, *X*-axis represents the mean normalized read count per gene. (B) Overall summary table describing results of RNA-Seq. "Significant" indicates genes with Benjamini and Hochberg adjusted *P*-value (adj. *P*) < .05. FC > 2 indicates genes with more than two-fold up- or downregulation. (C) Venn diagrams examining the overlap in upregulated (upper panels) and downregulated (lower panels) genes affected by each treatment.

10921 genes, respectively (Figure 2B).[8,10] LoA + B only altered the expression of 23 genes, although the majority of these (14) were affected more than two-fold (Figure 2B). We observed a significant overlap between genes affected by individual compounds and combined treatment (Figure 2C). In particular, 270 of the 376 genes upregulated by LoR were similarly affected by LoR + A, while 114 of the 199 downregulated genes were shared. Five out of 7 genes upregulated by LoB were also affected in LoB + R, while 7 out of 8 genes downregulated by LoB were similarly altered in LoB + R (Figure 2C).

We examined our RNA-Seq data for the expression of individual genes that are significantly altered by LoR, LoB, or LoA treatment to determine whether there was any additive effect in LoA + R or LoA + B. *SNX33*, *FOXJ2*, and *PBX1* were all increased in LoR treatment (Figure 3A). All 3 were similarly impacted in LoA + R, without any additive effects. *RCC1*, *SMARCE1*, and *ZNF837* were upregulated by LoB treatment (Figure 3A). *RCC1* and *ZNF837* were similarly impacted in LoA + B, but *SMARCE1* did not reach statistical significance. The lncRNA *AL021155.5* was the only gene significantly upregulated in LoA treatment. Expression was similarly altered in LoA + R and LoA + B with no observable additive effect of the combination treatment (Figure 3A). *CHPF2*, *CC2D1B*, and *TMEM214* were significantly downregulated by LoR treatment (Figure 3B). We observed a similar change in LoA + R for all 3 genes. *HTT*, *XRN2*, and *PITPNB* are downregulated in LoB, with the same alteration in LoB + R (Figure 3B). *PDXDC1* was significantly downregulated in LoR, LoB, LoA + R, and LoB + R (Figure 3B). Importantly, combination treatment did not increase the off-target effect on *PDXDC1* compared to the single compounds. Overall, combination treatment retained low off-target effects on all of the individual genes we analyzed.

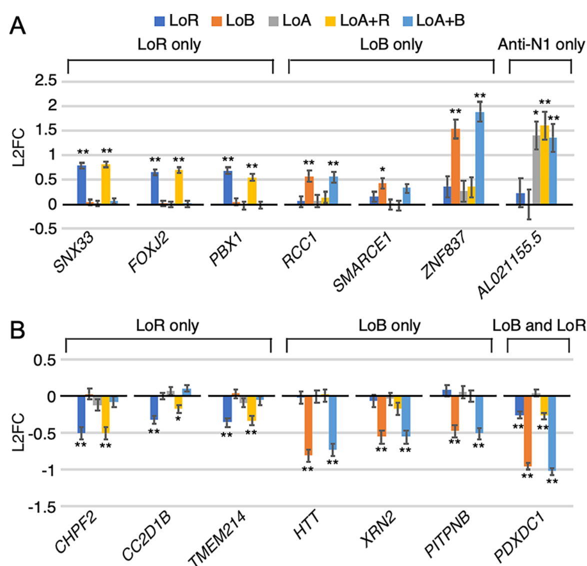


Figure 3. Effects of combination treatment on individual genes: (A) Expression of several candidate genes predicted by RNA-Seq to be upregulated by low doses of risdiaplam and/or branaplam or Anti-N1. Color coding of different treatments is indicated at the top. *Y*-axis represents $\log_2$ fold change (L2FC) compared to control. Genes are labeled at the bottom of the graph, whether they were affected by risdiplam, branaplam, or both, or anti-N1 is indicated at the top. Error bars represent the standard error of the mean (n = 3). $^*P < .05$, $^{**}P < .01$. Abbreviations: Bra, branaplam; Ris, risdiplam. (B) Expression of several candidate genes predicted by RNA-Seq to be downregulated by low doses of risdiaplam and/or branaplam or Anti-N1. Coloring and labeling are the same as in (A).

Discussion

Here we report a side-by-side comparison of off-target effects of 3 splicing modulating compounds, including one ISS-N1-targeting ASO (Anti-N1) and 2 small molecules, risdiplam and branaplam. With direct significance to SMA therapy, all 3 compounds restore exon 7 inclusion in transcripts generated from endogenous *SMN2* (Figure 1). While high concentrations of these compounds cause severe perturbations of the transcriptome, low concentrations provide suboptimal *SMN2* exon 7 splicing correction with the benefit of reduced off-target effects.[8,10] All 3 compounds employ different mechanisms of action and require different optimal concentrations for *SMN2* exon 7 splicing correction.[9,12] Consistently, they show significant differences in off-target effects at both high and low concentrations. The differences in the mode of action of these compounds open avenues for potential synergistic effects at concentrations not detrimental to cellular metabolism. Indeed, we recently showed a synergistic effect of risdiplam and branaplam on *SMN2* exon 7 splicing correction at concentrations low enough to minimize the potential off-target effects.[10] Here we show a similar scenario of synergistic effect between Anti-N1 and risdiplam or branaplam on *SMN2* exon 7 splicing correction (Figure 1). The low concentrations of ASO and small molecules used to capture the synergistic effect did not escalate the perturbations of the transcriptome (Figures 2 and 3). Findings provide a strong incentive to develop a combined therapy for SMA using 2 approved therapies, nusinersen and risdiplam.

Remarkable progress has been made toward developing therapies for SMA as 3 approved drugs, including nusinersen, risdiplam and gene therapy are already available. However, these therapies have begun to show their shortcomings as treated patients remain wheelchair-bound and/or die due to unresolved complications.[7] Recent reports of off-target effects of SMA drugs and/or overexpression of SMN have further added to the concerns.[8,10,13,14] The initial belief that restoration of SMN in motor neurons is sufficient to cure SMA completely did not hold true as body-wide restoration of SMN appears to be an absolute requirement for overall peripheral development.[15] This places an additional burden on looking for therapies capable of peripheral deliveries at concentrations not detrimental to tissues. Combined therapies, including those described here, may offer novel avenues to address some of the prevailing concerns. Now that we have shown the synergistic effects of low doses of 2 splicing modulating compounds in the context of a cellular model of SMA, the stage is set for future studies in animal models of SMA and eventually in SMA patients.

Materials and Methods

Cell culture and treatments

GM03813 primary SMA patient fibroblasts were grown as described earlier.[8,10] All ASOs were obtained from Dharmacon Inc as described earlier.[8] Risdiplam and branaplam were purchased from MedChemExpress and stock solutions were prepared as described previously.[10] GM03813 cells were plated at a density of 1.1×10^6 cells per 10 cm cell culture dish. Sixteen hours later, cells were transfected with ASOs using Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions. Six hours after transfection, media was replaced with fresh media containing the indicated concentration of DMSO, risdiplam, or branaplam. Twenty-four hours later, cells were collected for RNA isolation using TRIzol Reagent (Life Technologies). ASO sequences are as follows: Anti-N1: 5′-A*mU*mU*mC*mA*mC*mU*mU*mU*mC*mA*mU*mA*mA*mU*mG*mC*mU*mG*mG-3′. 10mer: 5′-mU*mU*mG*mC*mC*mU*mU*mU*mC*mU-3′. m indicates 2′ O-methyl modification and * indicates phosphorothioate modification of the ASO backbone.

Reverse transcription and PCR (RT-PCR), library generation and RNA-Seq

RT-PCR and RNA-Seq were carried out as previously described.[8,10] RNA-Seq data have been submitted to NCBI under the BioProject accession numbers PRJNA695152 and PRJNA758038.

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Author Contributions

RNS conceived the idea. EWO performed experiments and analyzed data. RNS and EWO wrote the manuscript.

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