The introduction of Ligand for Enhanced Nuclear Delivery of Antisense Oligonucleotide

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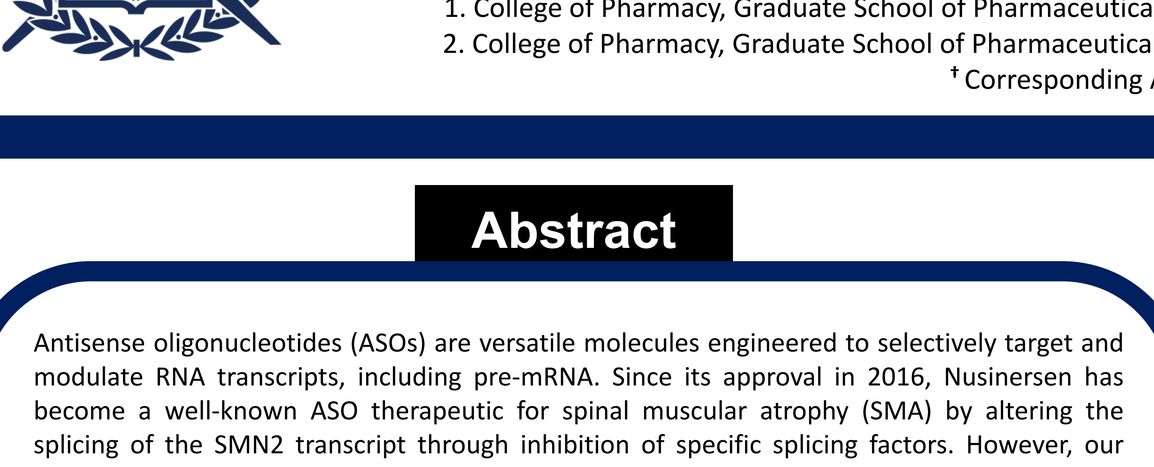
Abstract

Antisense oligonucleotides (ASOs) are versatile molecules engineered to selectively target and modulate RNA transcripts, including pre-mRNA. Since its approval in 2016, Nusinersen has become a well-known ASO therapeutic for spinal muscular atrophy (SMA) by altering the splicing of the SMN2 transcript through inhibition of specific splicing factors. However, our limited understanding of ASO delivery into cells and the nucleus has constrained efforts to minimize toxicity and enhance efficacy with low-dose treatments. We investigated a novel delivery strategy combining steroid receptor-mediated pathways with advanced lipid nanoparticle (LNP) technology to improve ASO therapeutic efficacy. Specifically, we employed Hexyl 2-Hydroxyethyl sulfide (HHES) ionizable lipid-based LNPs alongside corticosteroid-conjugated, locked nucleic acid (LNA)-modified Nusinersen to exploit glucocorticoid receptor-mediated mechanisms. This strategy increased efficacy by 1.5-fold at maximum dosage and maintained ~92% exon 7 inclusion with an 80% dose reduction. These findings highlight the potential of this combinatory approach for ASO drug delivery in clinical applications.



Results & Discussion

ASO conjugation with small molecule

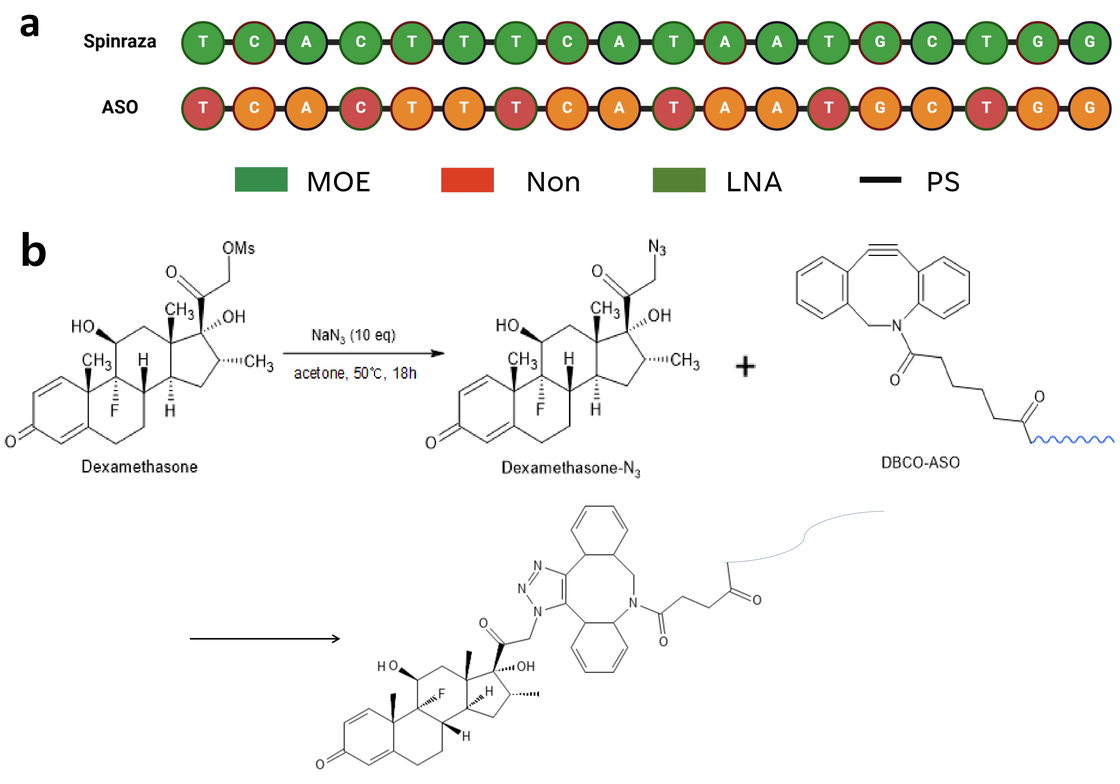


Figure 1. (a) The sequence and chemical modifications of Spinraza (Nusinersen) and small molecule-conjugated ASO. Locked Nucleic Acid (LNA) was applied in small molecule ASO as it has a higher affinity for target mRNA compared to MOE modification. (b) Small molecule and ASO conjugation scheme. DBCO and azide was introduced into ASO and small molecule, respectively, to conjugate the two molecules via click chemistry.

Confirming the conjugation using MALDI-TOF

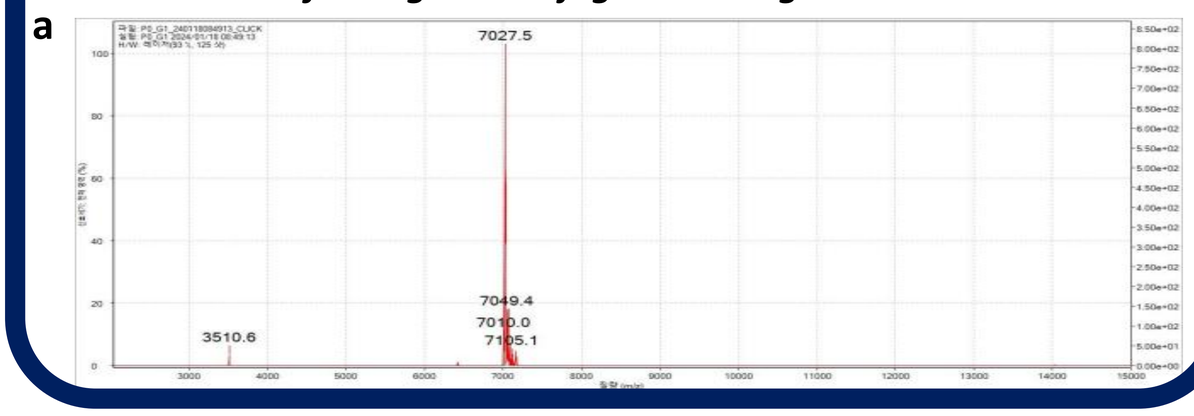


Figure 2. (a) MALDI-TOF analysis result. After removing any unreacted azide-small molecule, the conjugation product was confirmed using MALDI-TOF analysis. The mass spectrum obtained showed a peak of m/z 7027.5. This is almost similar to the expected molecular weight of the conjugate, 7028.78 (Molecular weight of DBCO-ASO=6611.4, azide-small molecule=417.48), confirming that small molecule was properly conjugated to ASO.

Fluorescence Polarization Assay

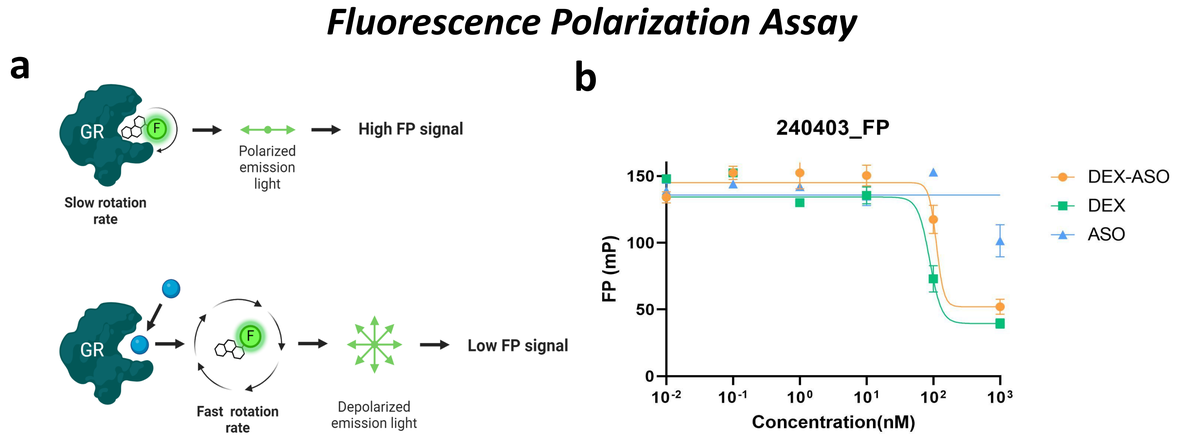


Figure 3. To validate that the conjugated ASO does not interfere with the binding of small molecule to the glucocorticoid receptor (GR), a fluorescence polarization (FP) assay was performed. (a) Principle of fluorescence polarization assay. When ligand binds to a receptor, it rotates slowly, generating a high polarization signal. Upon addition of competing ligands, ligand dissociates from the receptor, resulting in increased rotation speed and decreased polarization signal. (b) Fluorescein-labeled small molecule was first mixed with GR, and serially diluted competing ligands (small molecule-ASO or just small molecule) were added. As expected, ASO did not exhibit any change in FP values with increasing concentration. In contrast, both small molecule-ASO and just small molecule displayed a similar sigmoidal curve with increasing ligand concentrations, indicating their binding to the receptor with comparable binding affinity. The IC50 values were 111.7nM and 88.49nM, respectively.

Real Time-PCR

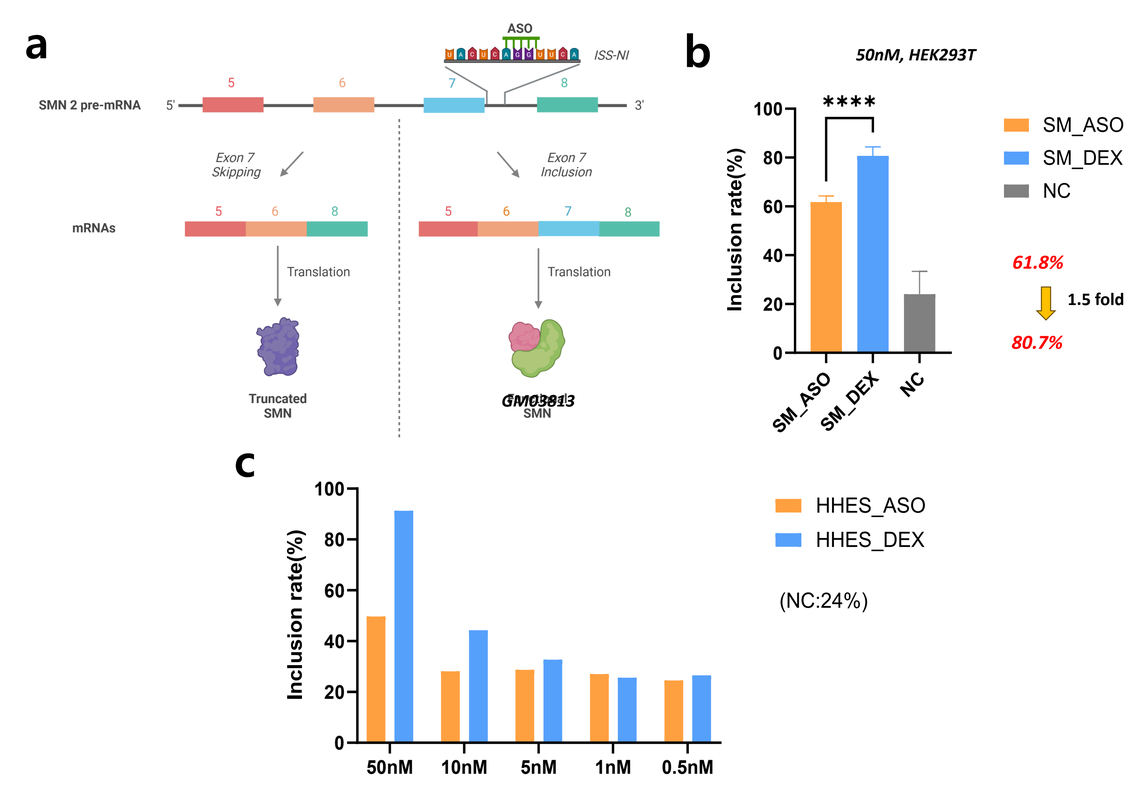


Figure 4. Lipid Nanoparticles encapsulating small-molecule-ASO and ASO were transfected to HEK293T cells (50nM) and their effects were evaluated using RT-PCR. (a) The ASOs bind to ISS-N1, a splicing inhibitory sequence in intron 7 of SMN2 pre-mRNA, and replace the splicing inhibitors, thereby allowing the production of functional SMN protein. (b) Total RNA was extracted on Day 2 and analyzed via RT-PCR to compare the amounts of full-length and exon 7-skipped mRNA transcripts. ASO showed an average inclusion rate of 61.78% whereas small molecule-ASO demonstrated superior efficacy, with an inclusion rate of 80.72%, approximately 1.5 times higher. (c) RT-PCR result of transfection with HHES LNPs encapsulating either small-molecule ASO or ASO in GM03813, an SMA patient-derived cell line. At 50nM, small molecule-ASO achieved nearly twice the exon 7 inclusion rate compared to control ASO. This suggests that small molecule could enable a substantial reduction in the required ASO dosage for effective splicing modulation.

Summary

In this study, azide and DBCO were introduced to small molecule and ASO, respectively, and they were conjugated through click reaction. The conjugate was possible to be confirmed through MALDI-TOF analysis. Later, it was demonstrated by FP that conjugated ASO does not interfere with the binding of small molecule to a glucocorticoid receptor. ASO and small-molecule ASO were transfected to HEK293T cells / GM03813 and RT-PCR was performed to quantify full-length and exon 7-skipped mRNA transcripts. Calculating the exon 7 inclusion rate using the ratio of these two transcripts, it was found that small molecule-ASO can increase inclusion rate by about 1.5 times. These results indicate that small molecule enhances the nuclear transport of ASO and the resulting increase in splicing modulation.