Going nuclear: Improved antisense oligonucleotide activity through conjugation with a nuclear importer

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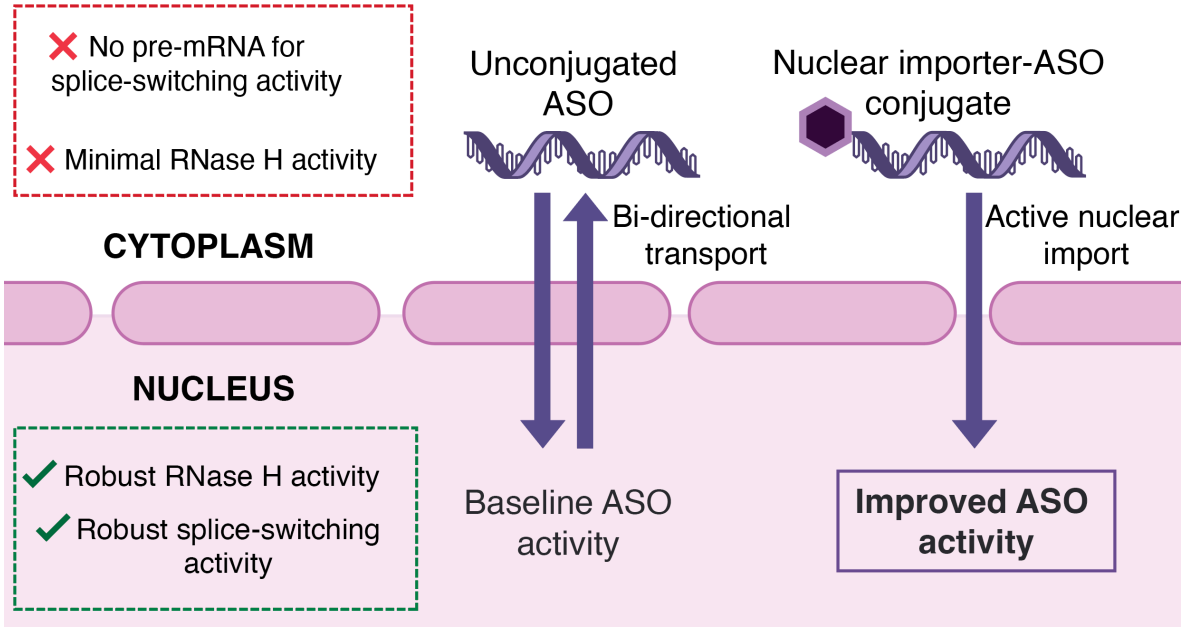
Abstract

Antisense oligonucleotides (ASOs) are a promising class of therapeutics designed to modulate gene expression. Both key mechanisms of action for ASOs operate in the nucleus: splice-switching ASOs modify pre-mRNA, processed in the nucleus, and mRNA-degrading ASOs require RNase H, an enzyme predominantly active in the nucleus. Therefore, to achieve maximal efficacy, ASOs require efficient nuclear delivery. Current ASO therapeutics shuttle in and out of the nucleus inefficiently. In this work, we have synthesised ASO conjugates for active nuclear import, by covalent conjugation with a potent small-molecule nuclear importer, (+)-JQ1. (+)-JQ1 is a well-characterised high-affinity binder for members of the BET bromodomain family of proteins and was recently shown to transport cytoplasmic proteins into the nucleus. Our (+)-JQ1-ASO conjugates outperformed their unmodified counterparts for both splice-switching and mRNA knockdown in the nucleus, at all concentrations tested. In particular, we improved the performance of Oblimersen, a BCL-2 ASO drug that failed phase-III clinical trials, showing that this therapeutic may merit re-evaluation. This work shows that the covalent modification of ASOs with a small-molecule nuclear importer can significantly improve target engagement and pave the way for more effective therapeutics.



Introduction

Nucleic-acid drugs have the potential to revolutionize how we treat a wide range of diseases.[1] Among these drugs, antisense oligonucleotides (ASOs) have attracted significant attention for their ability to provide precise control over translation.[2] ASOs have several mechanisms of action, including RNase H-mediated degradation of mRNA bound to DNA-based ASOs, modulation of pre-mRNA processing, and steric hinderance of ribosome binding.[3] This RNA-level intervention allows for a targeted approach to correct gene dysregulation associated with various pathological conditions, thus providing a specific and effective therapeutic strategy.

A critical aspect of ASO effectiveness consists in their ability to localize within the nucleus (**Figure 1**).[4] This is because ASOs operate primarily in the nucleus, through pre-mRNA splicing and RNase H-recruitment. Current ASO therapeutics are shuttled in and out of the nucleus: an inherently inefficient process, especially for large, negatively charged molecules like ASOs.[5] While there is a pool of ASOs in the nucleus that exert their gene-modulating effects, the constant shuttling is suboptimal. Enhancing the nuclear import and accumulation of ASOs has been postulated to significantly improve their target engagement and, consequently, their therapeutic efficacy. Previous work to improve nuclear delivery has employed nucleic acid conjugates with small molecules[6], aptamers[7], and peptides[8]. Conjugates generated with the small molecule double-stranded DNA-binding dye Hoechst[9] exhibited minimal and inconsistent improvements in gene-knockdown efficacy: targets with the same mechanism of action were knocked down to different degrees. Incorporating the nucleolin aptamer AS411 in a splice-switching oligonucleotide sequence has been shown to yield very modest splice switching improvements[10]. Efforts utilizing ASO conjugates with nuclear localization signal (NLS) peptides have faced significant toxicity and efficacy challenges[11,12]. This demonstrates the need to explore simpler and more effective alternatives for enhanced nuclear accumulation of ASOs.

A recent study demonstrated the potential of a bi-functional compound containing the small molecule (+)-JQ1 warhead to induce the nuclear localization of cytoplasmic proteins[13]. (+)-JQ1 is a widely studied potent binder for members of the BET bromodomain family of proteins.[14] While most of these proteins have primary roles in the nucleus[15, 16], they can perform secondary functions in the cytoplasm, especially in the context of cellular signalling and stress responses[17, 18]. Thus, BET bromodomain proteins display an intermediary shuttling state between the nucleus and cytoplasm.

In this work, we have synthesised novel covalent (+)-JQ1-ASO conjugates aimed at increasing the active nuclear import of ASOs. Our approach utilizes a single covalent (+)-JQ1 ligand modification to effect the nuclear enrichment of ASOs via interactions with BET bromodomain proteins. Our (+)-JQ1-ASO conjugates are superior to unconjugated ASOs, for both mRNA knockdown and splice-switching mechanisms (**Figure 1**). Moreover, we demonstrated that the improved efficacy of (+)-JQ1-ASO conjugates is dependent on binding to (+)-JQ1 target proteins, as excess small molecule (+)-JQ1 could outcompete them. By manipulating cellular localization through this mechanism, we pave the way for the development of more effective ASO therapeutics. The ability to actively augment the nuclear accumulation of ASOs, without increasing cellular toxicity, constitutes a significant advancement in the field. This advancement has the potential to improve therapeutic outcomes and expand the applicability of nucleic acid-based drugs.

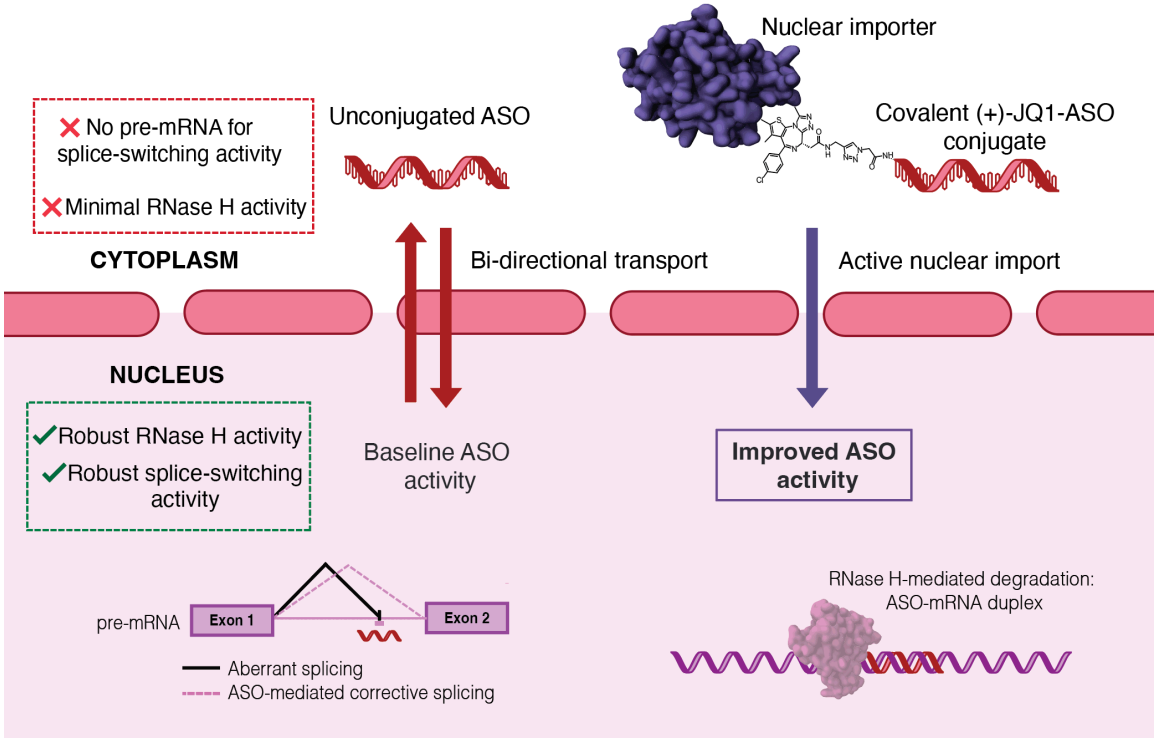


Figure 1. Schematic demonstrating the improved activity of (+)-JQ1-ASO conjugates over unconjugated ASOs. (+)-JQ1-ASO conjugates improve splice-modulation and RNase H-mediated knockdown through increased nuclear accumulation.

Results

To test the activity of (+)-JQ1-ASO conjugates, we initially chose a splice-switching ASO (SSO). The 18mer SSO we used contained a fully phosphorothioated (PS) backbone and all sugars with a 2'-OMe modification (**Figure 2a**). We used the gold-standard SSO sequence developed for the HeLa pLuc/705 cell line[19], which expresses a luciferase-encoding gene interrupted by a mutated ß-globin intron. The mutation generates a 5'-splice site which activates a cryptic 3'-splice site, resulting in incorrect mRNA splicing and non-functional luciferase production (**Figure 2b**). The SSO binds to the mutant 5'-splice site and promotes the exclusion of the aberrant intron, restoring the pre-mRNA splicing to produce functional luciferase.

To synthesise the covalent (+)-JQ1-SSO conjugate, we used copper-catalysed click chemistry. We synthesised alkyne-modified (+)-JQ1 in two steps, starting from the commercially available (+)-JQ1 ligand. First, the boc group of (+)-JQ1 was deprotected to yield the (+)-JQ1 acid. This was followed by a hydroxybenzotriazole (HoBT) and (3-Dimethylamino-propyl)-ethyl-carbodiimide hydrochloride (EDC-HCl)-mediated coupling with propargylamine, resulting in the formation of (+)-JQ1-alkyne. 5'-azide-SSO was prepared through an azidoacetic acid-N-hydroxysuccinimide (NHS) ester functionalisation of a 5'-terminally amine-modified SSO. The final (+)-JQ1-SSO conjugate was prepared using copper-catalysed click conjugation of the (+)-JQ1-alkyne with the 5'-azide-SSO (**Figure 2c**). We achieved >90% yields for all bioconjugation reactions performed and >95% purity following HPLC purification (**SI Figure 1, 2**).

To test our (+)-JQ1-SSO conjugates in the HeLa pLuc/705 cell line, we compared their activity to the well-studied SSO, without the 5' (+)-JQ1 modification. Cells were treated with varying concentration (25-200 nM) of either SSO (unconjugated and (+)-JQ1 modified) for 24-hours and quantified by luminometry, serving as a measure of splice correction and SSO efficacy. Our (+)-JQ1-SSO conjugate showed 2.0, 1.8, 1.9, and 1.7 fold higher splice-switching activity compared to the unconjugated SSO, at 25, 50, 100, and 200 nM respectively (**Figure 2d**). Covalent addition of (+)-JQ1 did not significantly increase the toxicity of the SSO at any concentration, relative to its unconjugated form – assessed through total protein production quantified by BCA (**SI Figure 3**) and Cell-Titer Glo (**SI Figure 4**).

To confirm that the improved splice switching was induced by BET protein-mediated nuclear import rather than non-specific binding to cellular proteins, we carried out a competition assay with excess small molecule, (+)-JQ1. At low concentrations of (+)-JQ1 (5 nM) the enhanced activity of the (+)-JQ1-SSO conjugate at 25 and 50 nM was effectively inhibited (**Figure 2f**). Consistent with the idea that the (+)-JQ1-ASO conjugate acts via an interaction with BET proteins, even higher concentrations of 200 nM (+)-JQ1 were required to inhibit the enhanced activity of the (+)-JQ1-SSO at 100 nM and 200 nM (**Figure 2g, SI Figure 5**). Thus, we demonstrated that splice-switching ASO activity can be doubled via a specific interaction with the BET proteins for enhanced nuclear import.

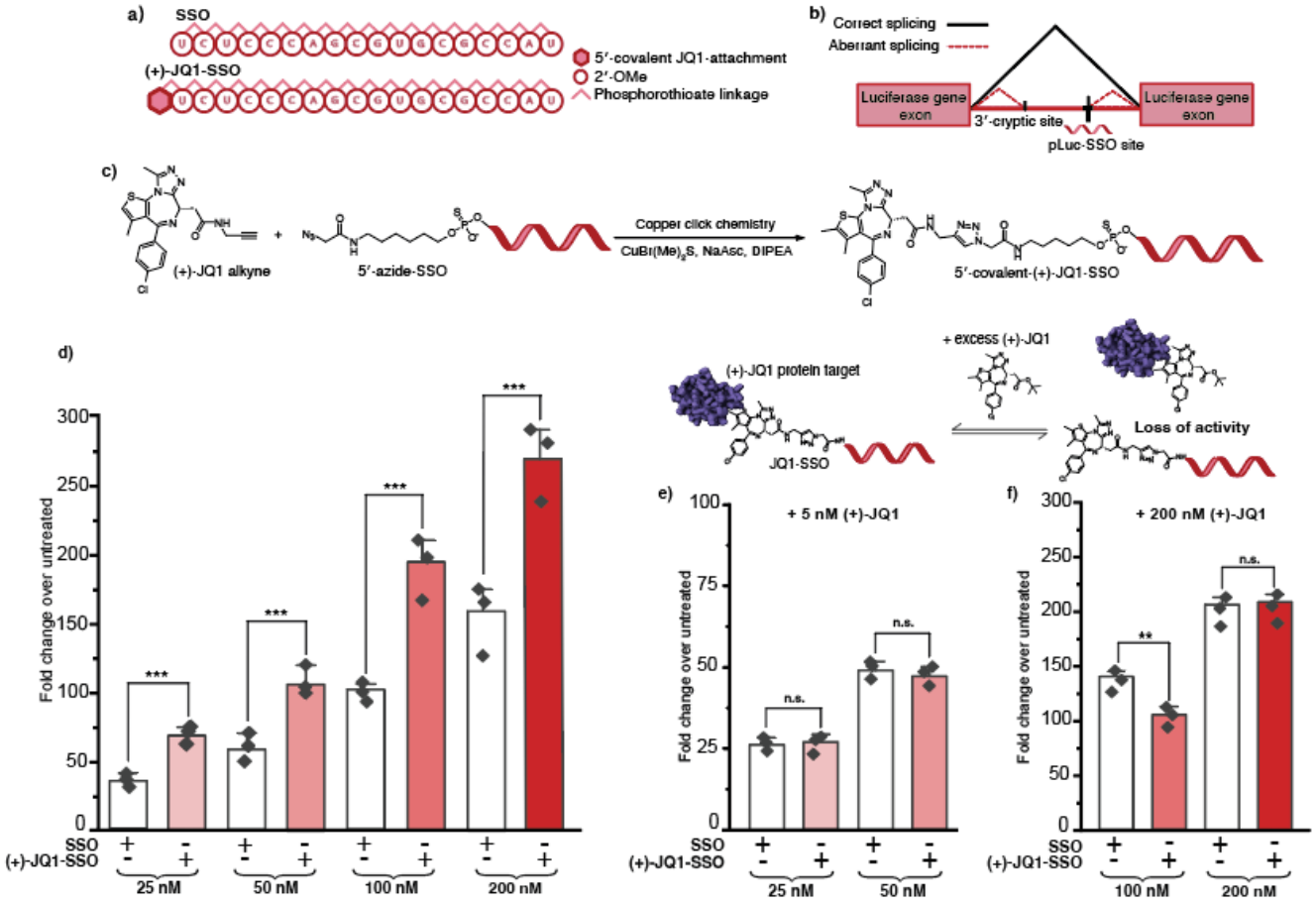


Figure 2: Covalent (+)-JQ1-SSO modification enhances slice-switching activity. **a**) Sequence and chemical modifications for SSO used in the HeLa pLuc 705 cell line. **b**) Splice-switching mechanism in HeLa pLuc 705 cells. **c**) Synthesis of (+)-JQ1-SSO conjugate using copper-catalysed click chemistry. **d**) Luminescence values for SSO and (+)-JQ1-SSO activity, transfected with lipofectamine 2000, at 24 hours at concentrations indicated. In all cases, luciferase activity was measured and normalised to untreated cells. Competition assay between (+)-JQ1-ASO conjugate and excess small molecule, (+)-JQ1 at **e**) 5nM and **f**) 200 nM. Error bars represent standard deviation. ** represents $p<0.05$, *** represents $p<0.01$, n.s. represents p values that are not significant.

Once we achieved improvement in splice-switching activity, we wanted to test whether this approach could be extended to RNase H-mediated gene knockdown (**Figure 3a**). Our test system was a 20mer ASO that targets the gold-standard knockdown target metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), a nuclear-enriched long non-coding RNA.[20] MALAT1 plays key roles in gene regulation and metastasis in cancer.[21,22] Implementing the current state-of-the-art in ASO design, this MALAT1-ASO had a gapmer design, containing a fully PS backbone with terminal wings of five 2'-methoxy-ethyl (MOE) sugar modifications (**Figure 3b**). In the gapmer design, the central region of DNA oligonucleotides are recognized by RNase H, while the flanks of 2'-modified sugars are RNase H-inactive, but enhance nuclease stability and target binding.[23] As with the SSO, we prepared the (+)-JQ1 conjugate utilising copper-catalysed click chemistry, using the same (+)-JQ1-alkyne and a 5'-azide-modified MALAT1 gapmer. The 5'-azide MALAT1 gapmer was also prepared using azidoacetic acid-NHS ester functionalisation of a 5'-terminally amine-modified gapmer ASO. Following copper-catalysed click chemistry, the (+)-JQ1-MALAT1 gapmer was produced in >90% reaction yields and >95% purity, after HPLC purification (**SI Figure 6, 7**).

To test the activity of the (+)-JQ1-MALAT1 gapmer conjugate, we measured MALAT1 transcript levels using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) at 24 hours, comparing the knockdown to the unconjugated MALAT1 gapmer, normalised to the house keeping gene GAPDH. As with the SSO conjugate, the (+)-JQ1-MALAT1 gapmer conjugate outperformed the unconjugated MALAT1 gapmer at all tested concentrations. We measured 20.1%, 30.2%, 56.8%, and 54% less transcript with the (+)-JQ1 modified gapmer treatment, compared to the unmodified gapmer, at 5, 50, 100, and 200 nM respectively (**Figure 3c**). Again, similar to the SSO, this enhanced activity was found to depend on specific (+)-JQ1-BET bromodomain protein interactions, as it was lost in the presence of excess quantities of small molecule (+)-JQ1. At 200 nM (+)-JQ1, the enhanced activity of the (+)-JQ1-MALAT1 gapmer at 50-200 nM was completely inhibited (**Figure 3d, SI Figure 8**). Furthermore, no increase in toxicity was observed for the (+)-JQ1-MALAT1 gapmer

conjugate compared to the unconjugated MALAT1 gapmer at all concentrations (**SI Figure 9**). This demonstrated that (+)-JQ1 conjugation can effectively increase both the splice-switching and gene-knockdown activity of ASOs.

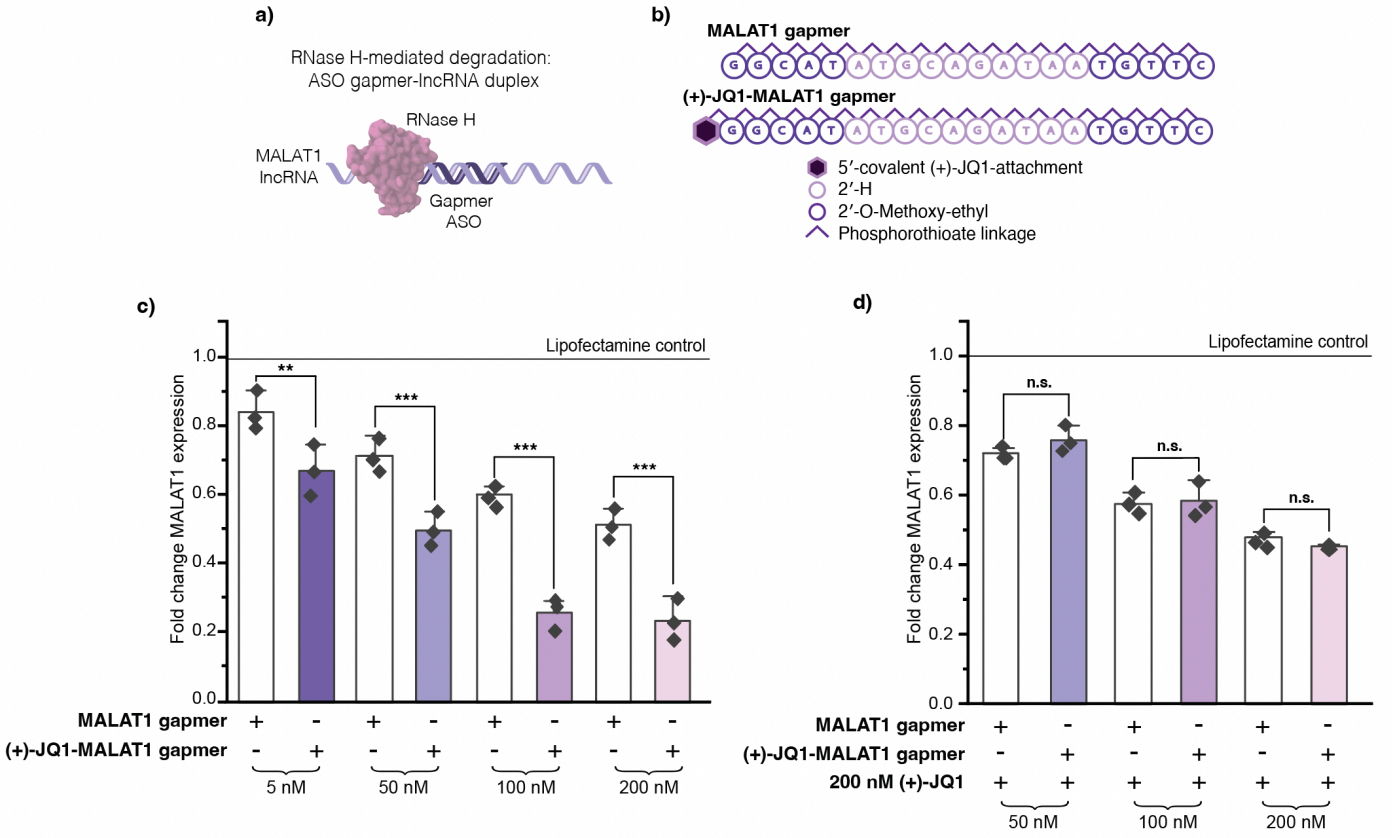


Figure 3: Covalent-(+)-JQ1 modification enhances RNase H-mediated knockdown. **a**) Mechanism of RNase H-mediated degradation of lncRNA MALAT1 localised in the nucleus. **b**) Sequence and chemical modifications for MALAT1 gapmer used. **c**) RT-qPCR data for MALAT1 knockdown upon lipofectamine transfection of (+)-JQ1- and unconjugated-gapmer in HEK293T cells for 24 hours at concentrations indicated. **d**) MALAT1 knockdown observed in competition assay in the presence of 200 nM (+)-JQ1. Error bars represent standard deviation. ** represents $p<0.05$, *** represents $p<0.01$, n.s. represents p values that are not significant.

While MALAT1 ASOs are being evaluated for their therapeutic effect, Oblimersen (G3139), an 18-mer ASO containing a fully PS backbone designed to target apoptosis factor BCL-2, reached phase III clinical trials.[24] BCL-2 is a crucial inhibitor of apoptosis that is overexpressed in various cancers.[25] Despite promising phase I-II results as a sensitiser for chemotherapy, G3139 failed to show efficacy in multiple phase III trails.[26] As this drug was well tolerated by patients[27], the limiting factor is likely target engagement and efficacy. Therefore, given that G3139 functions as an RNase H-active ASO (**Figure 4a**), like the MALAT1 gapmer, we aimed to measure whether a (+)-JQ1-G3139 conjugate could result in a more potent drug molecule (**Figure 4b**).

We synthesised the (+)-JQ1-G3139 conjugate using the same copper-catalysed click methodology as the previous ASOs. The 5'-azide-G3139 was synthesised from the commercially-obtained 5'-amine PS ASO using azideoacetic acid-NHS ester functionalisation, and the (+)-JQ1 conjugate was synthesised through a copper-catalysed click reaction with the (+)-JQ1-alkyne (**SI Figure 10, 11**). We transfected ASOs into HEK293T cells and measured the BCL-2 transcript and protein levels, using RT-qPCR and western blotting, respectively, after 24 hours. Our (+)-JQ1-G3139 conjugate dramatically outperformed the unconjugated G3139 ASO at all concentrations tested. We measured 43.9%, 51.3%, 50.9%, and 64.5% less transcript using the conjugated (+)-JQ1-G3139 compared to the unconjugated G3139, at 50, 100, 200, and 500 nM respectively (**Figure 4c**). We also observed a concomitant marked reduction in comparative protein levels, especially at lower ASO concentrations (**Figure 4d**). We then conducted a competition assay using excess small molecule (+)-JQ1, similar to the approach used with the other conjugates. At 5 nM (+)-JQ1, the enhanced activity of (+)-JQ1-G3139 at 100 nM showed a significant reduction, and at 200 nM (+)-JQ1, the enhanced activity of (+)-JQ1-G3139 was fully inhibited. As observed with the previous (+)-JQ1 conjugates, no increase in cellular toxicity was observed for (+)-JQ1-G3139, compared to the unconjugated ASO (**SI Figure 12**).

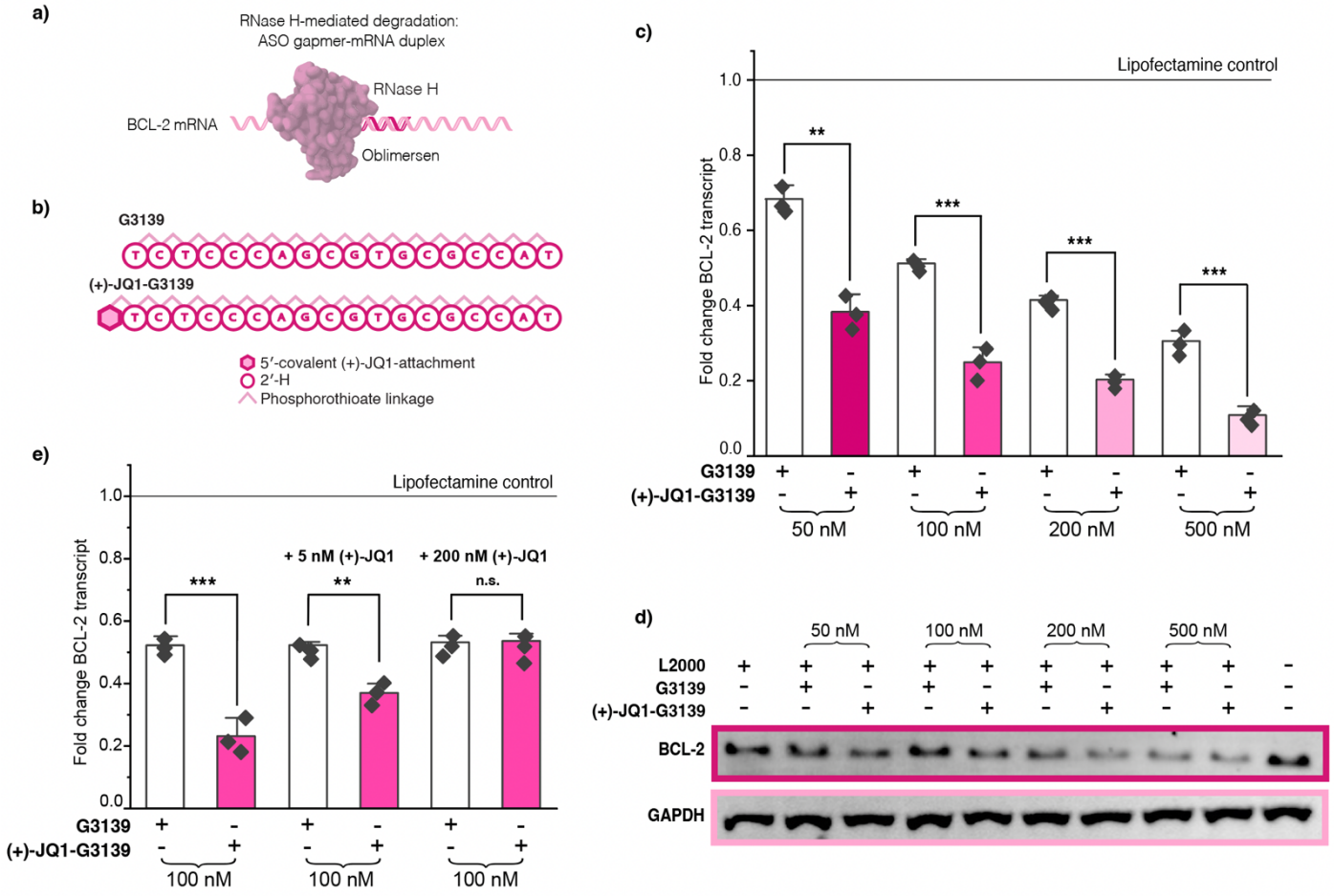


Figure 4: (+)-JQ1-G3139 outperformed the unconjugated ASO. **a**) Mechanism of RNase H-mediated degradation of BCL-2 mRNA. **b**) Sequence and chemical modifications for Oblimersen (G3139) used. **c**) RT-qPCR data of BCL-2 knockdown upon (+)-JQ1-G3139 and unconjugated-G3139 lipofectamine transfection in HEK293Ts for 24 hours at concentrations indicated. **d**) Western blot of BCL-2 levels upon treatment with G3139 and (+)-JQ1-G3139 upon transfection with lipofectamine at 24 hours at concentrations indicated. Normalised to GAPDH expression levels. **e**) Reduction of enhanced BCL-2 knockdown observed in competition assay in the presence of 5 nM and 200 nM (+)-JQ1. Error bars represent standard deviation. ** represents $p<0.05$, *** represents $p<0.01$, n.s. represents p value p values that are not significant

Discussion/Future perspectives

Our work demonstrates that covalent conjugation of the small molecule (+)-JQ1 to ASOs can enhance their activity in their two most prominent mechanisms of action: splice switching and RNase H-mediated gene knockdown. These findings build upon the previous literature surrounding the development of (+)-JQ1 as a nuclear importer. Our data underscores the importance of precisely targeting therapeutic agents to their site of action. By inducing ASO enrichment in the nucleus, we have improved their functional efficacy and therapeutic potential. In this way, we have shown that nuclear localization is crucial for maximizing the therapeutic potential of ASOs for splice switching and RNase H-mediated knockdown.

Our approach is simple, versatile, and broadly applicable, as evidenced by the successful enhancement of ASO activity across different backbone chemistries, different targets, and different mechanisms of action. Thus, our technology is robust and potentially applicable to a wide range of therapeutic scenarios. Notably, we have taken Oblimersen, an 'almost-therapeutic' ASO, and used our approach to dramatically improve its therapeutic efficacy. This improvement suggests that our work has the potential to transform other suboptimal ASO drugs into more effective therapeutics.

The modular nature of this technology opens exciting possibilities for future applications. By demonstrating that small molecules can be potent effectors of ASO cellular compartmentalization and thus, functionality, we pave the way for the development of advanced therapeutic strategies through small molecule conjugation. The integration of small molecules to manipulate cellular environments and target sites offers a promising approach to enhance therapeutic outcomes. Our work aligns with the burgeoning field of bi-functional molecules, which combines distinct functionalities into a single entity.[28] This intersection with bi-functional technologies allows our approach to be integrated into the broader context of therapeutic innovation, by leveraging the synergies between small-molecule ligands and nucleic acid-based therapeutics.

In summary, our study provides a compelling demonstration of how small molecules can be harnessed to enhance ASO activity in multiple mechanisms of action, through improved nuclear localization. The modular nature of this technology, combined with its compatibility with emerging bi-functional modalities, positions it as a promising platform for future therapeutic advancements.

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Author Contribution

D.K., T.A.M., and M.J.B. designed the project. D.K. designed, performed, and analysed the experiments, with contributions from T.M. and M.J.B. All authors wrote the paper.

Conflicts of interest

T.A.M. is a shareholder and consultant for Dark Blue Therapeutics. D.K. and M.J.B. declare no conflict of interest.

Data availability

All the data generated in this study are available within the article, the supplementary information, and figures. Source data will be made available on Zenodo upon acceptance of the manuscript.

Supplementary Information

Materials and Methods and Supplementary Figs. 1-12.