Engineering antisense oligonucleotides for targeted mRNA degradation through lysosomal trafficking

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Abstract

Antisense oligonucleotides (ASOs) can modulate gene expression at the mRNA level, providing the ability to tackle conventionally undruggable targets and usher in an era of personalized medicine. A key mode of action for ASOs relies upon RNase H-engagement in the nucleus, however, the site for mature mRNA is the cytoplasm. This disconnect limits the efficacy and biomedical applications of ASOs. In this paper, we have established a new mechanism of action for achieving potent and targeted mRNA knockdown by leveraging a lysosomal degradation pathway. To achieve this, we employ autophagosome-tethering compound (ATTEC) technology that utilises bifunctional small molecules for lysosomal trafficking. In this manner, to achieve degradation of target mRNA located in the cytoplasm, we conjugated an ATTEC warhead, ispinesib, to RNase H-inactive ASOs. These fully 2′-O-Methylated RNase H-inactive ASOs have higher chemical stability and tighter mRNA binding than conventional 'gapmer' sequences, but cannot be recognised by RNase H. Our RNase H-inactive ASO-ispinesib conjugates produced a higher degree of knockdown than even state-of-the-art RNase H-active 'gapmer' ASOs. Using lysosomal trafficking antisense oligonucleotide (LyTON) technology, we knock down Menin (MEN1), a promising clinical target in leukemias. Engineered to degrade mRNA independent of RNase H recognition, LyTONs will enable gene silencing using oligonucleotide chemistries with higher chemical stability, tighter mRNA binding affinity, and improved cell delivery profiles. This will enable us to target a wider range of disease-relevant mRNA, potentially leading to the development of new therapies.

Introduction

Nucleic acid drugs are promising therapeutic modalities due to their unique ability to target genetic pathways with exquisite specificity[1]. Antisense oligonucleotides (ASOs) lead the charge with over 10 drugs approved in the past two decades[2]. ASOs are short synthetic strands of DNA designed to modulate gene expression by binding to target mRNA through base-pair complementarity. Approved ASO drugs operate through two main mechanisms: the ASO-RNA duplex can either modulate pre-mRNA splicing or trigger RNase H-mediated degradation[3]. However, despite their success, approved ASOs target a narrow spectrum of diseases and their key mechanisms of action are predominantly localised within the nucleus.

Pre-mRNA splicing requires a specialized ASO mechanism active in the nucleus[4]. This process requires DNA-based ASOs to bind pre-mRNA in the nucleus and ultimately influence splicing into mature mRNA. This mechanism is a powerful strategy for treating genetic diseases caused by splicing defects. In contrast, general mRNA knockdown mechanisms are constrained by limited RNase H recruitment[5] – RNase H is enriched in the nucleus, whereas mature mRNA is located in the cytoplasm. Thus, RNase H recruitment is unreliable as its expression levels vary not only with cellular localisation but also with cell and tissue type[6]. One approach to improve target engagement—and thus the efficacy—of ASO-based gene knockdown therapies is to address this localization mismatch between the effector pathway and its molecular target. Conjugation with a nuclear importer has been shown to increase the activity of splice-switching oligonucleotides and RNase H-active ASOs[7]. However, this mechanism may not be desirable for different mRNA targets. RNase L-mediated cytoplasmic degradation of viral RNA has been achieved through the covalent modification of ASOs with endogenous RNase L ligands[8]. However, fine control over RNase L activity is key; uncontrolled activation of this potent cytoplasmic anti-viral effector pathway can lead to global translational shutdown[9], amplification of cellular stress responses, and cell death[10]. This demonstrates the need to develop new methods to harness a selective cytoplasmic ASO pathway for targeted degradation of mRNA.

Lysosomes serve as the primary degradation compartment within cells[11], capable of breaking down a wide range of biomolecules[12,13,14]. This functionality has been harnessed for protein degradation using antibodies, such as LYTACs (lysosome-targeting chimeras)[15,16] bispecific aptamer chimeras[17], and small molecules with approaches such as MrTACs (methylarginine targeting chimeras)[18]. These methods all employ ligands or chemical tags to direct proteins to the lysosome for degradation. Inspired by these strategies, we investigated whether lysosomal trafficking could be exploited for targeted mRNA degradation. For this purpose, we utilized ispinesib, a small molecule originally developed to inhibit the mitotic kinesin Eg5 protein as an anti-cancer drug[19,20]. Ispinesib has also been employed as a warhead in bi-functional molecules designed to degrade target proteins via lysosomal trafficking[21]. The proposed mechanism for these bi-functional molecules involves LC3-binding within the autophagosome, which subsequently fuses with the lysosome to mediate protein degradation. This activity was lost in the presence of inhibitors targeting various steps of autophagy or lysosomal trafficking. Bi-functionals with this mode of action are named as autophagosome-tethering compounds (ATTECs)[22].

By conjugating the small molecule ispinesib to ASOs, we developed lysosomal trafficking antisense oligonucleotides (LyTONs) that can selectively knockdown mRNA through transport to the lysosome (**Figure 1**). These RNase H-inactive LyTONs outperformed RNase H-active gapmer ASOs for mRNA knockdown. Inhibition of lysosomal acidification, via bafilomycin, resulted in the loss of LyTON activity, demonstrating LyTON-mediated transport of mRNA to the lysosome was responsible for knockdown. To illustrate the promise of this technology, LyTONs were developed to target Menin (MEN1), a promising clinical target involved in transcriptional regulation over cellular differentiation and proliferation in leukemias[23]. The MEN1 LyTONs resulted in a dramatic protein-level knockdown following treatment.

By leveraging lysosomal trafficking, this approach provides a powerful mechanism for degrading mRNA without interfering with native cellular processes or triggering widespread cellular damage. The ability to degrade disease-relevant mRNAs in the lysosome will improve target engagement and enable a higher degree of knockdown, as nuclear localisation and subsequent RNase H recruitment will no longer a limiting factor. Currently, all nucleic acid-mediated gene knockdown technologies rely upon DNA (ASOs) or RNA (small interfering RNA, siRNA) backbones for enzymatic recruitment and activity[24]. In contrast, our LyTONs can be employed with alternative oligonucleotide chemistries that offer higher potency and more favourable pharmacological profiles. Thus, we believe that LyTONs will allow for the targeting of a broader spectrum of clinically-relevant gene targets, expanding our toolkit for developing innovative nucleic acid therapeutics.

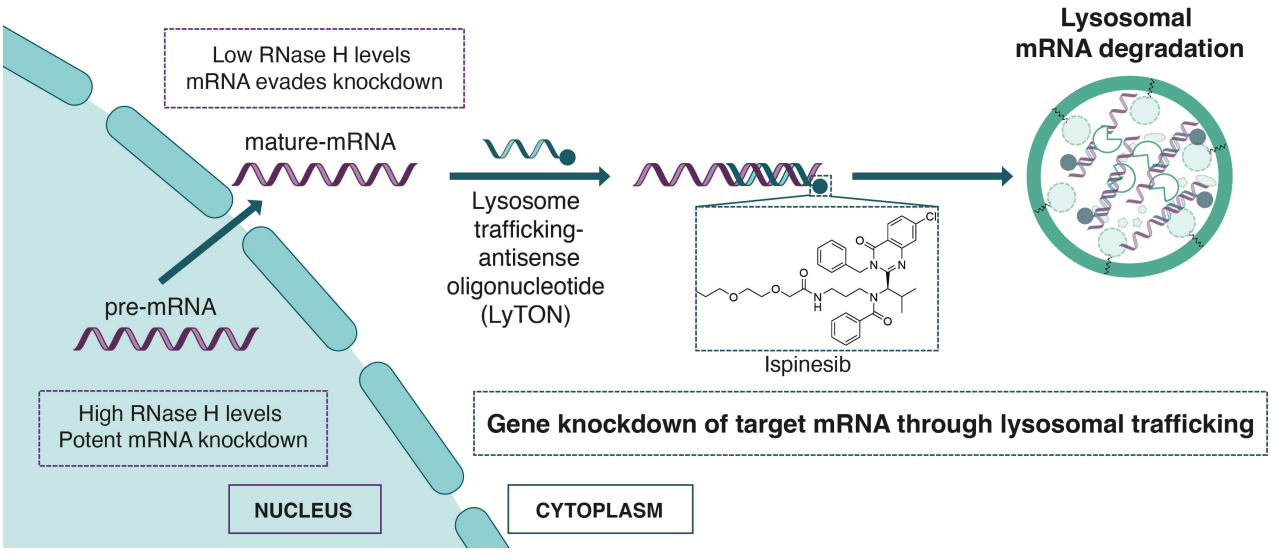

Figure 1: Ispinesib-ASO conjugates act as lysosomal trafficking antisense oligonucleotides (LyTONs), enabling targeted mRNA degradation in the lysosome.

Results

To test whether we could use the ATTEC-warhead ispinesib for lysosomal trafficking and subsequent lysosomal degradation of target mRNA (**Figure 2a**), we initially opted to attach it to an ASO with a fully 2'-O-Methyl (2'-OMe) sugar modified-backbone. This chemistry is known to be RNase H-inactive, and would allow us to assess the Ispinesib-ASO without interference from RNase H-mediated degradation. We chose a previously validated ASO sequence designed to target cytoplasmic NCL1 mRNA[25] (**Figure 2b**). A 5'-dibenzocyclooctyne (DBCO) NCL1 ASO was synthesised by reacting a 5′-terminally modified-amine 2'-OMe ASO with excess DBCO-*N*-hydroxysuccinimide (NHS) ester. We then used strain-promoted azide-alkyne click (SPAAC) chemistry[26] to couple commercially available ispinesib-azide to the 5'-DBCO 2'-OMe ASO (**Figure 2c**), achieving >90% yields for all bioconjugation reactions performed and >95% purity post HPLC purification (**SI Figure 1, 2**).

NCL1 transcript levels were measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in HEK293T cells at 24 hours. We compared NCL1 transcript levels upon NCL1 2'-OMe LyTON treatment to the unconjugated NCL1 2'-OMe ASO, all normalised to the housekeeping gene GAPDH. As expected, the NCL1 2'-OMe ASO showed poor knockdown, as it is only able to sterically block the target mRNA. The NCL1 2'-OMe LyTON far outperformed its unconjugated counterpart at all concentrations tested (**Figure 2d**). We measured 46.9%, 59.1%, 55.0%, less transcript upon treatment with the NCL1 2'-OMe LyTON conjugate, compared to the unconjugated NCL1 2'-OMe ASO, at 50, 100, and 200 nM respectively.

This NCL1 ASO sequence was originally designed for RNase H-mediated knockdown as a 'gapmer'. The current state-of-the-art for ASO modification chemistry is the 'gapmer' design comprised of wings of five bases containing 2'-methoxyethyl sugars and a central DNA region[27] (**Figure 2b**). Thus, we also sought to compare the activity of the NCL1 2'-OMe LyTON to the previously validated NCL1 'gapmer' ASO. As expected, at 100 nM, the NCL1 gapmer performed better than the RNase H-inactive NCL1 2'-OMe version (**Figure 2e**). However, the NCL1 2'-OMe LyTON performed significantly better than even the NCL1 gapmer, with 34.9% less transcript observed (**Figure 2e, SI Figure 3**). This indicated that degrading mRNA via a lysosomal pathway might be more efficient for gene knockdown than relying upon RNase H recruitment, primarily, in the nucleus.

Additionally, we synthesised a "dual activity" conjugate with ispenisib conjugated to the NCL1 gapmer, harnessing both RNase H-mediated degradation and lysosome trafficking. Synthesis was carried out as above, from a 5′-terminally amine-modified gapmer (**SI Figure 4, 5**). This dual activity NCL1 gapmer LyTON still outperformed the NCL1 gapmer with 40.7% less transcript at 100 nM (**Figure 2e, SI Figure 6**). While the NCL1 gapmer LyTON appeared to have performed better than the NCL1 2'-OMe LyTON, this difference was not significant (**SI Figure 7**) This indicated that we may have reached a plateau in gene silencing efficiency, likely based on the affinity of the ASO sequence to bind its target mRNA. We measured little to no significant differences in the toxicity profiles of the unconjugated and ispinesib-conjugated oligonucleotides, assessed by Cell Titer Glo (**SI Figure 8**) and RNA transcript levels of key housekeeping genes (**SI Figure 9**). This lines up with our initial expectations, as our treatments are in the sub-micromolar range, well below concentrations that result in toxicity associated with kinesin spindle protein (Eg5) inhibition (the canonical target of ispinesib).

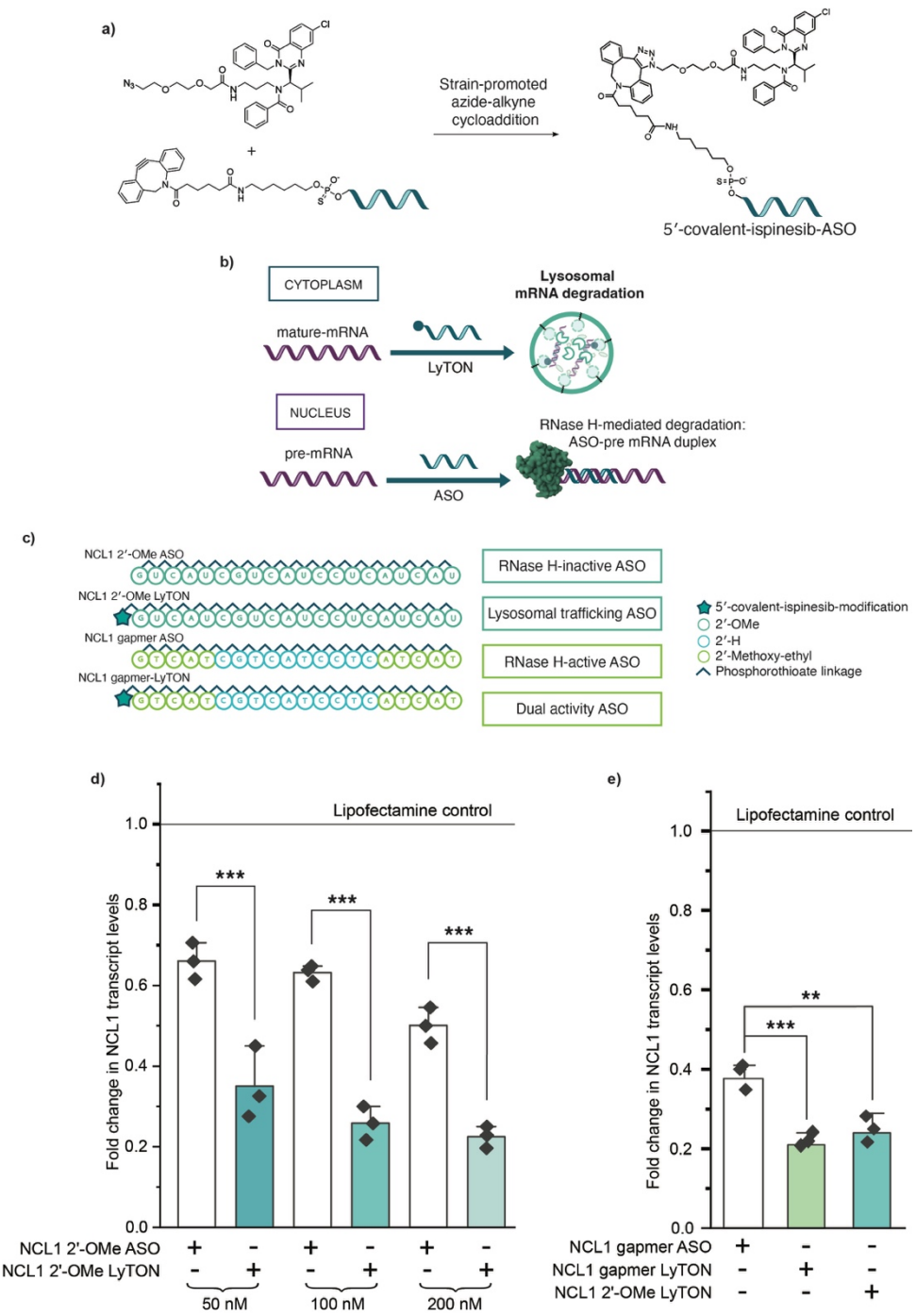

Figure 2: Covalent ispinesib modification of RNase H-inactive 2'-OMe ASO enables knockdown of target mRNA. **a**) Synthesis of covalent ispinesib-ASO conjugates using strain-promoted azide-cyclooctyne cycloaddition. **b**) Proposed mechanism of action for the lysosome trafficking-ispinesib ASO (LyTON) conjugates for mRNA knockdown, in contrast to the established RNase H mechanism for mRNA knockdown of ASOs. **c**) Sequence and chemical modifications of NCL1 ASOs used in the HEK293T cell line for targeting NCL1. **d**) RT-qPCR data for NCL1 knockdown upon lipofectamine transfection of NCL1 2'-OMe LyTON and unconjugated-NCL1 2'-OMe ASO in HEK293T cells at concentrations indicated. **e**) RT-qPCR data for NCL1 knockdown upon lipofectamine transfection of NCL1 gapmer, NCL1 gapmer LyTON and NCL1 2'-OMe LyTON in HEK293T cells at concentrations indicated. Three biological replicates in **c)** and **d)** are shown as diamonds for each condition (each from three technical replicates). The vertical bars represent the mean and the

error bars the standard deviation. ** represents p<0.05, *** represents p<0.01, n.s. represents p values that are not significant.

These results demonstrated that we could achieve significant reduction of target mRNA with NCL1 2'-OMe LyTON treatment, which could not be attributed to standard RNase H activity. Next, we sought to confirm whether the mechanism of mRNA degradation involved the lysosome, as expected. Bafilomycin, a well-characterised inhibitor of lysosome activity[28,29], was used (**Figure 3a**). Bafilomycin inhibits vacuolar V-ATPase and prevents lysosomal acidification – disrupting basal lysosomal flux. If the NCL1 2'-OMe LyTON was trafficking the target mRNA to the lysosome, treatment with bafilomycin would result in loss of knockdown activity (**Figure 3a**). First, we treated the HEK293T cells with bafilomycin for 24h and confirmed the inhibition of lysosomal activity through an increase in LC3-II levels-(**SI Figure 10**). Cell toxicity was observed with 100 nM bafilomycin, therefore, the lower 10 nM concentration (without any cell toxicity effects) was chosen for further studies with the ASOs (**SI Figure 11**). The knockdown of NCL1 transcript levels was then measured upon transfection with the NCL1 gapmer, NCL1 gapmer LyTON, NCL1 2'-OMe ASO, and NCL1 2'-OMe LyTON in the presence and absence of bafilomycin (**Figure 3b**). As expected, the bafilomycin treatment did not affect the activity of the NCL1 gapmer or NCL1 2'-OMe ASO. However, the enhanced mRNA knockdown activity of both the NCL1 2'-OMe and NCL1 gapmer LyTONs were completely lost in the presence of bafilomycin, showing comparable activity to the parent unmodified NCL1 2'-OMe ASO or NCL1 gapmer, respectively. This confirmed that LyTONs relied upon lysosomal degradation for activity, and upon inhibition of normal lysosomal flux only acted as a conventional ASOs.

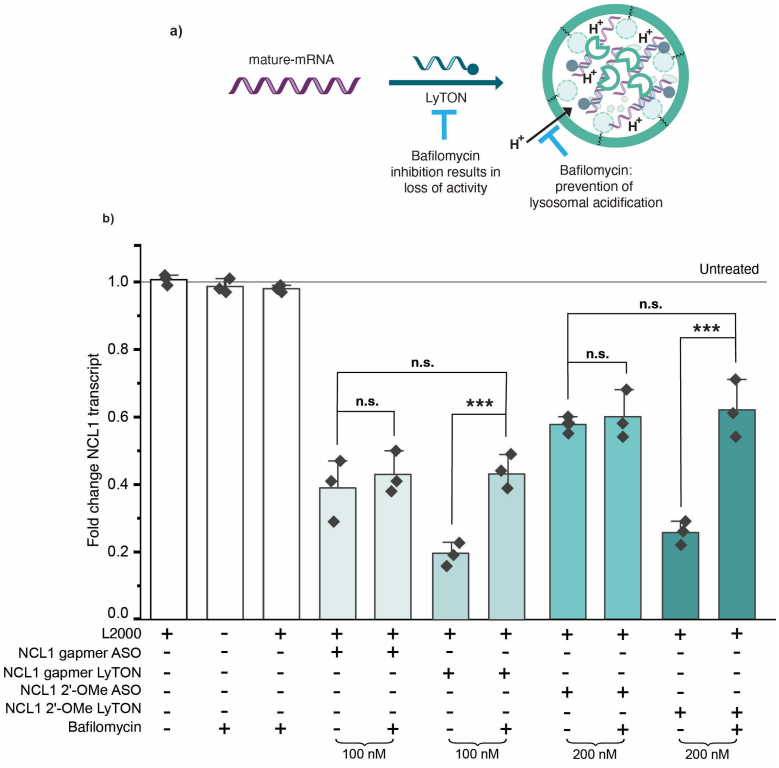

Figure 3: LyTON-warhead ispinesib unlocks a new mechanism of ASO-mediated mRNA degradation via lysosomal trafficking, validated using bafilomycin. **a**) Mechanism of bafilomycin-mediated lysosomal inhibition in relation to LyTON activity. **b**) RT-qPCR data for NCL1 knockdown upon lipofectamine transfection of NCL1 gapmer ASO, NCL1 gapmer LyTON, NCL1 2'-OMe ASO, and NCL1 2'-OMe LyTON in HEK293T cells in the presence or absence of 10 nM bafilomycin, at the concentrations indicated. Three biological replicates are shown as diamonds for each condition (each from three technical replicates). The

vertical bars represent the mean and the error bars the standard deviation. ** represents $p<0.05$, *** represents $p<0.01$, n.s. represents p values that are not significant.

After establishing the chemistry and mechanism of action of LyTONs, we decided to target an exciting therapeutically-relevant gene of interest. For this, we chose the MEN1 gene that encodes for the protein, Menin – which plays a particularly significant role in MLL-rearranged leukemias. Menin acts as a scaffold for oncogenic MLL fusion proteins resulting in the transcriptional activation programs for leukemic cell survival and growth[30]. Menin also has a key role in KRAS-driven cancers[31]. Inhibition of Menin has been shown to disrupt such cancer-sustenance pathways, leading to reduced proliferation and increased apoptosis[32,33]. Small-molecule Menin inhibitors have shown early clinical promise, however, recent clinical studies have found emerging resistance and patient relapse due to somatic mutations in MEN1[34]. The structural mutations in Menin occur within a key region of the inhibitor-binding pocket essential for stabilizing Menin inhibitor binding but not required for interaction with MLL. These small molecule drug resistance challenges, which manifest at the protein level, can potentially be circumvented by targeting MEN1 mRNA using ASOs.

A well-established MEN1 ASO sequence was chosen and obtained with all 2'-OMe-modified sugars[35] (**Figure 4a**). As with the NCL1 LyTONs, ispinesib was conjugated using SPAAC chemistry (**SI Figure 12, 13**). MEN1 transcript levels were measured after transfection of the MEN1 2'-OMe LyTON using RT-qPCR in HEK293T cells at 24 hours, comparing the knockdown to the unconjugated MEN1 2'-OMe ASO, normalised to the housekeeping gene GAPDH (**Figure 4b**). The MEN1 2'-OMe LyTON outperformed the MEN1 2'-OMe ASO at all concentrations tested. We measured 36.8%, 49.8%, 55.5%, less transcript upon treatment with the MEN1 2'-OMe LyTON conjugate compared to the unconjugated MEN1 2'-OMe ASO, at 50, 100, and 200 nM respectively. We also observed a marked reduction in Menin protein levels upon MEN1 2'-OMe LyTON treatment, in a concentration dependant manner (**Figure 4c, SI Figure 14**). Protein levels showed minimal changes upon treatment with the MEN1 2'-OMe ASO. The MEN1 2'-OMe LyTON also exhibited improved knockdown at both the RNA and protein levels compared to the established gapmer chemistry. This effect was observed across all tested concentrations upon transfection in HEK293T cells for 24 hours, and assessed using RT-qPCR (Figure **4d**) and western blotting (Figure **4e**). We then sought to again confirm the mechanism of action for the ispinesib-ASO conjugates, using bafilomycin treatment. As with the NCL1 LyTON, treatment with bafilomycin resulted in the complete loss of enhanced mRNA knockdown for the MEN1 2'-OMe LyTON (**Figure 4f**). Only steric blocking activity on par with the parent MEN1 2'-OMe ASO was observed. Furthermore, this loss of activity upon treatment with bafilomycin was also observed at the protein level (**Figure 4g, SI Figure 15**). We also observed little to no significant differences in the toxicity profiles of the unconjugated and ispinesib-conjugated MEN1 ASOs, as above, assessed through Cell Titer Glo (**SI Figure 16**) and transcript levels of key housekeeping genes (**SI Figure 17**).

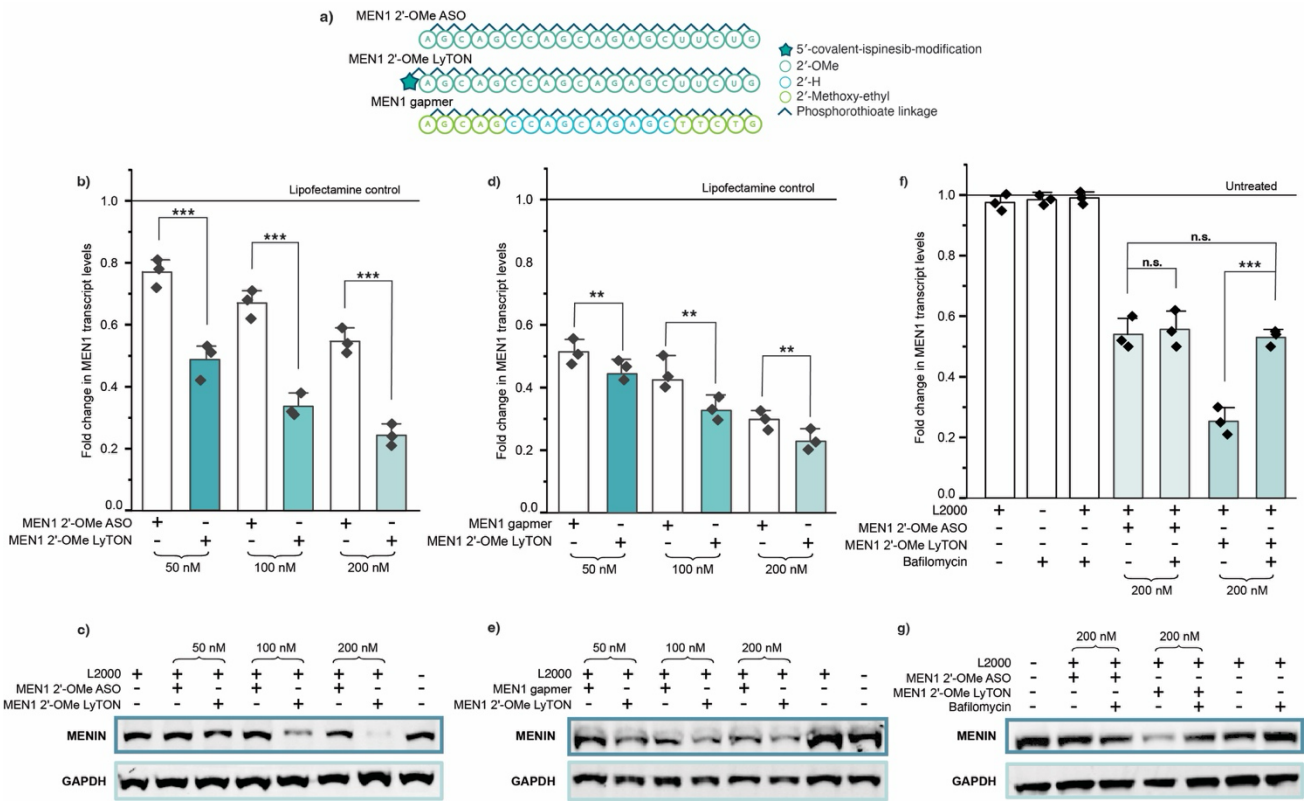

Figure 4: MEN1 2'-OMe LyTON treatment results in significant knockdown of MEN1 mRNA and protein. **a**) Sequence and chemical modifications of ASOs used to target MEN1. **b**) RT-qPCR data and **c**) Western blot for MEN1/Menin knockdown upon lipofectamine transfection of MEN1 2'-OMe ASO and MEN1 2'-OMe LyTON ASO in HEK293T cells at concentrations indicated. **d**) RT-qPCR data and **e**) Western blot for MEN1/Menin upon lipofectamine transfection of MEN1 gapmer ASO and MEN1 2'-OMe LyTON ASO in HEK293T cells at the concentrations indicated. **f**) RT-qPCR data and **g**) Western blot for MEN1/Menin knockdown upon lipofectamine transfection of MEN1 2'-OMe ASO and MEN1 2'-OMe LyTON ASO in HEK293T cells in the presence or absence of 10 nM bafilomycin, at the concentrations indicated. Three biological replicates in **b**), **d**), and **f**) are shown as diamonds for each condition (each from three technical replicates). The vertical bars represent the mean and the error bars the standard deviation. ** represents $p<0.05$, *** represents $p<0.01$, n.s. represents p values that are not significant.

Discussion/Future perspectives

Targeted mRNA degradation is a powerful strategy for therapeutic intervention: targeting the "undruggable", allowing for personalised medicine with "n-of-1" therapies, and, potentially, circumventing traditional small molecule drug resistance mechanisms. Currently, mRNA knockdown can only be achieved through RNase H recognition of DNA-based ASOs or RNA-induced silencing complex (RISC) formation with siRNA. Our work introduces an entirely new mechanism for targeted mRNA degradation. By conjugating ispinesib, an ATTEC warhead, to ASOs, target mRNA is trafficked to the lysosome for subsequent degradation. These lysosomal trafficking antisense oligonucleotides (LyTONs) are modular and versatile. These conjugates can be generated through simple post-synthetic modification chemistry in high yields with high purity. The therapeutic potential of LyTONs was demonstrated by targeting Menin, an exciting target in cancer medicine with effective knockdown at the protein level.

RNase H-active ASOs are predominantly functional in the nucleus, as this is where the highest levels of RNase H preside. However, mRNA is shuttled out of the nucleus for translation. While siRNA carry out cytoplasmic

degradation of mRNA, they are even more difficult to deliver into cells than ASOs[36,37]. Our approach leverages a lysosomal degradation pathway that improves target engagement, and thus, can potentially enable therapeutic targeting of more clinically-relevant genes using ASOs. Using our LyTON technology, we will be able to rethink the design of gene-silencing oligonucleotides. Without the need for incorporating DNA-containing sequences for RNase H recognition or RNA-containing sequences for RISC formation, many alternative oligonucleotide chemistries could be explored for nucleic acid drug development–allowing for ASO chemistries with higher chemical stability, higher target affinity, and improved delivery profiles for gene knockdown applications. A key future goal will be to fully characterise the binding partner of ispinesib for lysosomal trafficking[38,39,40,41]. This should enable the development of improved LyTONs through rational ligand design.

In summary, our work represents a significant advancement in the field of targeted mRNA degradation. By harnessing lysosomal trafficking through the design of a bi-functional ispinesib-ASO conjugate, we offer a powerful new strategy for selective mRNA degradation. This approach holds great promise in precision medicine, particularly in expanding the use of diverse oligonucleotide chemistries for gene silencing. With its modular design and capacity for future advancements, this technology could emerge as a powerful platform for nucleic acid-based therapeutic strategies.

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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. 1-18.