

Nebulisation of Antisense Oligonucleotide (ASO) Danvatirsen

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Summary

Antisense Oligonucleotides (ASOs) present a new paradigm in personalised medicine, facilitating treatments designed to selectively silence disease-driving genes. Inhalation offers a promising non-invasive method of delivering these novel medicines. Key factors, such as high viscosity, shear-induced degradation, and potential for aggregation present major challenges for aerosol delivery and device compatibility. To date, only two ASOs are in development for inhaled delivery. Nebulised delivery may offer a direct non-invasive route to target the lungs more effectively. In this study, we investigate the feasibility of nebulising Danvatirsen, a 16 mer gapmer ASO at clinically relevant dose of 20mg/ml, using Nebu-Flow's novel technology. Structural and biological integrity of the ASO was confirmed post-nebulisation. These findings support the potential for high-dose ASO delivery via the inhalation route for local delivery to the lungs and treatment of respiratory disorders.

Key Message

A prototype acoustic nebuliser was used to successfully deliver Danvatirsen, a gapmer ASO, at therapeutic concentration. Structural integrity was preserved post-nebulisation as confirmed by native Poly-acrylamide Gel Electrophoresis (PAGE), and Liquid Chromatography-Mass Spectrometry (LC-MS). These data support the feasibility of inhaled ASO delivery for clinical evaluation.

Introduction

Antisense oligonucleotides (ASOs) have emerged as a compelling therapeutic modality, enabling the alteration of gene expression across a wide range of diseases. Their potential for delivery via inhalation offers a novel strategy to treat pulmonary conditions, minimising systemic exposure and improving the therapeutic index [1]. Despite growing interest in pulmonary ASO delivery, significant formulation challenges remain — particularly at therapeutically relevant concentrations. ASOs are large, highly charged molecules with a propensity to aggregate and exhibit elevated viscosity at high concentrations [2]. To improve stability and activity, ASOs incorporate chemical modifications such as stabilised sugars, and a phosphorothioate (PS) backbone in place of the natural phosphodiester (PO). The ratio of PS:PO bonds typically seen in manufactured ASOs is >95%. These adaptations enhance systemic stability and pharmacokinetics by increasing nuclease resistance and target binding affinity, providing improved bioavailability in the lung. However, they also increase molecular rigidity and intermolecular interactions—leading to elevated viscosity, aggregation and potential delivery inconsistencies at high concentrations [3].

Danvatirsen, an ASO targeting STAT3, has shown antitumor activity in patients with lymphoma and non-small cell lung cancer. STAT3 is overexpressed in tumour cells and plays a central role in tumour immune evasion and inflammation [4]. Danvatirsen is a single stranded 16-mer cEt/DNA gapmer ASO with a phosphorothioate (PS) backbone and 5-methyl base modifications. It is composed of constrained ethyl (cEt)-modified ribonucleotides at the flanking ends and a central core of deoxynucleotides designed to recruit RNase H1 for targeted mRNA degradation of STAT3 transcript [4].

ASOs are typically administered via intravenous (IV) or subcutaneous (SC) injection at high doses ranging from 100-300mg per dose. High dosage is necessary due to rapid clearance, broad tissue distribution and low bioavailability when delivered systemically. Danvatirsen has been previously dosed at 200mg IV weekly in clinical trials [5]. In animal models, aerosolised LNA-gapmer achieved effective lung knockdown with doses as low as 0.19 µmol/kg (~1 mg/kg) via intratracheal (IT) delivery, roughly 20–25-fold lower than systemic doses [6].

Traditional nebulisers may impact drug stability through cavitation, hydrodynamic shear, or temperature changes. Based on our previous studies, we have shown that Nebu-Flow technology can deliver oligonucleotides with negligible damage or degradation as well as intact bioactivity [7].

To address the challenges of nebulising small biological entities such as ASOs, we explore the feasibility of nebulising Danvatirsen at 20 mg/ml using Nebu-Flow technology. The primary aim was to evaluate

structural integrity and biological activity post-nebulisation. Native PAGE was used to immediately confirm ASO structure, with Liquid-Chromatography Mass Spectrophotometry (LC-MS) tests confirming these findings.

Experimental method

Nebulisation experiments ($n=3$) were conducted using RNase-free Phosphate Buffered Saline (PBS)) containing Danvatirsen (MedChemExpress, Cat. No. HY-145729A) at 20mg/ml. A total of 500 μ L of Danvatirsen was nebulised using a prototype Nebu-Flow acoustic nebuliser. The aerosol was collected using a Next Generation Impactor (NGI) (Copley Scientific Ltd, UK) operating at 15L/minute at room temperature. The NGI was modified with Parafilm (HS234526B) from Fisher Scientific to facilitate recovery of low volume biologics as previously described. This method showed negligible loss or adsorption of nucleic acids to the parafilm [8].

Briefly, parafilm was placed over each cup surface to collect droplets and nebulisation experiments were performed. After nebulisation, 50 μ L of PBS was added to each NGI cup to maximise ASO recovery. Our previous study using parafilm modified NGI showed negligible effect on aerosol droplet distribution and no specific binding with DNA or RNA [8].

Two controls were used to benchmark ASO recovery: (i) an unnebulised control consisting of the original 20 mg/mL Danvatirsen solution without nebulisation, to establish baseline concentration; and (ii) an extraction control prepared by diluting 500 μ L of unnebulised 20 mg/mL Danvatirsen with 400 μ L PBS, simulating dilutions occurring during the NGI sample recovery process.

Samples from NGI stages 1–3 and 4–MOC were pooled to represent aerosol sizes above and below 3.3 μ m, respectively. Samples were then diluted 1:100 and analysed using a Tecan 200 M Nano+ platereader (Tecan AG, Switzerland) to determine ASO concentration and purity spectrophotometrically. Structural integrity was investigated via native or non-denaturing PAGE. Danvatirsen (10ng per lane) was loaded on a 20% TBE polyacrylamide gel (EC63155) followed by SYBR Gold (S11494) staining to visualise intact oligonucleotide bands. Polyacrylamide gels and SYBR Gold stain were obtained from ThermoFisher.

Nebulised samples from the NGI were pooled and structural integrity of ASO was analysed by LC-MS analysis. A parafilm NGI control was performed, whereby an unnebulized ASO sample was played on to a parafilm-laden cup in the NGI for 10 minutes, before being recovered. Mass spectrometry (MS) compatible ion-pairing reversed phase liquid chromatography (IPRP LC) methods were used for the analysis of Danvatirsen in solution. Briefly, the nebulised samples were diluted to a concentration of 0.5 mg/mL in water and 5 and 2 μ L were injected for analysis. A Waters Acquity BEH C18 column (50mm*2.1mm, 1.7 μ m, p/n 186002350) was used for all analysis. These methods were used for stability analysis, before and after nebulisation. The determination of purity and impurities were based on UV detection and as a characterization test by mass spectrometry. The ratio of phosphorothioate to phosphodiester bonds (PS:PO) was used to assess degradation in the sample after nebulisation.

Results

Danvatirsen at 20 mg/mL was successfully nebulised using Nebu-Flow's technology across three independent runs. Figure 1 (a) shows the concentration of ASO recovered from the NGI, in comparison to an unnebulised control sample and an extraction control. The unnebulised control represents the original Danvatirsen solution without processing. The extraction control simulates dilution recovery losses from the NGI process by diluting unnebulised Danvatirsen in the same manner as nebulised samples, helping account for dilution and handling losses.

Recovered ASO concentrations from NGI cups 1–3 and 4–MOC were lower than the unnebulised sample, as expected due to dilutions introduced during recovery. Combined recovery from both sets of NGI stages was approximately 124 ng/ μ L, which, when compared to the extraction control (113 ng/ μ L), indicates good retention of material and minimal degradation during nebulisation. Notably, a higher concentration was recovered from cups 4–MOC, which represent aerosols with aerodynamic diameters <3.3 μ m—highlighting that clinically relevant, lung-depositing particle sizes can be achieved using this approach.

This supports the feasibility of delivering Danvatirsen via inhalation at therapeutic concentrations, with structural integrity maintained. The PAGE analysis (Figure 1 b) shows a single well-defined band at ~16 bp for both fractions from NGI cups 1-3 (>3.3 μ m) and cups 4-MOC (<3.3 μ m), with no evidence of

degradation or shearing observed, and the ASO moiety remaining intact. This indicates that molecular stability of the ASO is maintained throughout the nebulisation process. Furthermore, structural integrity was confirmed at higher resolution using LC-MS. Analysis of nebulised samples showed minimal changes to the purity or degradation of the ASO molecule post nebulisation —illustrating that the nebulisation process did not compromise ASO stability.

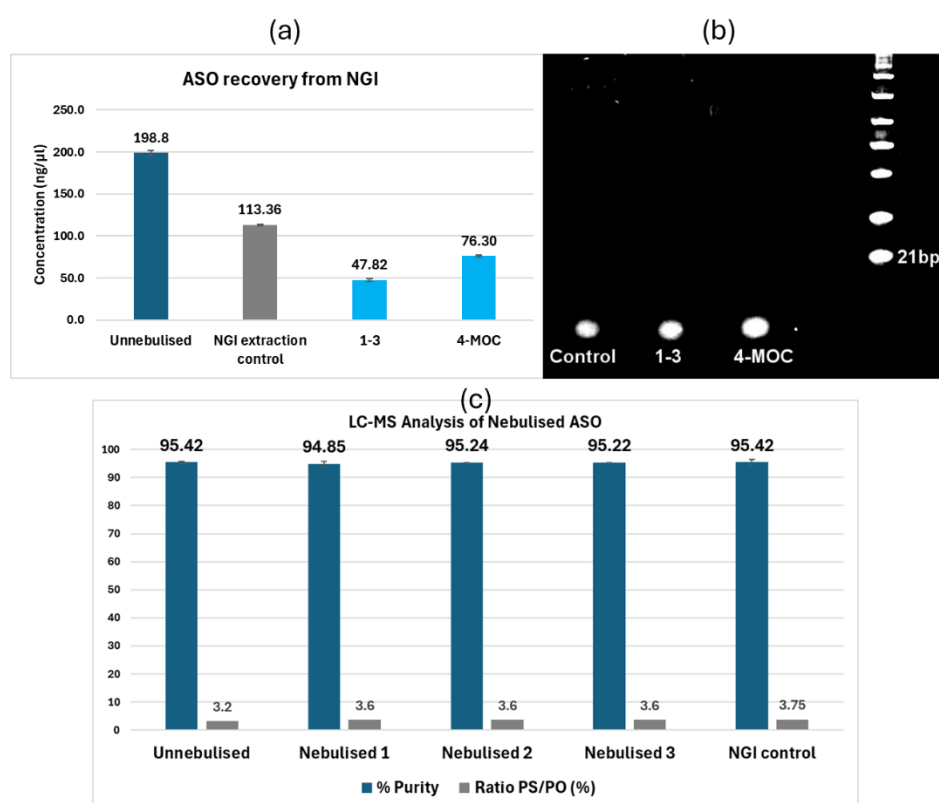


Figure 1 – (a) Recovered fraction of nebulised ASO across the NGI in comparison to unnebulised and extraction control (left). Results are expressed as mean $\pm$ standard deviation ($n = 3$)

(b) Integrity of nebulised Danvatirsen ASO assessed via native polyacrylamide gel electrophoresis. Lane 1: control unnebulised Danvatirsen. Lane 2: Nebulised fraction recovered from cups 1-3 of NGI. Lane 3: Nebulised fraction recovered from cups 4-MOC of NGI. Lane 4: dsRNA ladder (New England Biolabs, N0363S) with a 21 bp reference band at the base.

(c) – Results from LC-MS analysis of nebulised samples, with 3 distinct nebulised samples showing similar purity to unnebulised sample (95%). No significant increase in the ratio of PS:PO was observed post nebulisation. Results are expressed as mean $\pm$ standard deviation ($n = 3$).

Discussion

To our knowledge, this is one of the first studies to explore the nebulisation of a clinically relevant dose of cEt-modified gapmer ASO. Pulmonary drug delivery offers a non-invasive route for treating respiratory and systemic diseases, but delivering biologics remains challenging due to their sensitivity to physical stresses during nebulisation.

Danvatirsen, a generation 2.5 antisense oligonucleotide has shown *in vivo* efficacy without the need for a liposomal or nanoparticle carrier [3]. This carrier-free composition provides a foundation for effective nebulisation into ultrafine droplets capable of reaching the deep lung.

Nebu-Flow technology enables the delivery of clinically relevant doses of Danvatirsen into a fine aerosol while preserving structural integrity. Oligonucleotide structure was confirmed to be intact in aerosolised particles with aerodynamic diameters above and below 3.3 μm . In addition, LC-MS studies have shown

that Danvatirsén survives nebulisation with no signs of degradation or conformation changes or loss to the molecule itself.

There are, however, limitations to this study. Comprehensive aerosol characterisation—such as droplet size distribution, mass reconciliation and fine particle fraction—was not performed (due to the limited volume of the samples). Despite these current gaps, the preservation of structural integrity post-nebulisation supports the potential for retained biological activity. Assays to determine biological activity of nebulised Danvatirsén are ongoing and will be presented at a later stage.

The successful nebulisation of Danvatirsén at 20 mg/mL demonstrates that clinical concentrations of antisense oligonucleotides (ASOs) can be effectively aerosolised. This work addresses a key limitation in the development of inhaled oligonucleotide therapeutics: the ability to deliver chemically modified ASOs at concentrations relevant for clinical dosing.

Conclusion

Overall, this study provides early evidence that inhalation delivery of a clinically relevant dose of ASO is technically feasible, using specialised nebulisation platforms. These findings contribute to the translational pathway for inhaled nucleic acid therapeutics, particularly in the treatment of pulmonary diseases such as lung cancer and inflammatory airway disorders.

References

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