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In vitro evaluation of the potential use of snake-derived peptides in the treatment of respiratory infections using inhalation therapy: A proof of concept study

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ABSTRACT

Inhalation therapy using nebulisers is an attractive non-invasive route for drug delivery, particularly for the treatment of lung infections with anti-inflammatory and anti-microbial compounds. This study evaluated the suitability of three snake-derived peptides (termed Sn1b, SnE1 and SnE1-F), which we have recently shown have potent anti-inflammatory and bacteriostatic activities, for nebulisation using a vibrating mesh nebuliser (VMN). The effect of nebulisation on peptide concentration, stability and function were assessed, prior to progression to aerodynamic particle size distribution, and *in vitro* drug delivery in simulated adult spontaneous breathing and mechanical ventilated patient models. When nebulised, all three peptides exhibited similar functions to their non-nebulised counterparts and were found to be respirable during simulated mechanical ventilation. Based on the assessment of the droplet distributions of nebulised peptides using a Next Generation Impactor (NGI) demonstrated that if administered *in vivo* each peptide would likely be delivered to the lower airways. These data suggest that nebulisation using a VMN is a viable means of anti-microbial / anti-inflammatory peptide delivery targeting microbial respiratory infections, and possibly even systemic infections.

1. Introduction

Inhalation therapy is an attractive means of a non-invasive targeted antimicrobial therapeutic strategy for lung infection. Availing of direct drug delivery to the affected site can reduce unwanted systemic side effects and lessen the required dose, which is beneficial for expensive drugs such as synthetic antimicrobial peptides (AMPs) (Mahlapuu et al., 2016). Nebulised drug delivery offers several advantages over other types of inhaled drug delivery as it occurs independently of breathing and eliminates the requirement for inhalation-actuation synchronisation (Clark et al., 2020). Other approaches include the use of oxygen-containing nanobubbles that can exert a range of beneficial physiologic and pharmacologic effects that include tissue oxygenation, as well as tissue repair mechanisms, anti-inflammatory properties, and antibacterial activity (Afshari et al., 2021). Drugs that are currently available for nebulisation include the antibiotics tobramycin, gentamicin, aztreonam and colistimethate sodium, and mucoactive agents recombinant human deoxyribonuclease (rhDNase) and hypertonic saline (NICE 2017). Although there are few nebulised peptides currently in pre-clinical testing or clinical trials for respiratory disease, several cationic AMPs and pro-drug AMPs have been shown to be successfully nebulised without significant loss of activity in vitro (Forde et al., 2019; Wang et al., 2016). Nebulised SPX-101, an ENaC inhibitor, and α1-proteinase inhibitor have been tested in Phase two clinical trials (Couroux et al., 2019; Gaggar et al., 2016).

Nebulisation is achieved using three different types of nebulisers: jet, ultrasonic and vibrating mesh (Carvalho and McConville, 2016; Le Brun et al., 2000). Vibrating mesh nebulisers (VMNs) used in the present

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study are small, portable, and efficient devices, exhibiting little drug wastage and do not typically generate substantial heat (Ari, 2014; Arzhavitina and Steckel, 2010; Beck-Broichsitter et al., 2012; Dhand, 2010). In addition to superiority in spontaneous breathing simulations, vibrating mesh technology was also deemed "most efficient" compared to jet nebulisers, ultrasonic nebulisers and pMDIs at different positions within *in vitro* models of both spontaneous breathing and mechanical ventilation (Berlinsk and Willis, 2013; Fernández Fernández et al., 2021; Naughton et al., 2021). Further, VMN are closed circuit nebuliser systems, which prevent the release of both medical aerosols and patient-derived bioaerosols to the local environment, which is an important consideration in the COVID-era (Joyce et al., 2021; Fink et al., 2020; O'Toole et al., 2021; Mac Giolla Eain et al., 2022).

AMPs are naturally occurring peptides produced by plants and animals with a broad spectrum of activity against bacteria, viruses, fungi and parasites (Zasloff, 2002). The snake-derived bradykinin-potentiating peptide (BPP-10c) has been shown to decrease angiotensin II by inhibiting ACE, increasing bradykinin-related effects on the bradykinin 2-receptor and increasing nitric oxide-mediated effects and may at as an effective anti-SARS-COV-2 drug (Gouda and Mégarbane, 2021). We have recently demonstrated they that AMPs originally derived from Hydrophis cyanocinctus (Sea snake) (termed Sn1) hold therapeutic potential for infection due to their potent antimicrobial and anti-inflammatory activities (Carlile et al., 2019; Creane et al., 2021). Thus, assessing the therapeutic potential of these snake-derived peptides and others such as those derived from the King Cobra *Ophiophagus hannah* (termed SnE1 and SnE1-F) in the context of inflammatory lung disease is therapeutically relevant and, therefore, here we assessed their suitability for delivery by nebulisation. The effect of nebulisation on peptide concentration, stability and function were assessed, in addition to aerodynamic particle size determination and their evaluation in *in vitro* simulated adult spontaneous breathing and mechanical ventilation patient models, which are representative of the potential patient interventions that would facilitate aerosol delivery of therapeutics.

2. Methods

2.1. Peptide synthesis

Sn1, SnE1 and SnE1-F peptides were manufactured by Fmoc synthesis in free acid form upon request by GL biochem (Shanghai, China). Batches of peptide were at least 95% purity and validated by high performance liquid chromatography (HPLC).

2.2. Peptide analysis

The Pierce Quantitative Fluorometric Peptide Assay (Thermo-Fisher Scientific) was employed to quantify the total concentration of synthetic peptide samples and carried out as per the manufacturer's instructions. For peptide samples obtained from particle size determination (MMAD - median mass aerodynamic diameter) and *in vitro* simulated adult spontaneous breathing and mechanical ventilation experiments, two-fold dilution series (1000-7.8 μg/mL) were produced using corresponding peptides as standards. A volume of 10 μL of standard or sample was added to each well of a black 96-well plate (Thermo-Fisher Scientific). In the absence of light, 70 μL of fluorometric peptide assay buffer was added to each well, followed by 20 μL of fluorometric peptide assay reagent. The plate was incubated in the dark at room temperature with agitation for five minutes. Fluorescence was measured using Ex/Em at 390 nm/475 nm using a Varioskan Lux (Thermo-Fisher Scientific). A standard curve was generated for each assay by plotting fluorescence values of standards against their respective concentrations. For samples from MMAD and simulation model testing, the sample peptide concentrations were determined by linear regression analysis. For all other samples, the concentration (μM) of each sample was quantified by linear regression analysis, and concentration in μg/mL calculated using the following equation:

Concentration (μg/ml) = Peptide molecular weight (Da) × Concentration (μM)/1000.

2.3. Peptide delivery and nebulisation

Nebulisation of the peptides and their delivery via the various ventilators including their particle size determination is outlined in Fig. 1. An aliquot of 0.5 mL of 0.5 mg/mL of Sn1, SnE1 and SnE1-F peptide (reconstituted in sterile saline) was nebulised using an Aerogen Solo VMN as per the manufacturer's instructions. Before and after nebulisation of each peptide, a 2 mL dose of saline was nebulised, followed by 2 mL of autoclaved distilled water. All solutions were nebulised as follows: the nebuliser was placed on top of an open 50 mL Falcon tube, secured with a layer of Parafilm (Sigma), and a small hole pierced using a needle to allow pressure equalisation during nebulisation. The plug of the nebuliser cup was opened, and the solution carefully pipetted into the medication cup. The controller cable was connected to the nebuliser and switched on. Upon completion of nebulisation, the nebuliser was switched off and the controller disconnected from the nebuliser. The Falcon tube was sealed and stored on ice until the experiment was complete. The samples were then centrifuged at 2594 x g for one minute to collect the sample at the bottom of the tube. The peptide concentration was measured via the Pierce Quantitative Fluorometric Peptide assay and compared to non-nebulised controls. The antimicrobial and anti-inflammatory activities of the nebulised peptides were tested using an RDA and an *in vitro* LPS-induced inflammation model using THP-1 monocyte-derived macrophages, respectively.

2.4. Radial Diffusion Assay (RDA) and anti-inflammatory activity

The impact of nebulisation on the antimicrobial (RDA) and anti-inflammatory (impact on THP-1 macrophage LPS-induced IL-6 production) activities was carried out as previously described (Creane, S.E et al, 2021). For the RDA analysis, a 100 μL aliquot of an overnight culture of P. aeruginosa 27853 or S. aureus 25923 was diluted in Mueller Hinton Broth (MHB) and incubated aerobically at 37 ∘C for three hours to obtain a mid-logarithmic culture. The culture was centrifuged, washed twice with 10 mM sodium phosphate buffer and the optical density at a wavelength of 600 nm (OD600 nm) adjusted to 0.4–0.5 in sterile 10 mM sodium phosphate buffer. An aliquot of 100 μL of this bacterial suspension (equating to approximately 5×10^6 bacterial cells) was added to 10 mL of molten base agarose (21 mg MHB powder, 1 g agarose, 20 μL Tween 20, 100 mL sodium phosphate buffer (10 mM), inverted, poured onto a square Petri dish (Sarstedt, Nümbrecht, Germany) and allowed to solidify. Wells of diameter 2.3 mm were punched into the agarose using a suction pump. A two-fold serial dilution (concentrations indicated in Fig.s) of peptide was added to the plate, with 3 μL of each test concentration added per well. The insect AMP, cecropin A (100 μg/mL) (Merck, Gillingham, UK), and vehicle controls, were included as positive and negative controls, respectively. The Petri dish was incubated upright at 37 ∘C for three hours to allow diffusion of test compounds into the agarose. Then, 10 mL of molten high nutrient overlay agarose (4.2 g MHB powder, 1 g agarose, 100 mL distilled water) was poured over the base agarose, allowed to solidify and incubated aerobically at 37∘C overnight. The following day, 5 mL of conditioning medium (10 mL acetic acid (Merck, Gillingham, UK), 2 mL DMSO, (Merck, Gillingham, UK), 88 mL distilled water) was added to the Petri dish for 10 min with gentle rotation to prevent any further bacterial growth. The conditioning medium was replaced with Coomassie brilliant blue stain (2 mg Coomassie Brilliant Blue R250, 27 mL methanol, (Merck, Gillingham, UK), 15 mL 37% formaldehyde, 63 mL distilled water) and incubated overnight at room temperature with gentle rotation. Inhibition of bacterial growth was indicated by circular areas devoid of colour. Measurement of the diameter of these areas was performed using a x8 measuring eyepiece (Flubacher, Wingate, UK). The Petri dish was

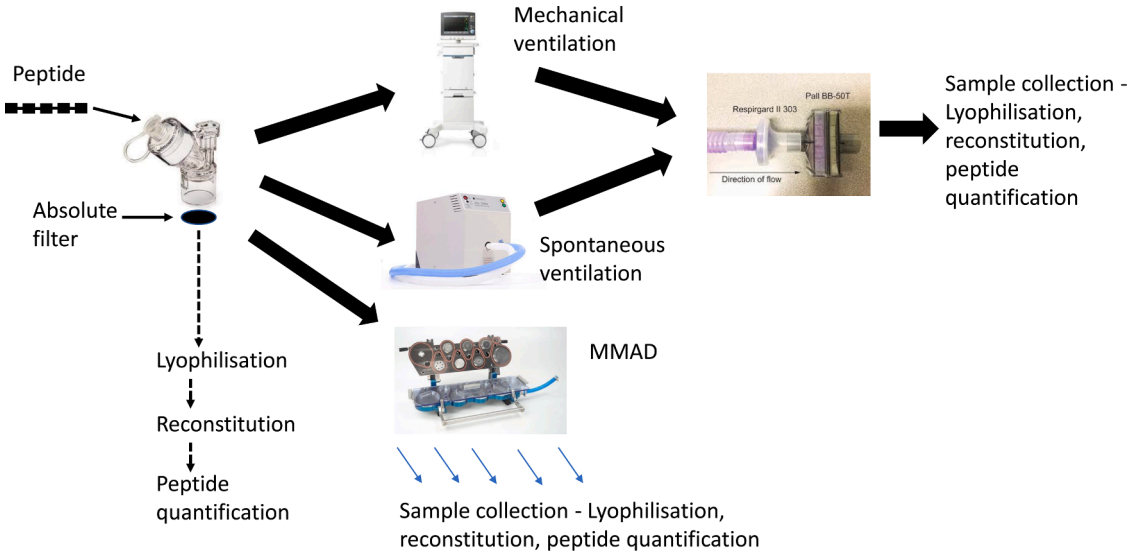

Fig. 1. Overview of peptide nebulisation procedures. Pictorial overview of the various experimental steps including quantification of peptides following nebulisation via the Aerogen VMN, nebulisation via the normal (spontaneous) and mechanically-ventilated simulators and nebulisation for Mass median aerodynamic diameter (MMAD) testing.

imaged using a Syngene G:box and GeneSnap software. Minimal inhibitory concentration (MIC) values were determined by linear regression of the size of inhibition zones versus the log concentration of the peptides.

For analysis of anti-inflammatory activity, THP-1 cells were seeded at a density of 2.5×10^5 cells/well in a 24-well plate and differentiated to THP-1 monocyte-derived macrophages by culturing under standard cell culture conditions (37∘C/5% CO_2) in the presence of 160 nM phorbol 12-myristate 13-acetate (PMA) (Merck, Gillingham, UK) for 72 h. The cell supernatant was replaced with 1 mL of fresh media and incubated for a further 24 h prior to experimentation. Immediately prior to cell stimulation, cell supernatants were replaced with 500 μL of fresh RPMI (+10% FBS, 2 mM L-glutamine). The cells were incubated with peptide and/or 100 ng/mL *Pseudomonas aeruginosa* LPS (Serotype 10, Source strain ATCC 27316) (Merck, Gillingham, UK) for 16 h under standard cell culture conditions. The cell supernatants (100μl) were collected after 24 hours for IL-6 and IL-8 analysis using commercially available ELISAs (Thermo Fisher Scientific, Loughborough, UK) and raw data analysed using GraphPad Prism.

2.5. Nebulisation of peptides in an in vitro simulated adult mechanical ventilation

A 1 mL dose of 1 mg/mL of each peptide, Sn1b, SnE1 and SnE1-F (reconstituted in distilled saline), was nebulised using a VMN (Solo, Aerogen, Ireland) positioned between the wye and the endotracheal tube (ETT) (8.0 mm, Flexicare, UK) of a dual limb circuit (Fisher & Paykel, New Zealand) during simulated adult mechanical ventilation using a Servo-i Ventilator (Maquet, Sweden). A capture filter (Respirgard 303EU, Vyaire, US) was positioned between the ETT and a test lung (Intersurgical, UK). Humidification was supplied by an Ultipor heat and moisture exchanger (Pall Medical). A standard adult mechanical ventilation breathing pattern was employed: 15 breaths per minute, 500 mL tidal volume and inspiratory to expiratory ratio of 1:1. Each experiment was replicated at least three times. Time to nebulisation cessation was recorded. The deposited fraction of the peptide was captured on the filter was eluted using 5 mL of deionised water. The eluent was transferred to a 50 mL Falcon tube, freeze-dried and reconstituted in a smaller volume of saline to concentrate peptide samples to a detectable concentration for the Pierce Quantitative Fluorometric Peptide assay.

2.6. Nebulisation of peptides in an in vitro simulated spontaneous breathing adult patient

Experiments to assess the ability to deliver nebulised peptides in an *in vitro* spontaneous breathing adult model were performed at Aerogen, Galway. To determine the total dose of peptide reaching the airways during simulated adult spontaneous breathing, an adult head model (LUCY) was connected to a breathing simulator (ASL5000, Ingmar Medical, US) set to mimic a normal adult breathing pattern: 15 breaths per minute, 500 mL tidal volume and inspiratory to expiratory ratio of 1:1. A filter (Respirgard 303EU, Vyaire, US) was connected at the level of the trachea of the head model to allow collection of nebulised peptides. A 1 mL dose of 1 mg/mL of Sn1b, C2, SnE1, SnE1N or SnE1-F was nebulised using an Aerogen Solo VMN in combination with a filtered mouthpiece/aerosol chamber (Ultra, Aerogen, Ireland). A supplemental gas flow rate of 2 L/minute was used. All testing was replicated three times. Time to nebulisation cessation was recorded. Peptide was eluted from the filter using 5 mL deionised water and eluent transferred to a 50 mL Falcon tube. The samples were freeze-dried and reconstituted in a smaller volume of saline to concentrate peptide samples to a detectable concentration for the Pierce Quantitative Fluorometric Peptide assay.

2.7. Particle size determination (MMAD)

The MMAD of the peptides was characterised using a Next Generation Impactor (NGI) (Copley Scientific, UK) in combination with an Aerogen Solo VMN using a 1 mL dose of 1 mg/mL Sn1b, SnE1 or SnE1-F. Each experiment was replicated three times. The NGI has seven stages, five of which are in the range 0.5 to 5 μm, and a micro-orifice collector (MOC), acting as a final filter. A vacuum air flow of 15 L/minute was passed through the NGI, forcing the aerosol droplets through a series of nozzles with reducing jet diameters corresponding to their likely deposition sites in the respiratory tract. As the aerosol is forced through the NGI, only droplets below the nozzle diameter will progress to the subsequent stage. Droplets that are too large to pass through will impact onto the collection plate. Time to nebulisation cessation was recorded. At each stage, the resultant sample was collected on the corresponding collection plate, which was eluted with 5 mL of deionised water and transferred to a 50 mL Falcon tube. Peptide samples were then freeze-dried and reconstituted in a smaller volume of saline to concentrate peptide samples to a detectable concentration for the Pierce

Quantitative Fluorometric Peptide assay. The percentage of the total sample dose at each stage of the NGI was determined.

2.8. Intranasal peptide treatment in an acute S. aureus lung infection

C57bl6 mice were placed under 4 % vaporised isofluorane anaesthesia (AbbVie Ltd, Maidenhead, UK), supplemented with 2.5 L/min of oxygen. Once the breathing rate decreased to a satisfactory state as determined by visual observation, each mouse received an intranasal instillation of 20 μl of sterile PBS or OD600nm = 5 (equating to 0.3-3.6×107 CFUs) of S. aureus Newman Strain. Three hours later, the mice were anaesthetised and received an intranasal instillation of 20 μl of sterile saline or 600 μM SnE1. A further three hours later, mice were sacrificed by pentobarbital overdose. Bronchoalveolar lavage (BAL) of the lung was carried out in all mice with a total volume of 600 μl of sterile PBS which was centrifuged at 500 x g for 10 minutes at 4°C to form a cell pellet. The cell pellet was resuspended in 100 μl of PBS (5×10^6 cells/ml) and a small volume diluted in 0.4% trypan blue (Sigma) to allow total cell number enumeration. Cytospins were prepared by placing a Shandon filter card (Thermo Scientific) between a Superfrost Plus Adhesion slide (Thermo Scientific) and a Shandon double cytofunnel (Thermo Scientific), held together by a Shandon cytoclip (Thermo Scientific). Up to two samples (100 μl each) were added per cytospin and centrifuged in a Shandon Centrifuge 2 (Thermo Scientific) at 63 x g for five minutes. Slides were allowed to air dry overnight. The slides were fixed in methanol (Sigma) for 20 minutes and allowed to air dry. The slides were immersed in May-Grünwald stain (Merck, Hertfordshire, UK) for eight minutes, immersed twice in PBS to remove excess stain and allowed to dry. The slides were then immersed in 10% Giemsa stain (Merck, Hertfordshire, UK) for eight minutes and placed in distilled water twice to remove excess stain. Finally, the slides were immersed in clearene (Leica Surgipath) for three minutes and coverslips applied using DPX mountant (Sigma). Slides were imaged at multiple fields of view using a Leica DM5500 microscope (Leica Microsystems, Milton Keynes, UK) using x40 objective lens magnification. Differential cell counts were performed by enumerating at least 400 cells per sample.

3. Results

3.1. The effect of nebulisation on peptide concentration

To evaluate the possibility that the peptides may bind to the nebuliser, we initially assessed whether nebulisation altered peptide concentration. As shown in Fig. 2, nebulisation of Sn1, SnE1 and SnE1-F peptides did not significantly alter peptide concentration. Sn1b exhibited a slight reduction in mean percentage of dose concentration following nebulisation, with mean percentages of 90% of the original peptide dose concentration detected while 80.5% of SnE1 peptide concentration was detected. Only SnE1-F generated a mean percentage of at least 100% (Fig. 2).

3.2. Nebulisation does not impair antimicrobial or anti-inflammatory activities

Though vibrating mesh technology generates less thermal and shear stress than older technologies such as jet nebulisation, VMNs are still capable of generating heat and shear stress with the potential to alter and/or damage peptide structure and function (Hertel et al., 2014); therefore, it was important to determine if nebulisation would affect peptide function. The antimicrobial activity of nebulised and non-nebulised Sn1, SnE1 and SnE1-F peptides against *P. aeruginosa* 27853 and *S. aureus* 25923 were tested using RDAs. All nebulised peptides resulted in similar MIC values to non-nebulised counterparts against *S. aureus* 25923 and *P. aeruginosa* 27853 (Table 1). Similarly, to clarify if nebulisation of peptides would affect anti-inflammatory function, nebulised Sn1, SnE1 and SnE1-F peptides were compared to non-nebulised counterparts using a cell-based inflammation model. When incubated with THP-1 monocyte-derived macrophages in the presence of LPS, nebulised Sn1b, SnE1 and SnE1-F generated significance reduction or trends toward reduction in IL-6 production in response to LPS (Fig. 3). These observations show that nebulisation did not impair the antimicrobial or anti-inflammatory properties of these peptides.

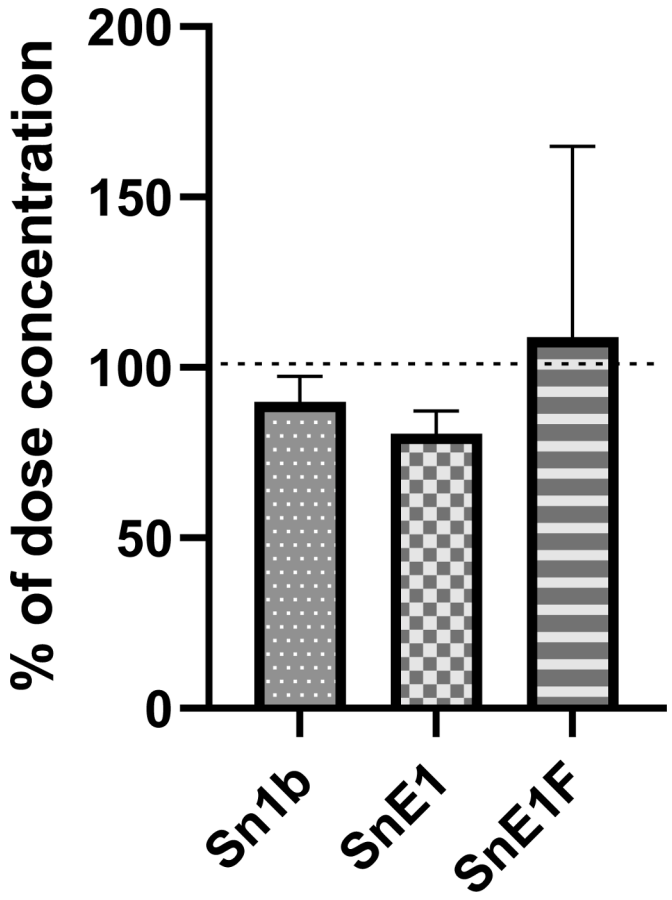

Fig. 2. Percentage of dose concentration of peptides following nebulisation. Peptides were nebulised using an Aerogen Solo VMN and peptide concentration before and after nebulisation measured using the Pierce Fluorometric Quantitative Peptide assay. The mean percentage (±SD) of original peptide dose concentration is displayed. One sample t test performed where possible, comparing to a hypothetical value of 100%. Experiments were performed at least N=3.

Table 1
MIC values of nebulised and non-nebulised peptides against *P. aeruginosa* 27853 and *S. aureus* 25923. MIC values (μM ± SEM) of nebulised and non-nebulised peptides against *P. aeruginosa* 27853 and *S. aureus* 25923. MIC values were determined using RDAs. Experiments were performed at least N=3.

Peptide	MIC value (μM ± SEM) against *Staphylococcus aureus* 25923		MIC value (μM ± SEM) against *Pseudomonas aeruginosa* 27853	
	Non-nebulised	Nebulised	Non-nebulised	Nebulised
Sn1b	5.48 (±1.66)	7.85 (±1.62)	3.61 (±0.818)	5.15 (±1.10)
SnE1	1.27 (±0.268)	1.74 (±0.602)	0.724 (±0.217)	0.936 (±0.301)
SnE1-F	0.937 (±0.743)	0.568 (±0.267)	0.513 (±0.508)	0.574 (±0.427)

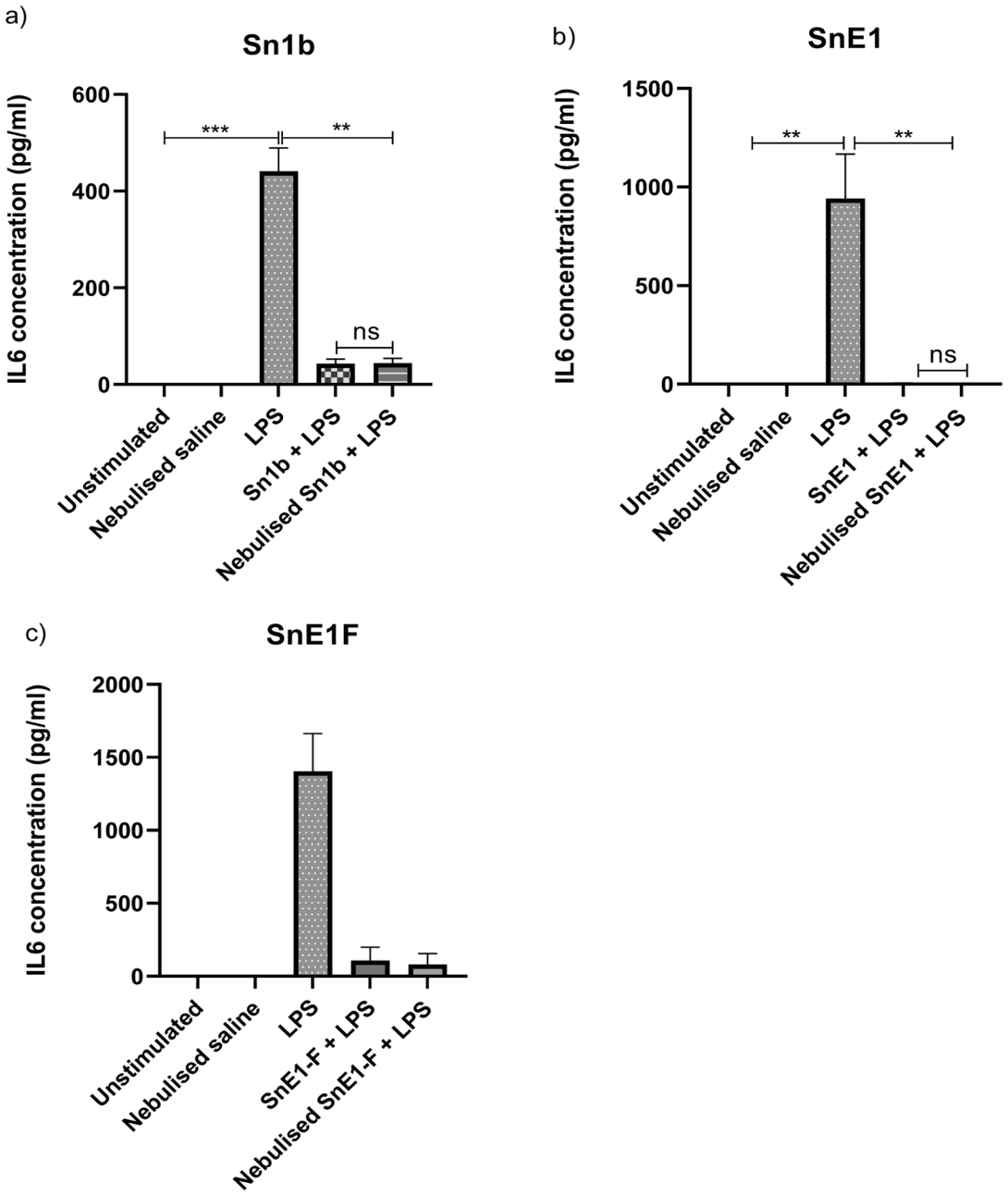

Fig. 3. IL-6 concentration in the supernatant of THP-1 monocyte-derived macrophages following incubation with nebulised and non-nebulised peptides and/or LPS. THP-1 monocyte-derived macrophages were incubated with nebulised or non-nebulised (a) Sn1b, (b) SnE1 and (c) SnE1-F and LPS for 16 hours. Cell supernatants were collected, and IL-6 levels measured via ELISA. Kruskal Wallis with Dunn's Multiple Comparison **p<0.01, ***p<0.001.

3.3. Estimated peptide delivery during simulated adult spontaneous breathing and mechanical ventilation

To estimate the total dose of peptide delivered to the airways via nebulisation, the peptides of interest were tested in lung model simulations to mimic typical adult breathing and mechanical ventilation scenarios. Sn1b resulted in the highest delivered peptide in the simulated adult spontaneous breathing model, with a mean tracheal dose of 19.7 % indicating that this peptide was successfully delivered to the airways at the test concentration (Fig. 4). SnE1 and SnE1-F were virtually undetectable in this model, suggesting these peptides did not reach the airways at the tested concentration (Fig. 4). SnE1-F demonstrated the highest delivery in the mechanically ventilated adult model, with 56.7 % of the administered dose reaching the airways (Fig. 4).

3.4. Peptide aerosol droplet size analysis

Nebulisation generates a heterogeneously sized population. The Mass median aerodynamic diameter (MMAD) indicates the aerodynamic diameter at which half of the aerosolised drug mass is above and half is below a stated diameter. Quantification of Sn1, SnE1 and SnE1-F peptide concentration at each stage allowed identification of the heterogeneity of the droplet distribution. Droplet size testing determined that Sn1b, SnE1 and SnE1-F produced MMAD values within the range of 4.01-4.59 μm (Table 2; Fig. 5). Given that the ideal droplet diameter range for deposition in the lower airways is 0.5-5 μm, these findings suggest that the peptides could be successfully delivered to the lower airways (Pilcer and Amighi, 2010). The duration for complete nebulisation of 1 mL of 1 mg/mL of peptide was less than five minutes for Sn1b, and less than three minutes for the SnE1 peptides with SnE1-F demonstrating a similar

Figure 4

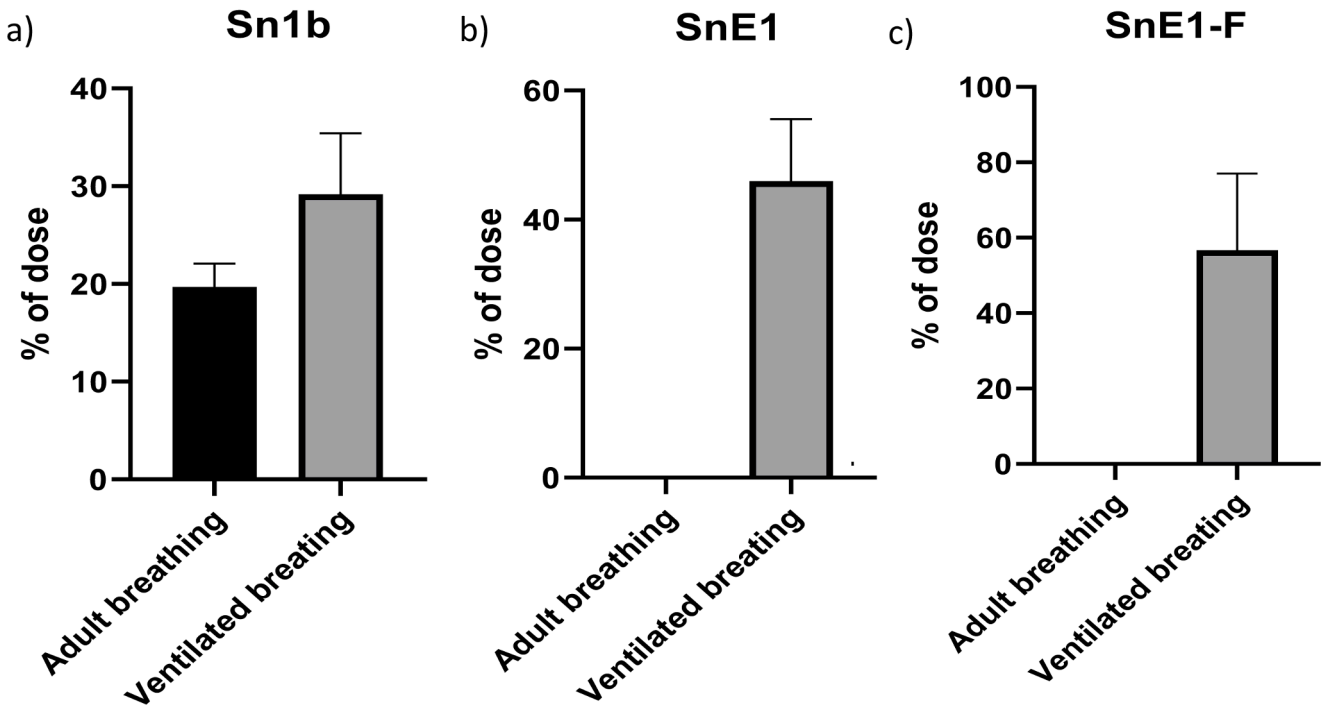

Fig. 4. The percentage of dose of peptides when nebulised in normal adult breathing and mechanically ventilated breathing simulations. Percentage of dose (mean ± SD) detected by Pierce Quantitative Fluorometric peptide assay when 1 ml of 1 mg/ml of (a) Sn1b (b) SnE1 and (c) SnE1-F was nebulised into normal adult breathing or mechanically ventilated breathing simulations. Experiments were performed at least N=3.

nebulisation time to the parent SnE1 (Table 2).

3.5. *In vivo evaluation of SnE1 in an acute S. aureus lung infection model*

To determine if direct delivery of peptides could reduce inflammation in a mouse model of lung infection, SnE1 was directly instilled into the lungs of mice infected with *S.aureus*. Although there was no reduction in bacterial burden in mice treated with peptide (data not shown), the intranasal delivery of SnE1 into the lungs of wild-type mice infected with *S. aureus* resulted in significant reductions in total number of BAL cells, neutrophils and macrophages (Fig. 6).

4. Discussion

This study demonstrates that it is possible to aerosolise Sn1, SnE1 and SnE1-F peptides using a VMN and that nebulisation does not alter the function of the peptides analysed. It also shows delivery during simulated adult spontaneously breathing and mechanical ventilation is feasible and likely to reach deep into the lung airways.

During nebulisation, a peptide drug solution is subjected to interfacial, shear and thermal stresses, which can lead to protein aggregation and/or degradation (Hertel et al., 2014; Smyth and Hickey, 2011). It is widely accepted that these factors are less pronounced in VMN compared to jet and ultrasonic nebulisers (Ari, 2014; Waldrep and Dhand, 2008). For the peptides examined in this study, peptide concentration was not increased by nebulisation, ruling out the "up-concentration" effect frequently observed in jet and ultrasonic nebulisation (Beck-Broichsitter et al., 2012). There was a slight decrease in the percentage of dose concentration of Sn1b and a trend towards a reduced percentage of dose concentration of SnE1, suggestive of peptides binding to the nebuliser. However, SnE1-F resulted in a mean percentage of dose concentration of approximately 100%, suggesting this peptide exhibit no binding to the nebuliser. These observations are similar to those described by Forde et al. (Forde et al., 2019), where nebulisation of the pro-drug of specific AMPs using the same VMN did not affect MIC values against *P. aeruginosa* PAO1 and clinical isolates.

We have previously shown that Sn1b, SnE1 or SnE1-F possess antimicrobial activity against *P. aeruginosa* 27853 and *S. aureus* 25923 (Creane et al., 2021). We now show that nebulisation does not affect the antimicrobial activity of these peptides. To determine if nebulisation had any effect on anti-inflammatory activity of Sn1, SnE1 and SnE1-F, nebulised peptides were tested using an in vitro LPS-induced inflammation model using THP-1 monocyte-derived macrophages. When each nebulised peptide was incubated with THP-1 monocyte-derived macrophages in the presence of LPS, similar IL-6 levels were detected in cell supernatants compared with non-nebulised counterparts with nebulised and non-nebulised counterparts exhibiting significant reductions in IL-6 (Fig. 3). These observations indicate that nebulisation did not compromise peptide anti-inflammatory activity, a finding also observed by Sweeney et al. (Sweeney et al., 2019), whereby pro-inflammatory activity of interferon-γ was conserved post-nebulisation with two types of VMN.

The heterogenic composition of aerosol produced by nebulisation and resulting MMAD is affected by many variables, including nebuliser type. Some drugs exhibit similar MMAD values when tested in different nebulisers; for example, nebulisation of human DNase I using a VMN resulted in an MMAD of 4.3 μm, and 4.2 μm using a jet nebuliser (Johnson et al., 2008). Moreover, for some drugs droplet distributions can vary greatly between nebuliser types; for instance, comparison of several nebulisers using tobramycin demonstrated that some did not successfully produce an aerosol while others had highly variable MMAD profiles, many of which were not within an ideal diameter range and

Table 2
The mean percentage of dose of peptide in each compartment of an NGI, MMAD values and nebulisation times. Percentage of dose (%) ±SD of Sn1b, SnE1 and SnE1-F collected in each compartment, resulting MMAD (n=3), and time taken for complete nebulisation when 1 ml of 1 mg/ml of each peptide was nebulised into an NGI. Experiments were performed at least N=3.

Compartment	Cut-off (μm)	Mean percentage of dose (SD) Sn1b	SnE1	SnE1-F
Nebuliser		3.12 (0.920)	7.53 (12.7)	12.9 (4.09)
T-piece		3.68 (2.61)	2.76 (0.881)	7.64 (1.76)
Throat		0.573 (0.353)	0.757 (0.687)	0.667 (0.464)
Stage 1	14.1	3.40 (0.692)	4.60 (1.62)	4.25 (1.08)
Stage 2	8.6	5.30 (1.41)	9.42 (4.73)	9.48 (2.16)
Stage 3	5.4	10.7 (1.46)	22.0 (1.37)	14.1 (0.731)
Stage 4	3.3	14.0 (1.95)	25.7 (3.16)	25.1 (3.90)
Stage 5	2.1	11.8 (3.00)	17.6 (0.901)	15.9 (7.51)
Stage 6	1.4	5.40 (0.802)	7.48 (1.78)	5.64 (3.17)
Stage 7	1.0	1.03 (0.647)	1.96 (2.25)	1.40 (0.531)
MOC		3.97 (3.29)	0.577 (0.580)	0.297 (0.0902)
Filter		1.14 (0.680)	2.43 (2.63)	6.06 (6.18)
MMAD (μm) (GSD)		4.01 (2.14)	4.59 (1.93)	4.42 (1.78)
Nebulisation time (seconds) (±SD)		257 (28.2)	139 (9.95)	134.8 (16.5)

unlikely to be delivered to the airways (Weber et al., 1994). It has also been reported that droplet diameter distributions can vary greatly depending on the nebuliser model employed (Smith et al., 1995). Here we found that the MMAD values of Sn1b, SnE1 and SnE1-F generated using the Aerogen Solo VMN were within the ideal range (0.5-5 μm) for delivery to the lower airway (Pilcer and Amighi, 2010). These values are comparable to that of interferon-γ when nebulised using an Aerogen Solo VMN (Sweeney et al, 2019). Additionally, for all peptides tested, the majority of the nominal dose was detected within stages three to six, which possess cut-off diameters within this ideal range for lung delivery. Therefore, this implies that the peptides are likely capable of deep lung delivery using the Aerogen Solo VMN.

Recent studies using a VMN in spontaneous breathing and mechanical ventilation models have demonstrated efficient drug delivery (Forde et al, 2019; Sweeney et al, 2019). Similar to these findings, peptide testing in the in vitro mechanically ventilated lung model suggested the vast proportion of our peptides would reach the lung. However, with the exception of SnE1-F, the majority of peptide nominal doses did not reach the model lung, likely due to the combined deposition of peptide in the breathing apparatus and the nebuliser. MacIntyre et al noted considerable deposition on the endotracheal tube when using a jet nebuliser during in vivo mechanical ventilation, with a total deposition of 35.6% of the inhaled dose ((MacIntyre et al., 1985). Similarly, O'Riordan et al observed deposition of labelled aerosol in the tracheostomy tube during in vivo mechanical ventilation; during inspiration, tracheostomy tube deposition was recorded as 10.4% of total patient deposition, and a further 28.1% upon expiration (O'Riordan et al., 1994). Drug loss to the breathing apparatus during in vivo mechanical ventilation tends to be higher than in vitro modelling as in vitro models do not account for expiration of inhaled aerosol (Berlinski and Willis, 2013); however, as demonstrated by O'Riordan et al, there is considerable deposition in the tracheostomy tube during inspiration alone (O'Riordan et al., 1994). Dugernier et al. reported in a human

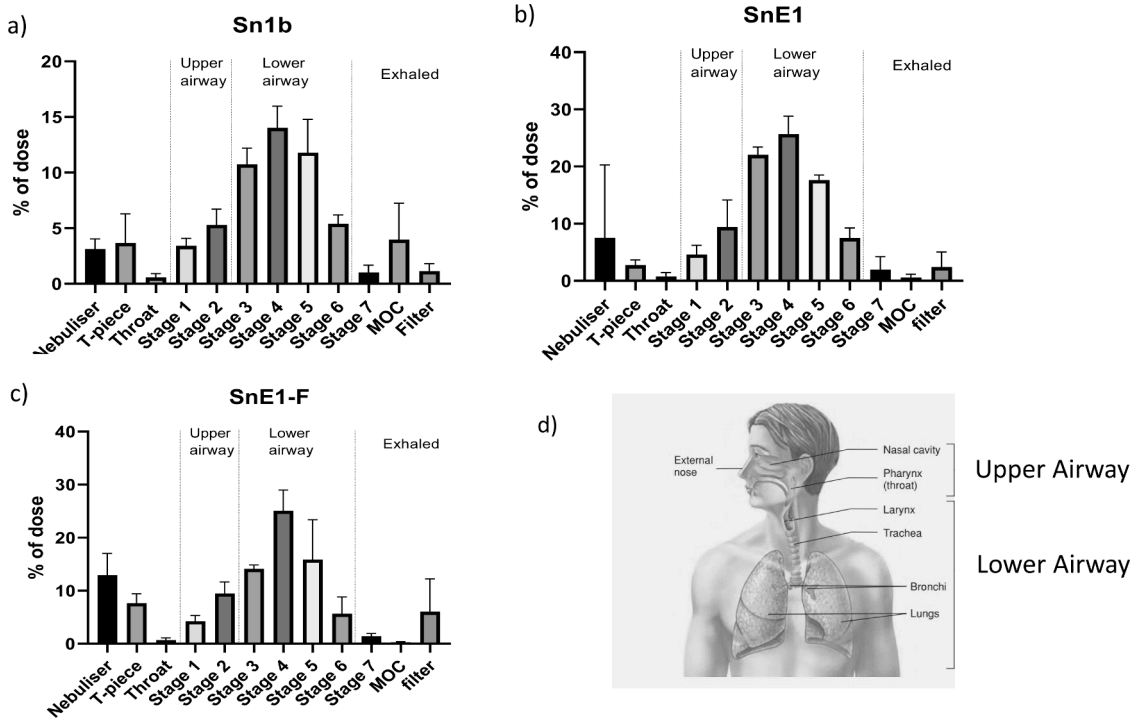

Fig. 5. The mean percentage of peptide dose at each stage when nebulised into an NGI. The mean percentage of dose (%) detected at each stage when 1 ml of 1 mg/ml of (a) Sn1b (b) SnE1 and (c) SnE1-F was nebulised into an NGI using an Aerogen Solo VMN. Peptide was washed off the collection cup at each stage with water and samples concentrated by lyophilisation and reconstitution in a lesser volume of saline. Peptide concentration at each collection cup was quantified using the Pierce quantitative fluorometric peptide assay. (d). Schematic demonstrating the upper and lower airway regions of the human respiratory tract (Adapted from Essentials of Anatomy and Physiology Brainkart.com). Mean percentage of dose ± SD. Experiments were performed at least N=3.

Figure 6

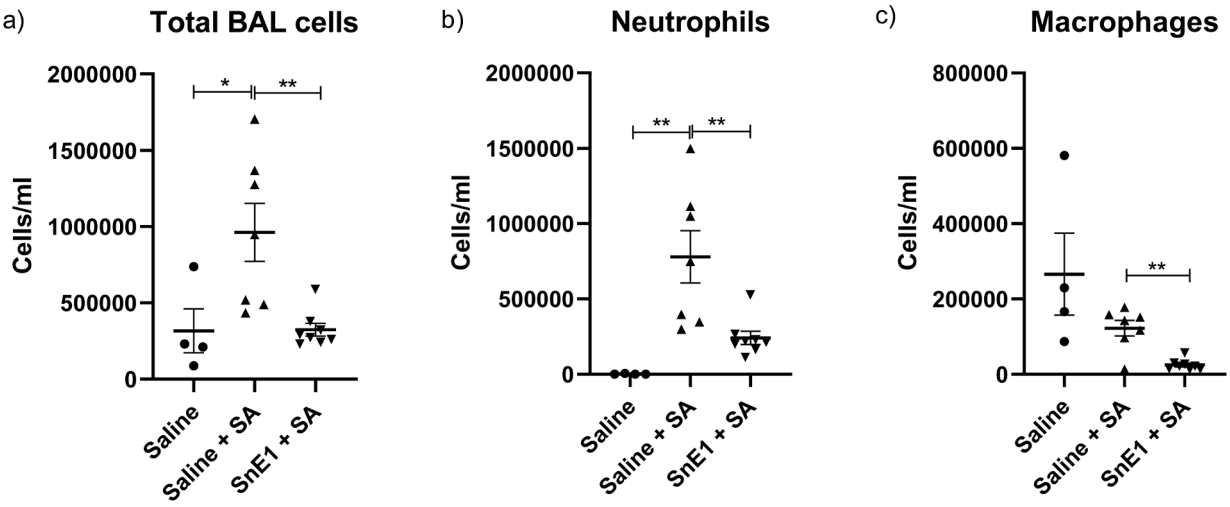

Fig. 6. Effect of SnE1 intranasal instillation on lung inflammatory cells in wild-type mice infected with S. aureus. BAL fluid was evaluated centrifuged and the cell pellet reconstituted in PBS. Cell counts were performed to get the total cell count (a) in each BAL sample. A sample of 100μl (5×10^5 cells) was then cytospun onto a glass slide, air dried overnight and stained with May-Grünwald stain followed by Giemsa staining. Neutrophils (b) and macrophages (c) were counted for each sample. Mann Whitney performed between groups. *$p<0.05$, **$p<0.01$. n=4-8.

scintigraphy that across various ventilation strategies the dose delivered to the expiratory port on the ventilator was approximately 23% of the nominal dose (Dugernier et al., 2016). Collectively considering these studies, it is reasonable to anticipate substantial drug loss to breathing apparatus during mechanical ventilation.

Many peptides are amphipathic in nature and readily bind to surfaces (Goebel-Stengel et al., 2011). Cationic peptides have been shown to be highly capable of binding to plastic (Chico et al., 2003). Factors such as net charge, hydrophobicity and amino acid chain length may impact binding capacity but are not reliable predictors (Sécher et al., 2022). Suboptimal formulation of antibodies has been shown to increase the potential of aggregation, but this is easily mitigated through simple formulation strategies. The three peptides tested in this study were highly cationic (+14-+15) which may aid peptide delivery by nebulisation. Using a computational stochastic lung model, Majid et al deduced that charged particles demonstrate enhanced deposition in the airways most likely due to electrostatic induction of the tissue of the airway wall, resulting in an attractive force (Majid et al., 2012). Azhdarzadeh and colleagues (Azhdarzadeh et al., 2014a, b, 2015) made similar observations *in vitro*, whereby the introduction of electrostatic charge on aerosolised particles enhanced delivery to paediatric and adult oral-extrathoracic and infant nasal-extrathoracic airway models. Therefore, peptide net charge may partially explain differing dose delivery of peptides in the breathing simulations.

Future studies will involve taking forward the 3 peptides into more definitive pre-clinical testing by nebulising each peptide into relevant models of lung inflammation and infection (mouse and human). Although we did not observe a reduction in bacterial load in our mouse infection data despite a reduction in inflammatory cell load (Fig. 6) this may require optimisation of peptide delivery to the lung in future studies and testing for the presence of these peptides in relevant compartments (blood, lung). In addition, although many of these peptides have unsurprising antimicrobial activity in vitro, less is known about why they have anti-inflammatory activity. Therefore, future testing will also include understanding how the anti-inflammatory mechanism of the snake peptides is exerted in cellular and animal models which also allow us to identify the active region(s) of each peptide by designing and testing shorted peptides based on the known sequences of these peptides

5. Conclusions

We have shown that nebulisation of antimicrobial and/or immunomodulatory peptides using an Aerogen Solo VMN is feasible and does not obviously impact stability or significantly alter peptide anti-inflammatory function. Peptide nebulisation produces a heterogeneous droplet distribution, the majority of which is likely to target the lungs. *In vitro* testing demonstrated that considerable proportions of nominal peptide doses are likely to reach the airways during mechanical ventilation. A substantial amount of Sn1b nominal dose is also likely to be delivered to the airways during spontaneous breathing. Further investigation may highlight areas for enhancing peptide delivery via nebulisation, such as breath-actuation droplet size optimisation and formulation optimisation.

Credit author statement

CCT, JPD, RMcL and Sw conceived of the project idea; SC conducted the research and investigation process, performed the experiments and collected the data; MJ and RMcL provided resources and helped with formal analysis; SC and MJ validated the findings; CCT and JPD drafted the manuscript

Data availability

Data will be made available on request.

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