Sweetening Inhaled Antibiotic Treatment for Eradication of Respiratory Biofilm Infection

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Abstract

The failure of chronic therapy with antibiotics to clear persistent respiratory infection is the key morbidity and mortality factor for patients with chronic lung diseases, due to the presence of biofilm in the lungs. It is hypothesised that the use of carbon sources, such as mannitol, could stimulate the metabolic activity of persister cells and restore their susceptibility to antibiotics. The current study aims to establish a representative model of *Pseudomonas aeruginosa* (Pa) biofilm lung infection, and investigate the effects of aerosolised mannitol on antibiotic efficacy, focusing on ciprofloxacin, in the eradication of the biofilm. A biofilm was cultured onto Snapwell inserts incorporated into a pharmacopeia deposition apparatus, the Anderson Cascade Impactor. Three formulations were nebulised directly onto the *Pa* biofilm, including: mannitol only, ciprofloxacin only and ciprofloxacin and mannitol combined. Antibacterial effectiveness was evaluated using colony-forming units (CFU) counts and scanning electron microscopy. Nebulisation of mannitol promotes the dispersion of the bacteria from the biofilm and demonstrated an enhancement of the antibacterial efficacy of ciprofloxacin compared to delivery of the antibiotics alone. The development of a representative lung model of bacterial biofilm could potentially be used as a platform for future pre-clinical screening. Furthermore, the co-administration of mannitol and ciprofloxacin could represent new strategy to improve antibiotic therapy.

Keywords: biofilm, mannitol, ciprofloxacin, inhalation, respiratory tract infection

1.0 Introduction

Lung infections are the number one cause of transmissible death worldwide. In fact, lower respiratory tract infections (RTIs) were reported to be the 3rd leading cause of death worldwide by the World Health Organisation.[1] To add to this, chronic obstructive pulmonary disease (COPD) and lung cancer are the 4th and 5th leading cause of death, respectively. The majority of these patients, together with other chronic lung disease patients including cystic fibrosis (CF), bronchiectasis (BE) and primary cilia dyskinesia (PCD), are persistently colonised by bacteria, such as *Pseudomonas aeurigonosa*, *Staphyloccocus aureus* and *Haemophillus influenza* in their lungs.[2-4] These pathogens play an important role in the maintenance of the vicious infection and inflammation cycles contributing to disease progression.

Standard treatment for persistent RTIs is limited to chronic administration of suppressive antibiotic treatment, primarily administered as inhalation, oral tablets or intravenous injections.[5] However, there are increasing evidence demonstrating that these pathogens are not only becoming less responsive to the current clinical antimicrobial agents, but it is now emerging with newer inhaled antimicrobial formulations as well.[6-8] The reduced or lack of response to these agents is due to pathogens acquiring resistance in the form of biofilms.[2-4] These biofilms comprise of communities of non-motile microorganisms and a subpopulation of persister cells that are characterised by reduced metabolic activity and are covered with extracellular polymeric matrices (EPS) that have developed high tolerance to antibiotic therapies. In fact, Worlitzsch *et al.* (2002) has shown that *P. aeruginosa* grows mainly in biofilms in the conductive zone, the region from the trachea to the terminal bronchiolus, whereas

very few planktonic bacteria are localised at the epithelial surface of the bronchi.[9] These characteristics make biofilms resistant to both the body defence mechanisms and to standard antibiotics, enhancing the development of chronic infections. Once a biofilm infection has colonised the airways of patients, it is difficult if not impossible to eradicate.

The use of conventional antibiotic therapies are only effective against metabolically active and rapidly dividing planktonic bacteria.[10] Therefore, initial infection of bacteria in the airways is often environmentally acquired can be cleared easily with treatment. Subsequent intermittent isolates eventually become an established chronic infection with biofilm formation. Unfortunately, use of one or more antibiotics often render cells tolerant and/or potentiate toxicity and increase unwanted side-effects. Given these limitations, the development of a strategy to treat and restore antibiotic sensitivity biofilm infection is a priority. It has been hypothesised in a recent study that, carbon sources to stimulate the metabolic activity of persister cells and restore their susceptibility to antibiotic treatments.[11] This concept is very interesting, since using current therapy as adjunct, coupled with a novel administration approach could help prevent antibiotic-tolerance and sensitise the low metabolic persister cells towards antibiotic treatment. Specifically, mannitol is particularly attractive for this purpose as it is a naturally occurring sugar alcohol, a potential carbon source that has been approved as an osmotic agent to assist mucus clearance in CF patients.[12]

However, another major challenge with the development of an effective inhalation therapy for biofilm treatment is the lack of pre-clinical biofilm models that could

accurately represent biofilm growth in the airways. Current biofilm models that were used to understand the complex nature of biofilm, can be divided into 2 district groups: (A) Static models, where biofilms are grown in a closed system with limited nutrients and aeration.[13] This models include microtitre plate based models where biofilms are grown on the bottom of the plates or grown on coupons placed in wells of the microtitre plates and more recently developed Calgary biofilm device. These models allow for rapid quantification of biofilm mass and viable cells enabling high throughput screening. (B) Dynamic models are continuous cultures where waste, metabolic by-products, dispersed and dead cells are continuously replaced by fresh medium.[14] Examples of some common flow models are drop flow biofilm reactors, centers for disease control (CDC) biofilm reactor and the rotating disc reactor. These models allow for more control over environmental flow and shear forces, enabling the characterisation of biofilm's physical and chemical resistance. However, these current models of biofilms are neither optimised, nor physiologically representative of the biofilm growth in the lung. Although biofilms are commonly referred to as solid-attached structures within a liquid environment, they predominantly grow at the air interface of the airways.[15] Because of the inability to reproduce these growth environments with current *in vitro* models, there is a significant lack of understanding in the influence of the different interface on biofilm development and treatment responses. The poor predictability and correlation of current *in vitro* biofilm assays impede the development of novel solutions to biofilm respiratory infections and discovery of effective drug molecules, but more importantly failure to translate findings to clinic. In addition to developing a more effective therapy to treat respiratory biofilm infection, this study also aims to develop an *in vitro* air interface biofilm that mimics the growth conditions in the airways using *P. aeruginosa* as the model bacterium.

Most *in vitro* studies investigating the therapeutic efficacies against biofilms involves the application of drug solution or suspension on top of the biofilm layers, which present a uniform concentration of drug over the surface of the biofilm. This is significantly different to *in vivo* processes in the lung where therapies are aerosolised and deposited as liquid droplets or solid particles.[16] Upon deposition of the aerosol compound, it is envisaged that the deposited liquid droplet will spread across the biofilm surface (dissolution into the biofilm EPS matrix in the case of dry powder) and create a concentration gradient across and through the biofilm layer. The non-uniform exposure across the biofilm is expected to affect drug efficacy and penetration profiles, which have not been investigated to date. Subsequently, in this study, the air interface biofilm was integrated in to a modified Andersen Cascade Impactor (ACI) to simulate realistic deposition of inhaled formulation that will mimic the *in vivo* processes following inhalation therapy.[17] The efficacy of the treatment was assessed in terms of antibiotic penetration, antimicrobial activity and particle-biofilm interactions. Hence, the main objective of this study was to determine the ability of this integrated model as a potential predictive tool to investigate the efficacy and penetration of co-delivering ciprofloxacin (an antibiotic) and mannitol (a mucolytic agent), as a potential inhaled combination therapy for the treatment of respiratory biofilm infection, providing an insight into the different variables that could influence clinical outcomes.

2.0 Materials and methods

2.1 Materials

Calu-3 cells were purchased from the American Type Cell Culture Collection (ATCC, Rockville, US). Ciprofloxacin HCl was used as supplied (MP; Biomedical Australasia Pty Limited, NSW, Sydney, Australia). Non-essential amino acids solution, CelLytic™ M Cell Lysis, protease inhibitor cocktail and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Sydney, Australia). Other cell culture reagents including trypsin-EDTA solution (2.5g/L trypsin, 0.5g/L EDTA), Dulbecco's Modified Eagle's Medium (DMEM, without phenol red and L-glutamine, including sodium bicarbonate and 15mM HEPES), Trypan blue solution (0.4% w/v), Phosphate buffered saline (PBS), L-glutamine solution (200mM), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS) were obtained from Invitrogen (Australia). Snapwell cell culture inserts (1.12 cm^2 polyester, 0.4µm pore size) were purchased from Corning Costar (Lowell, MA, USA) and all other sterile culture plastic wares were from Sarstedt (Adelaide, Australia). All solvents used were of analytical grade and were supplied by Biolab (Victoria, Australia).

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2.2 Characterisation of nebulised formulations

The three different nebulised solutions were prepared in deionised water containing ciprofloxacin alone (cipro, 20mg/mL), mannitol alone (150 mg/mL) and combined ciprofloxacin and mannitol (cipro-man) of the same concentrations. The formulations were nebulised using a PARI LC Sprint® jet nebuliser powered by the Pari Turbo Boy S compressor (Stranberg, Germany). 2 mL of the respective formulations were introduced in to the reservoir of the nebuliser prior to nebulisation. The aerodynamic particle size distributions of the nebulised formulations were assessed using the Andersen Cascade Impactor (ACI, Copley Scientific Limited, Nottingham, UK) according to methodologies as outline in the British Pharmacopeia. The flow rate was set to 15

L/min using a calibrated flow meter (Model 4040, TSI Model Instruments, Germany). The ranges of cut of diameters at this flow rate are: 12.4, 8.0, 6.5. 4.5, 2.9, 1.5, 1.0, 0.6 and 0 μm, for stages 1 through to the filter, respectively, with particles captured on any specific stage having an aerodynamic diameter less than the preceding stage.[18-19] Subsequently, the fine particle fine particle fraction (FPF, <5 μm), mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated from regression of log-linear plots of stage-size verses cumulative stage-deposition.

Briefly, the nebuliser mouthpiece was fitted to a rubber adapter to obtain a sealed attachment to the United States Pharmacopoeia (USP) throat connected to the ACI. Samples were nebulised at a flow rate of 15 L/min for approximately 1 minute. For the mass recovery assay, each ACI stage, adapter and USP throat were oven dried at 50 °C to evaporate the aqueous phase before cooling to ambient temperature. The components were then rinsed with either 10 mL of deionised water. Quantification of the ciprofloxacin and mannitol in these samples was achieved using a validated method coupled with High-Performance Liquid Chromatography (HPLC).

2.3 Validation of aerosol deposition on modified Andersen Cascade Impactor plate

A custom-made ACI plate that could accommodate the Snapwell inserts was designed and manufactured using a 3D printer to deposit the aerosols onto the biofilm, as previously described.[19-20] The modified plate, containing the Snapwell inserts replaced Stage 4, to collect particle size fraction between 2.9- 4.5 μm, correlating to deposition of

particles in the airway region of the lungs. The inserts were wetted with Mucin Type III at a concentration of 0.5 mg/mL to prevent particle bounce on the cell-free Snapwells.[21] After nebulisation of the formulation for 1 minute, the modified ACI plate was removed and each Snapwell insert was washed separately with water to obtain the total amount of drug deposited on the specific stage and on each well.

2.4 Quantification of drug by high performance liquid chromatography

2.4.1 Ciprofloxacin analysis

Quantification of ciprofloxacin was performed using the same HPLC method. This was achieved using a Shimadzu Prominence UFLC system equipped with the SPD-20A UV-Vis detector, LC-20AT liquid chromatograph and SIL-20A HT Autosampler (Shimadzu Corporation, Japan) and Phenomenax C18 column (5 µm, 150 X 3.9 mm). The mobile phase was a mixture of methanol and 0.1M sodium dihydrogen phosphate at a ratio of 30:70 (v/v), pH adjusted to 3.35 with phosphoric acid to allow ciprofloxacin separation. Standards were prepared in MilliQ water. HPLC was set according to the following conditions: detection wavelength 275nm, flow rate of 1.0 mL/min and an injection volume of 100 µL. Linearity was obtained between 0.05 and 100 µg/mL (R^2=0.99), with a retention time of 7.85 minutes.

2.4.2 Mannitol quantification

A refractive index detector (RID 10A refractive index detector, Shimadzu, Kyoto, Japan) was used to quantify mannitol. Deionised water was used as mobile phase and samples were injected into the HPLC system that was equipped with a Resolve C-18 column (5

μm, 150 X 3.9 mm, Waters, Milford, MA, USA). The HPLC was set to a flow rate of 1 mL/min and an injection volume of 100 μL resulting in a retention time of 3.5 minutes. A mannitol calibration curve was constructed using standard solutions with concentrations ranging from 0.05 to 10 mg/mL (R^2=0.99).

2.5 Transepithelial transport studies

Transepithelial transport studies were performed on fully differentiated Calu-3 epithelial cells on Snapwell inserts according to previously established protocol.[19] Experiments on cells were performed between days 14 and 21, from passages 35-42. The ACI with modified plates was used for deposition of the nebulised aerosols onto the Calu-3 cells. Three cell containing inserts were placed on to the modified plate with the remaining wells containing blank inserts. Aerosolisations of the formulations onto the cells were performed with the modified ACI plate placed on Stage 4 as described in Section 2.3. After the study, assessment of the Calu-3 monolayer's integrity was performed using transepithelial electrical resistance (TEER) measurements, as previously described using the epithelial voltohmmeter (EVOM, World Precision Instruments, USA).[19, 22] TEER measurements were performed on untreated-control cells and after the transepithelial transport studies were completed.

2.6 In vitro biofilm assays

2.6.1 Biofilm formation and quantification assay

The biofilm formation assay was performed in microtitre plates in which *P. aeruginosa* PAO1 was first allowed to attach to the surface of 96-well polystyrene plate. *P. aeruginosa* was grown overnight in cation adjusted Mueller-Hinton broth (CAMHB, Oxoid, Cambridge, UK) at 37 °C, shaken at 250 rpm and diluted 100-fold to 10^6 CFU/mL with CAMHB as determined by optical density (OD) and plate count assay. Subsequently, 100 µL aliquot of the culture was introduced into each well and the plates were incubated without shaking at 37 °C for 24 hours. The liquid was then gently removed and the wells rinsed twice with PBS before ciprofloxacin or mannitol treatment. The effects of different treatment were studied using a range of different concentrations of ciprofloxacin (0-800 µg/mL) and mannitol (0-20 mM). 100 µL of the treatment solution was added to the wells containing the biofilms and further incubated for 2 hours at 37 °C. After the treatment, 100 µL of culture containing the dispersed cells was transferred into a new 96-well plate. Subsequently, the OD was measured at 600 nm of the dispersed cells using a microtitre plate reader (Beckman, CA, USA)[23]. The same plate was also retained for biofilm staining with crystal violet. The wells were washed extensively with PBS and the remaining attached biofilm stained with crystal violet (CV), left for 1 hour before rewash and finally de-stained with ethanol-acetone. The OD was measured using a microtitre plate reader (Beckman, CA, USA) set at 595 nm. For each experiment, three replicates were performed and each experimental condition was repeated on three separate days. The amount of biofilm removal was calculated as a percentage of initial biofilm mass.

2.6.2 Air interface biofilm assay for drug deposition studies

For drug deposition studies on the biofilm, air interface biofilm assay was established by culturing *P. aeruginosa* on to Snapwell inserts. *P. aeruginosa* was first grown overnight in CAMHB at 37 °C, shaken at 250 rpm and diluted 100-fold to 10^6 CFU/mL with CAMHB, as determined by OD and plate count assay. Subsequently, 100 µL aliquot of the culture was introduced into the Snapwell insert. The bacteria containing Snapwell inserts were maintained on Trypticase soy agar for 3 days to obtain a reproducible colony-forming unit count (CFU) of 10^8 CFU/mL. The final inoculum size was verified by counting visible colonies by spread plate technique. The Snapwells containing the air interface biofilm were placed onto the modified and reusable ACI plates and subsequently incorporated into the ACI on Stage 4 to allow for deposition of the nebulised aerosols on the biofilm, as outlined in section 2.3. After the deposition of the aerosolised formulation, the Snapwells were carefully removed from the plate and transferred into a 6 well plate containing 1 mL of fresh pre-warmed Hank's Balanced salt solution (HBSS). Cells were maintained at 37°C in 5% CO_2 and 95% humidity throughout the experiment.

After drug deposition, the biofilm was evaluated for: 1) penetration of ciprofloxacin across the biofilm, 2) quantification of viable attached bacteria and dispersed bacteria from the biofilm and, 3) observation of biofilm structure. To evaluate the amount of ciprofloxacin penetration into and across the biofilm, samples of 200 µL were drawn every half an hour from the basolateral chamber and an equal volume of fresh HBSS buffer was added to replace the withdrawn sample. At the final time point, the insert was transferred to an empty well and the remaining bacteria cells were gently washed with HBSS buffer to collect any remaining drug and the samples were analysed using

HPLC. As for enumeration of viable bacteria, Snapwells were rinsed twice with PBS and together with the samples from the basolateral chamber were collected to account for the number of dispersed bacteria. Another 1 ml of PBS was added to the well and sonicated thoroughly for 30 s to account for bacteria remaining in the biofilm. Samples were then serially diluted and viable plate counting was performed.

The observation of biofilm structure was performed under a scanning electron microscope (SEM), whereby treated and untreated biofilm samples were rinsed twice with PBS and fixed in 4% paraformaldehyde overnight. The samples were then dehydrated through a series of graded ethanol baths placed onto SEM stubs. Samples were dried using a critical-point drier, gold coated and finally imaged using SEM (Zeiss Ultra, Oberkochen, Germany).

2.7 Statistical analysis

All results are expressed as mean ± standard deviation of at least three separate determinants. To determine significance between groups and control, unpaired 2-tailed t-tests were performed (quoted at the level of $p<0.05$). The diffusion of ciprofloxacin over time from the different formulations on the air interface Calu-3 cell and the biofilm models were statistically analysed to determine significance using Fit Factors described by Moore and Flanner, which was adopted, by the Food and Drug Administration guidance for dissolution testing in the industry.[21, 24-26] The model has independent approaches to compare release profiles for a test and reference formulation using

difference factor (f_1) and similarity factor (f_2). For the curves to be considered statistically different, arbitrary limits with $f_1 \geq 10$ and $f_2 \leq 50$ were chosen for analysis.

3.0 Results and discussion

3.1 Rationale for combined ciprofloxacin and mannitol

The establishment of biofilm by *P. aeruginosa* pathogen in the lungs make them resistant both to secondary defences and to standard antibiotics, boosting the development of chronic respiratory infection. In this study, it is proposed that mannitol could be used to disperse *P. aeruginosa* from the biofilm into active planktonic forms and subsequently restore their susceptibility to antibiotic treatments. Figure 1A and B demonstrates the effects of mannitol and ciprofloxacin on pre-formed submerged biofilm colonies after treatment with increasing drug concentrations. This is represented as the percentage of remaining attached biofilm biomass mass after 2-hours treatment. Generally, the increase in both ciprofloxacin and mannitol concentrations demonstrated reductions in biofilm mass adhered on the surface of the microtitre plates. This is expected with antibiotic treatment, whereby there was a more significant reduction in the remaining biofilm at the lower ciprofloxacin concentrations, compared with the higher concentrations. However, this effect starts to plateau at 400 μg/mL, with approximately 20% of the biofilm still remaining after treatment with higher concentrations of antibiotics, further reinforcing the notion of biofilm resistance that could contribute to persistent infection. Although mannitol does not have antibacterial activity, it was found (Figure 1C) that it was able to reduce the biofilm mass in a more gradual manner with increasing drug concentration. This observation suggests that mannitol could be used as a source of nutrients to stimulate the metabolic

activity of the bacteria leading to its dispersal from the biofilm and forming active planktonic bacteria.[11] Hence, the combination of mannitol with an antibiotic, ciprofloxacin in this specific case, could help reduce antibiotic-tolerance and restore antibiotic susceptibility of the low metabolic persister cells within biofilm towards antibiotic treatment preventing chronic infection.

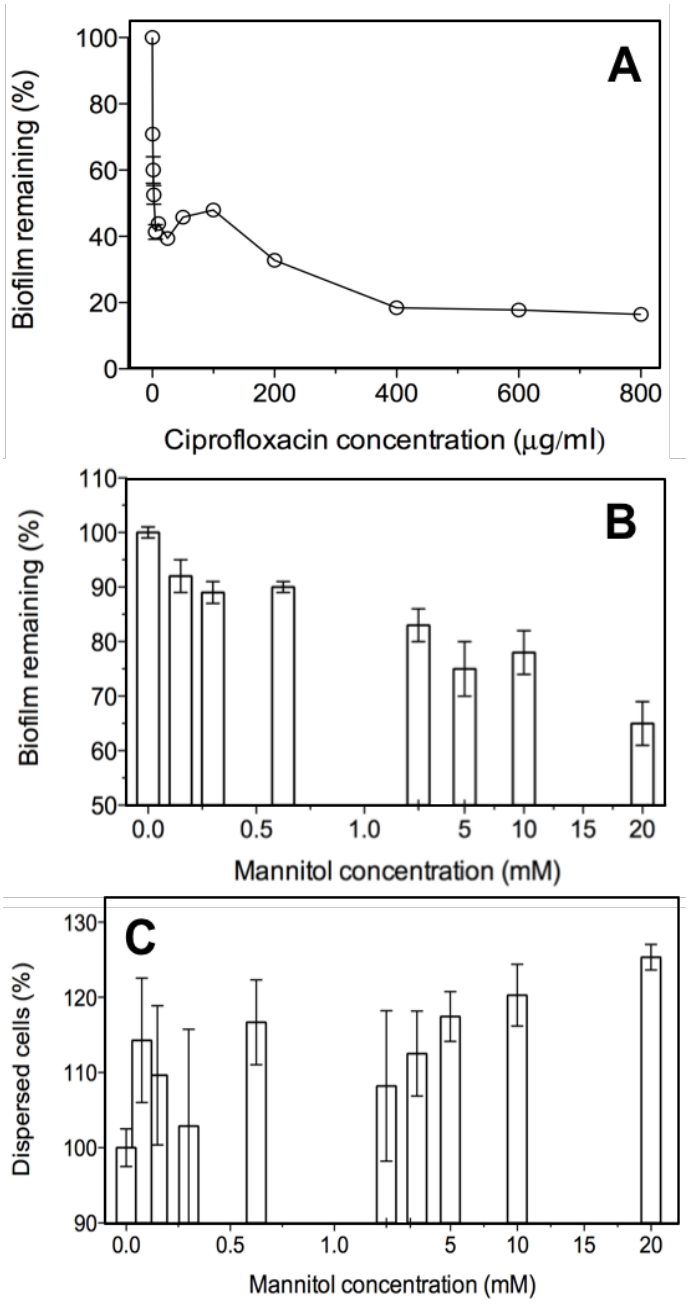

Figure 1: The effects of (A) ciprofloxacin and (B, C) mannitol at different concentrations on preformed biofilm colonies. Data represented as relative reduction or increase of

dispersed bacteria and biofilm colonies of *P. aeruginosa* PAO1 grown for 24 hours in the 96-microtitre plate wells supplemented with CAMHB.

3.2 Nebulised ciprofloxacin-mannitol formulation characterisation

The deposition of ciprofloxacin and mannitol on each stage of the ACI after nebulisation of the formulations is shown in Figure 2. Data are represented as the percentage of total emitted dose of drug that deposited in the mouthpiece, throat and each stage of the ACI over the emitted dose. The aerosol performance data are shown in Table 1. In general, the aerosol performance of the three different formulations were found to be within the range for respiratory delivery (1-5 μm)[27] and not significantly different (p>0.05) to each other. However, the ciprofloxacin only formulation yielded a significantly lower MMAD, with a higher emitted dose compared to the mannitol only and cipro-man combination formulations. This could be due to the higher density and viscosity of the aerosols generated from the mannitol only and cipro-man formulations that contain approximately 7-8 times more drug mass, resulting in an increased in aerodynamic size compared to the cipro only formulation.[28-29]

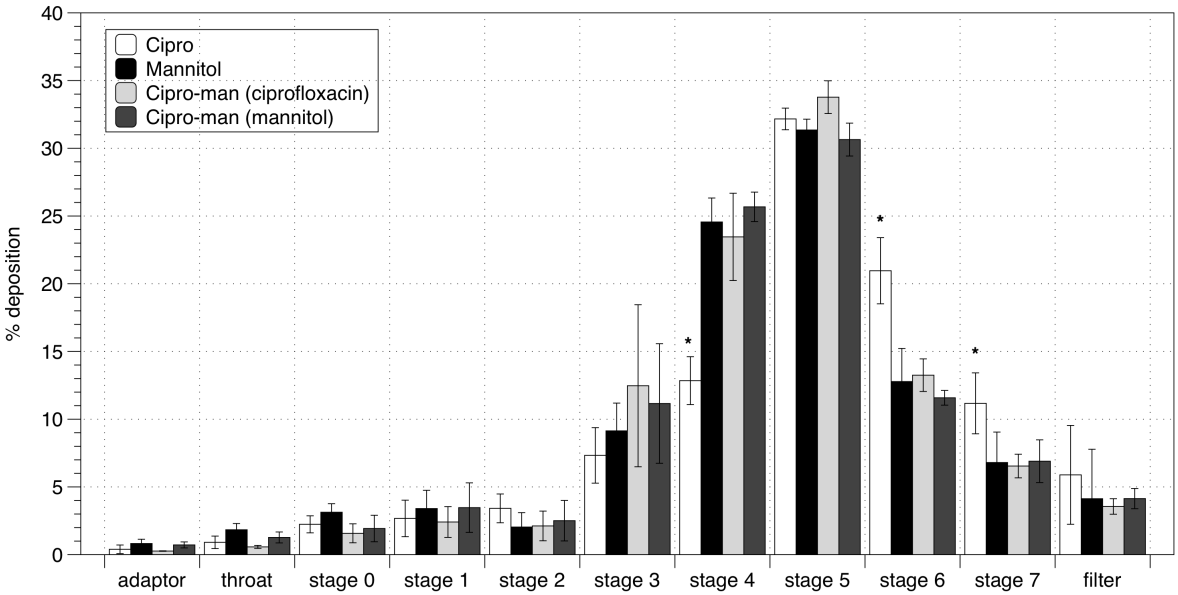

Figure 2: Anderson Cascade Impactor stage deposition data of nebulised ciprofloxacin

only (cipro), mannitol only (mannitol) and ciprofloxacin with mannitol (cipro-man) formulations (n=3, ± SD).

Table 1: The emitted dose (ED), fine particle fraction of ED (FPF), mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of nebulised formulations, as determined by the Anderson Cascade Impactor.

Formulation	**ED**	**FPF (%)**	**MMAD (μm)**	**GSD**
Cipro	3279.73 ± 484.42 μg	86.95 ± 0.82	1.83 ± 0.12*	2.43 ± 0.66
Mannitol	23.56 ± 7.02 mg	86.73 ± 1.38	2.61 ± 0.33	2.04 ± 0.09
Cipro-man - **ciprofloxacin** - **mannitol**	 2031.17 ± 254.83 μg 17.81 ± 1.96 mg	 90.93 ± 3.60 87.59 ± 6.16	 2.56 ± 0.16 2.70 ± 0.08	 2.05 ± 0.21 2.04 ± 0.08

*Significantly different to the other formulations, p-value < 0.05

3.3 Validation of stage deposition on conventional and modified plates

The custom-made ACI plate with up to 8 Snapwells was validated against the standard ACI plate on Stage 4 to capture aerosol particle size between 2.9 - 4.5 μm that would deposit in the bronchiolar regions of the lungs. In this region, Worlitzche *et al.* have shown that *P. aeruginosa* mainly grows in biofilms.[30] The amounts of ciprofloxacin and mannitol deposited on the modified plate and on each Snapwell, compared to the standard ACI plate, are shown in Figure 3. The amount of ciprofloxacin and mannitol collected from the standard unmodified ACI plate were found to be not statistically different, compared to the total drug collected on each of the Snapwells and the modified plate for the different formulations. Interestingly, the amount of ciprofloxacin

deposited was found to be equivalent in both formulations. This is attributed to the higher emitted dose coupled with the significantly lower percentage of ciprofloxacin deposition on Stage 4 of the ACI of the cipro only formulation, compared to the cipro-man formulation. This was also supported by evidences from a previous study that also demonstrated that the modified plate containing the Snapwells did not affect the deposition profiles of the formulations tested.[19] Results also showed that reproducible amounts of mannitol and ciprofloxacin could be deposited on each Snapwell. Therefore, deposition studies on the biofilm and epithelial cells that mimic the *in vivo* lung physiology can be confidently performed using the modified ACI since deposition profiles are equivalent to that of the standard ACI.

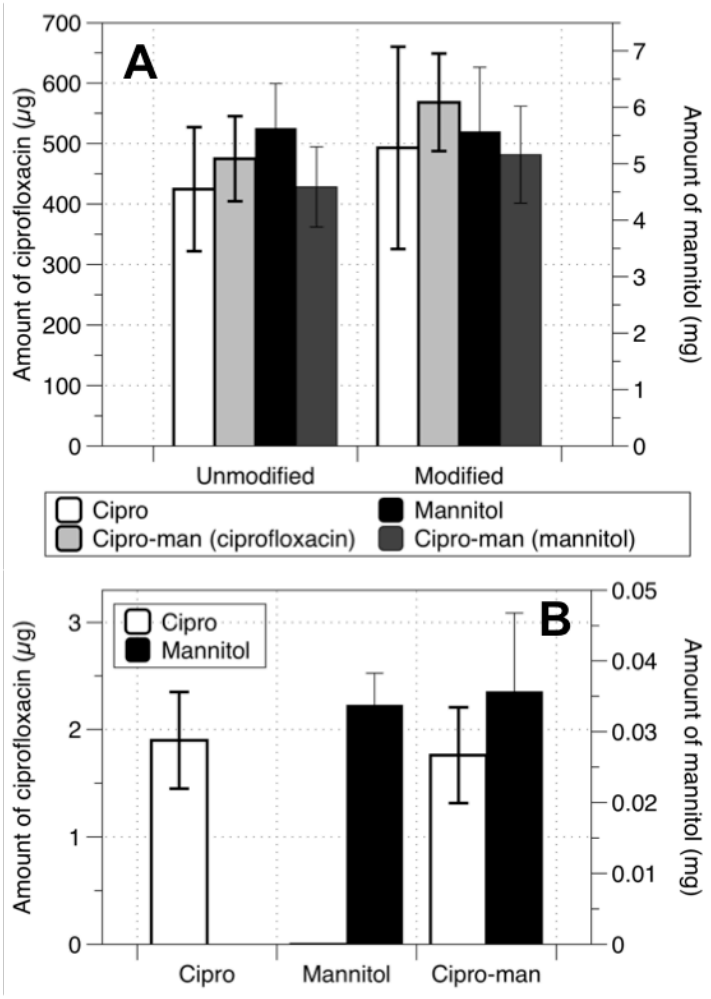

Figure 3: The amount of ciprofloxacin and mannitol deposited on: (A) standard unmodified and modified ACI plates and, (B) Snapwells from the 3 different formulations.

3.4 Transepithalial transport studies

The transepithelial transport profiles of ciprofloxacin over time of the nebulised ciprofloxacin only and cipro-man nebulised formulation, using the modified ACI, are shown in Figure 4A. Drug concentrations are expressed in terms of the percent total recovery throughout the experiments and data were plotted as mean cumulative percentage (± standard deviation) of drug transported across the Calu-3 cells over 4 hours. Equivalent amounts of ciprofloxacin were deposited onto the Calu-3 epithelial layer with an average of 2.10 ± 0.73 μg. It was shown that, approximately 96% of the drug was transported across the epithelial cells over the 4 hours long experiment, and there were no significant differences found between the ciprofloxacin transepithalial transport profiles of both the formulations (f_1= 4.84, f_2= 66.71). The rapid transport of nebulised ciprofloxacin formulations is attributed to the rapid diffusion of the drug into the epithelial lining fluid, followed by absorption across the epithelium bypassing the need for the rate-limiting dissolution process.[19] This was in good agreement with previous *in vivo* mice study where 99% of the instilled ciprofloxacin dose has been cleared from the lungs within the first hour.[31]

TEER measurements were performed after the experiment to measure the integrity of the cell monolayer and it showed a significant increase (760.61 ± 22.33 Ω.cm^2)

compared to control (686.13 ± 21.29 Ω.cm^2**)** (Figure 4B). The enhancement of tight junctions was also concurrent with results showed in previous studies[32-33], which resulted in a decrease in ciprofloxacin tranport after 1 hours of mannitol deposition. However, the mannitol constituent within the cipro-man nebulised formulation in this study did not affect the ciprofloxacin transport rate, possibly due to the differences in formulations and administration sequence.

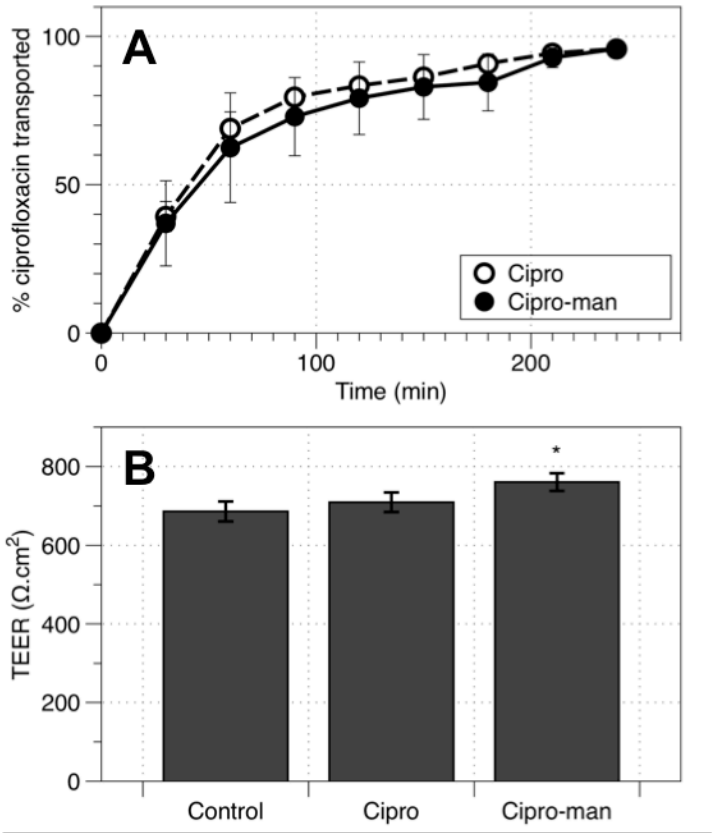

Figure 4: (A) Cumulative transport of ciprofloxacin from the cipro-man and ciprofloxacin only formulations normalised for initial drug deposition across the Calu-3 air interface cell model. (B) The effects of the formulations on transepithelial electrical resistance (TEER). (n=3, mean ± St. Dev., *Significantly different to control, p-value < 0.05)

3.5 Air interface biofilm assay

The different *in vitro* cell culture system provides a platform to study the individual processes and mechanisms responsible for drug efficacy, as well as interactions between the aerosol and biological components separately. Furthermore, the benefit of this novel set-up is the delivery and evaluation of inhalation antibiotics delivered at a clinical dose. It was found that the total amounts of ciprofloxacin deposited onto the air interface biofilm model from the different formulations was equivalent, with an average of 1.52 ± 0.38 µg. This was consistent with observations from drug deposition on cell-free Snapwell inserts, as well as on the air interface Calu-3 cells. It was hypothesised that mannitol could help enhance penetration of antibiotics through the biofilm. This hypothesis is based on the current understanding that mannitol will induce water flux, facilitating penetration of the antibiotics through the biofilm. In contrast to the transport study that demonstrated similar transport kinetic profiles, drug deposition and flux of drug on the air interface biofilm study (Figure 5) showed different behaviours for the two nebulised ciprofloxacin formulations (f_1= 22.89, f_2= 39.75). The ciprofloxacin only formulation had a higher diffusion rate across the biofilm compared to the cipro-man formulation. This was also in good agreement with a study by Tseng *et al.*[34] that demonstrated the relatively rapid penetration of ciprofloxacin across the bacterial biofilm. The nebulised cipro-man formulation had a slower antibiotic diffusion across the biofilm, with a significant proportion of ciprofloxacin remaining within the biofilm. This may be an advantage for the cipro-man formulation to allow higher concentrations of antibiotic to remain within the biofilm for enhanced antimicrobial activity.

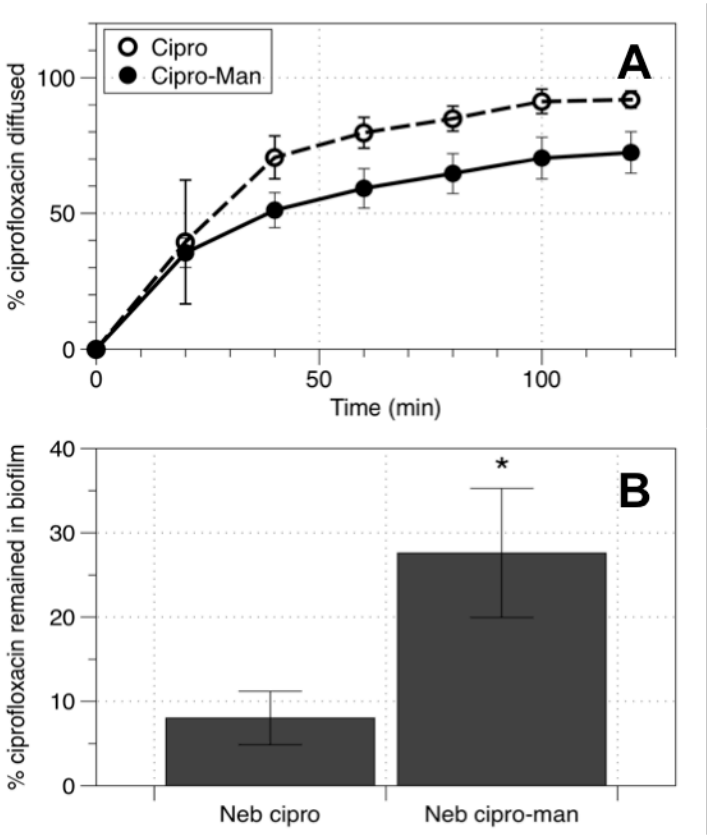

Figure 5: Percentage of ciprofloxacin from the cipro-man and ciprofloxacin only formulations normalised for initial drug deposition, (A) that has diffused across the biofilm air interface model and, (B) remained within the biofilm. (n=3, mean ± St. Dev., *Significantly different to nebulised ciprofloxacin, *p*-value < 0.05)

The presence of these drugs resistant and persister cells within biofilm are major health concern that lead to chronic colonisation, often associated with more frequent acute pulmonary exacerbations, accelerated lung function decline which require hospitalisation. Treatment efficacies on the biofilms were evaluated quantitatively by determining the colony forming unit (CFU) counts (Figure 6), and qualitatively using the scanning electron microscope (Figure 7). The CFU counts were performed on 2 occasions: bacteria remaining within the biofilm (Figure 6A) and bacteria dispersed from the biofilm (Figure 6B). It was found that nebulisation of ciprofloxacin was able to

significantly reduce the number of bacteria within the biofilm, the number of dispersed bacteria and total bacteria counts compared to untreated control, as well as nebulised mannitol. It was expected that ciprofloxacin would reduce bacteria load due to its antibacterial activity, but complete eradication of the biofilm was not achieved due to the resistant nature of biofilm. This is also in good agreement with previous studies demonstrating biofilm resistance towards ciprofloxacin.[35-36] Interestingly, the cipro-man formulation demonstrated an enhanced efficacy against the bacteria residing within the established biofilm matrix compared to the ciprofloxacin only formulation. However, the converse was observed with higher CFU counts of *P. aeruginosa* that was dispersed from the biofilm when cipro-man formulation was nebulised, compared to the antibiotic only formulation. Taking into account the total CFU counts, both dispersed and within the biofilm, cipro-man formulation had a 2-fold reduction in the number of bacteria compared to ciprofloxacin only, demonstrating superior antibacterial activity against *P. aeruginosa* biofilm. Parallel with these observations, nebulisation of mannitol alone showed a reduction in the number of bacteria within the biofilm with a concurrent increase in number of dispersed bacteria. In addition, the non-significant difference in the total CFU counts between the nebulised mannitol only compared to untreated control demonstrates that the mannitol itself does not have an antibacterial or growth promoting effects. Rather, the mannitol component may have assist the dispersion of the bacteria from the biofilm, making them more susceptible to antibiotics.

This is in good agreement with previous study which demonstrated that carbon sources such as mannitol, glucose and pyruvate could be used to stimulate the metabolic activity

of persister cells within biofilms of *Escherichia coli* and *Staphylococcus aureus* and restore their susceptibility to Gentamycin treatment.[11] This same concept could be applied in this study with mannitol activating the metabolic uptake of ciprofloxacin by *P. aeruginosa* within the biofilm matrix. It has been well established that the use of inhaled antibiotics is only effective against metabolically active and rapidly dividing bacteria. In biofilm, the presence of steep nutrient gradients can lead to nutrient starvation and subsequent decrease in overall metabolic activity, which further enhances bacteria tolerance to immune defences and antibiotic treatments.[37-38] Hence, this novel administration approach could help revert antibiotic-tolerance and sensitise the low metabolic persister cells towards antibiotic treatment, improving treatment of chronic infections in respitory diseases. In another study, it was found that a mix of simple sugars including mannose, fucose and galactose had a synergistic action with anti-pseudomonas antibiotics through inhibition of bacteria attachment to the epithelial layer, which limits lung damage and formation of unstable biofilm clusters that are susceptible to antibiotics.[39] Hence, mannitol could also serve to prevent formation of stable biofilm and persister cells.[12]

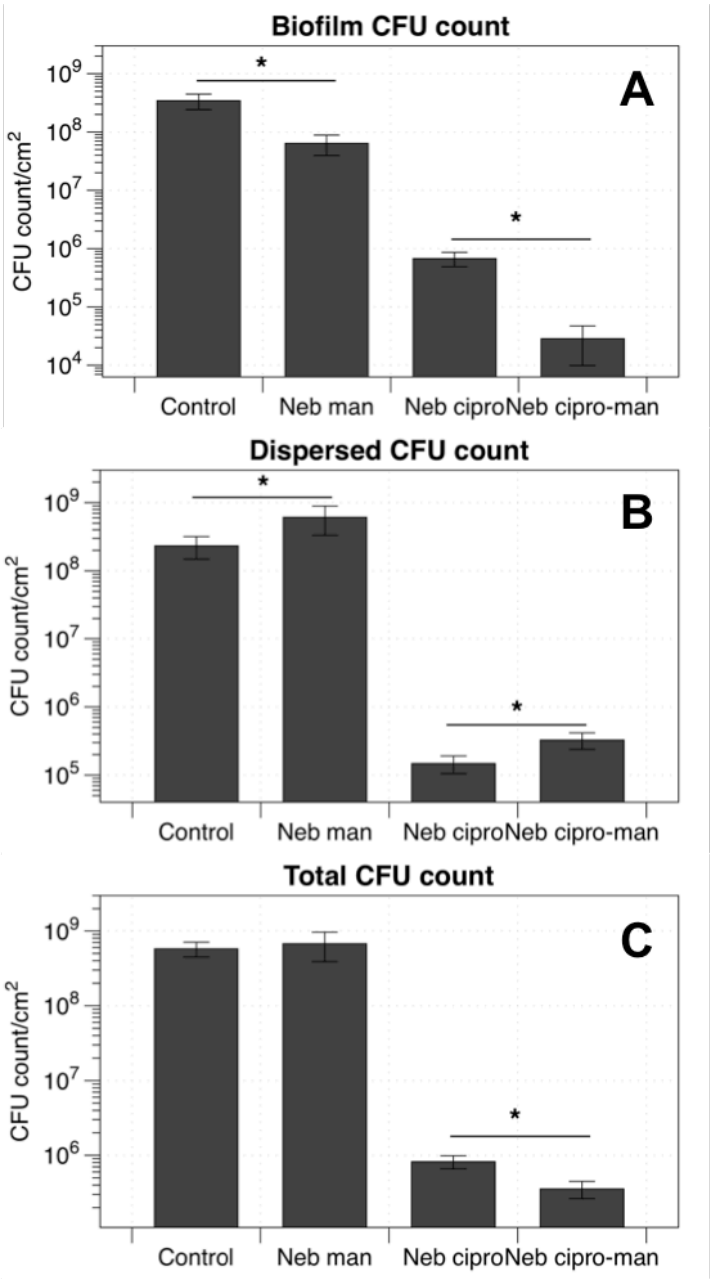

Figure 6: Colony forming units (CFU)/cm^2 of *P. aeruginosa* PAO1 after deposition of different nebulised formulation compared to control with respect to: (A) the bacteria remaining in the biofilm, (B) bacteria that has been dispersed from the biofilm and, (C) total bacteria from both within the biofilm and dispersed from the biofilm.

Scanning electron micrographs of untreated air interface biofilm compared to after the aerosol deposition of cipro-man are shown in Figure 7. The biofilm grown at the air

interface (Figure 7A) forms a pellicle film demonstrating organisation of bacteria with the matrix surface. This is in contrast to submerged biofilms, that seem to form columns and mushroom-like projections that are interconnected by water-filled channels.[40] Subsequent nebulisation of the cipro-man formulation through the modified ACI, demonstrated a non-homogenous deposition of the formulation aerosol on to the *P. aeruginosa* biofilm, where the number of bacteria at the site of aerosol deposition are considerably lower compared to areas with no drug deposition (Figure 7B). On the other hand, conventional approaches of drug solution and suspension on biofilm, will produce a homogenous effect across the entire biofilm surface that is significantly different to *in vivo* processes after inhalation therapy. The non-homogenous deposition pattern following treatment with inhaled formulation could have contributed to the difficulty in treating and eradicating biofilms that resides in the lungs. This further confirms that depositing nebulised aerosols onto the surfaces of air-interfaced *P. aeruginosa* biofilm using the modified ACI produces a representative model of the *in vivo* state for the evaluation of bacterial biofilm and drug interactions following inhalation, a model that could be easily translatable to the clinic.

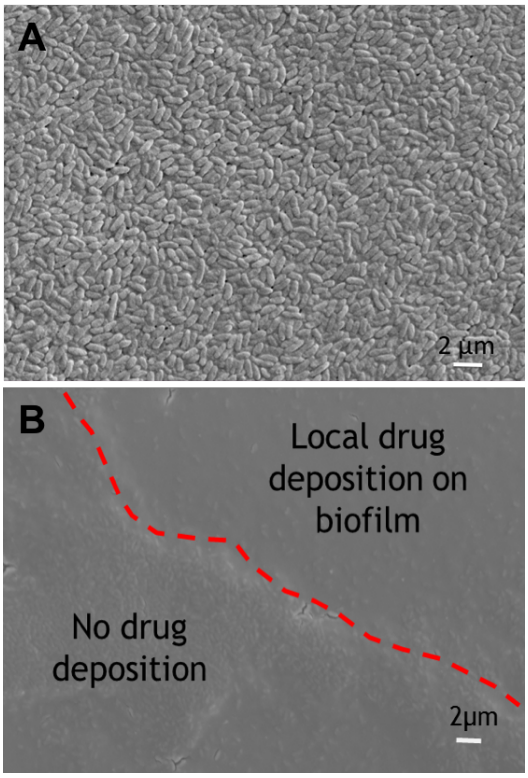

Figure 7: Scanning electron micrograph of the air interface biofilm: (A) Untreated control and, (B) after the deposition of cipro-man formulation.

4.0 Conclusions

In conclusion, the development of a representative air interface bacterial biofilm that simulates chronic infection in the airways could potentially be used as a future pre-clinical screening platform to correlate experimental results to clinical outcomes in a more predictive manner. The simulated *in vitro* model of respiratory infection in the bronchial region, consisting of a biofilm model integrated into a modified ACI, allows the simultaneous evaluation of antibiofilm efficacy and interaction between the bacteria biofilm and aerosols of novel inhaled therapies. Importantly, the co-administration of inhaled mannitol together with ciprofloxacin has been demonstrated to be a potential new therapeutic strategy to improve the efficacy of current antibiotic therapies. This

could be an important new approach in the way drugs could be administer to be more clinically efficacious in the treatment of chronic bacterial infections.

This system will be the foundation for more complex models that will focus on the development and validation of a co-culture airway epithelial cell model, with the biofilm grown on the apical surface of the epithelium.

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