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Inhaled dry powder liposomal azithromycin for treatment of chronic lower respiratory tract infection

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Abstract

A dry powder inhaled liposomal azithromycin formulation was developed for the treatment of chronic respiratory diseases such as cystic fibrosis and bronchiectasis. Key properties including liposome size, charge and encapsulation efficiency powder size, shape, glass transition temperature (Tg), water content and *in vitro* respiratory deposition were determined. Antimicrobial activity against cystic fibrosis (CF) respiratory pathogens was determined by MIC, MBC and biofilm assays. Cytotoxicity and cellular uptake studies were performed using A549 cells. The average liposome size was 105 nm, charge was 55 mV and encapsulation efficiency was 75%. The mean powder particle size d[v,50] of 4.54 µm and Mass Median Aerodynamic Diameter (MMAD) was 5.23 µm with a mean Tg of 76˚C and water content of 2.1%. These excellent physicochemical characteristics were maintained over one year. Liposomal loaded azithromycin demonstrated enhanced activity against *P. aeruginosa* clinical isolates grown in biofilm. The formulation was rapidly delivered into bacterial cells with >75% uptake in 1 hour. Rapid uptake into A549 cells via a cholesterol-dependent endocytosis pathway with no cytotoxic effects apparent. These data demonstrate that this formulation could offer benefits over current treatment regimens for people with chronic respiratory infection.

Keywords

Liposome; Azithromycin; Spray Drying; Dry Powder Inhaler; Respiratory Infection, Cystic Fibrosis

1. Introduction

People with chronic lung diseases such as cystic fibrosis and bronchiectasis often have chronic cough, sputum production and frequently develop acute infective pulmonary exacerbations. In addition to antibiotic treatment of acute exacerbations, patients are frequently prescribed long-term macrolides orally, such as erythromycin and azithromycin, as they have been

shown to reduce the frequency of pulmonary exacerbations [1, 2]. Long-term oral azithromycin treatment has been shown to be effective in reducing the frequency of pulmonary exacerbations in cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary disease (COPD) [3, 4]. This is thought to be due to the anti-inflammatory activity of this antibiotic where it suppresses the activation of NF-kB and the synthesis of proinflammatory cytokines IL-6 and IL-8 [4]. However, long-term oral azithromycin maintenance therapy may lead to the development of azithromycin resistance among respiratory pathogens, and other systemic adverse effects on hearing and liver function in addition to prolongation of QTc interval [5, 6]. A major advantage of inhaled therapy is the ability to achieve higher concentrations at the site of action compared to oral or intravenous doses. Pulmonary delivery of azithromycin may also minimize possible adverse effects associated with the oral treatment [7, 8]. Pulmonary delivery requires inhaled particles to be of optimum size in order to reach the alveolar epithelium, generally using particle size less than 5 µm to achieve this [9]. Currently approved inhaled antibiotics include tobramycin, aztreonam, levofloxacin and colistin which are indicated for the treatment of CF and reduce bacterial density in sputum, resulting in stabilisation of lung function. Clinical trials of these treatments in patients with CF and bronchiectasis have shown a decrease in Pseudomonas sputum bacterial load, but with mixed results in terms of improvement in exacerbation frequency or quality of life [10] [11] [12-14]. Macrolides are currently approved for use in inhalation and due to their dual anti-inflammatory and antibacterial action would represent a clinically relevant new treatment for chronic lung infection. With respect to drug delivery systems, cationic liposomes are known to be efficient in delivering drug molecules into bacterial cells and mammalian cells [15]. Therefore, formulating a liposomal azithromycin dry powder inhaler (DPI) formulation for local azithromycin pulmonary delivery could offer benefits over the current long-term oral azithromycin treatment regime in targeting drug to the site of action and minimizing possible systemic adverse effects, which would result in improved clinical outcomes for people with chronic respiratory infection. Preliminary experiments to optimize formulation were performed with clarithromycin before moving to azithromycin for further fine-tuning and characterisation. In this paper the development, characterization, stability and in vitro lung powder deposition testing of this spray dried powder formulation comprising liposomal azithromycin are described including antimicrobial activity, bacterial cellular uptake, cytotoxicity and cellular uptake.

2. Materials and methods

2.1. Materials

Azithromycin was purchased from Dexa-Medica (Palembang, Indonesia). Clarithromycin lactobionate powder for infusion (PhEur grade) was purchased from AAH (Bowmed Ibisqus, UK). SPC (Lipoid SPC, 100%) was purchased from Lipoid (Steinhausen, Switzerland). Ammonium Chloride, D (+) - lactose monohydrate (PhEur grade), L-leucine (PhEur grade), TPGS, dimethyl dioctadecyl ammonium bromide (DDAB), glycine, potassium phosphate monobasic (Reagent PlusTM, ≥99%), potassium acid phthalate, methanol (HPLC grade, ≥99.9%), acetonitrile (HPLC grade, ≥99.9%), sodium chloride (PhEur grade), and the 0.20 µm membrane filter (Whatman®) were purchased from Sigma–Aldrich. Citric acid monohydrate (PhEur grade) and sodium hydroxide pellets were obtained from Merck. Chloroform (99.2%),

tris (hydroxylmethyl) methylamine Aanlar (99%), sodium hydrogen carbonate (Ph. Eur grade), sodium acetate trihydrate AnalaR NORMAPUR® (Ph. Eur. grade), Sodium carbonate anhydrous (ACS grade), calcium chloride dihydrate (Ph. Eur grade), disodium hydrogen phosphate and phosophoric acid (85%) were purchased from VWR (Lutterworth, UK). α,α-Trehalose dehydrate (high purity, low endotoxin, FERRO Pfanstiehl Mayfield Heights, USA). Dialysis membrane (Spectra/Por 7: 10 kDa, 1 mL MWCO 10,000 regenerated cellulose Spectrum Laboratories, California, USA). Aluminium standard lids and aluminium hermetic pans/lids TA instruments – Waters LLC, New Castle, USA).

P. aeruginosa and *S. aureus* isolates used in this study were selected from the Halo Research Group repository and stored at −80 °C until required. These isolates had been previously cultured from airway samples from people with CF. *S. aureus* ATCC 29213 type strain was purchased from Kwik Stiks™ (St. Cloud, USA).

A549 human lung epithelial carcinoma cell lines were authenticated by short tandem repeat (STR) profiling carried out by the suppliers and routine testing in our laboratory showing that these cells were Mycoplasma-free.

Chlorpromazine hydrochloride (≥ 98%), genistein (≥ 98%), methyl-β-cyclodextrin (BioReagent), 4–well chamber slide (Nunc® Lab-Tek® II Chamber Slide™ system 4 wells, glass slide, 1.7 cm^2/well, sterile), and mounting medium were purchased from Sigma–Aldrich (UK). Minimum Essential Medium, Opti-MEM® Reduced Serum Medium, Roswell Park Memorial Institute (RPMI) 1640 medium, Fetal Bovine Serum and Gibco® and trypsin enzyme were purchased from Life Technologies (UK). CellTiter 96® solution cell proliferation assay was purchased from promega (USA). All aqueous solutions were prepared with freshly de-ionized water source (Elga, Purelab Maxima, United States).

2.2. Formulation preparation

For the majority of formulations, the mole ratio of liposome composition was SPC: DDAB: TPGS is 160:40:1 as described previously and prepared using the commonly-employed thin-film hydration method [16, 17]. The antibiotic concentration was chosen based on a previous study [18]. Lipids and drug were dissolved in chloroform:methanol (9:1, v/v) followed by solvent removal using a rotary evaporator (Rotavapor, R210 – BUCHI). The thin film was hydrated with 10 mM Tris-HCl buffer pH 7.4 containing the lyoprotectant trehalose dihydrate with or without the anti-adherent L-leucine according to the formulation requirements (compositions in Table S1) at 60°C with stirring at 500 RPM followed by sonication (10 sec ON, 5 sec Off, 40% amplitude) for 6 minutes (Fisher Scientific; USA). The drug loaded liposomal dispersions were spray dried (Mini-Spray-dryer, Büchi 190; Flawil, Switzerland) with inlet temperature 120°C, outlet temperature 85°C, pump setting 3.0 mL/min, nitrogen flow 600–650 Nl/h, aspirator power of 100%, and a 0.5-mm nozzle [19].

2.3. Physicochemical characterisation

Size and zeta potential were measured before and after spray-drying using a Malvern ZetaSizer (Malvern instruments, UK) in triplicate at 25°C. The spray dried liposomal formulations were reconstituted with de-ionized water before further dilution and analysis in 10 mM Tris-HCl buffer of pH 7.4. Values are reported as the Z-average diameter (d. nm), polydispersity (PI) ± sd and ZP (mV) ± sd respectively.

Drug quantification was determined using reversed phase HPLC analysis (1200 Series, Agilent Technologies G1322A Degasser, Agilent Technologies G1311A Quaternary HPLC pump, Agilent Technologies G1329A Autosampler, Agilent Technologies G1315A Absorbance Detector, Agilent Technologies G1316A Temperature Column Controller; Waldbronn, Germany) in conjunction with a Luna C-18 column (100A°, 150 X 4.6 mm 5 mm; Phenomenex, Cheshire, UK) at 40 or 50°C for azithromycin or clarithromycin, respectively. All samples were quantified at a wavelength of 210 nm and the injection volume was 50.0 µL. Clarithromycin samples were analysed in isocratic mode using a mobile phase of (60:40 v/v) methanol: 0.067 M potassium phosphate buffer of pH 3.5 and at a flow rate of 1.0 mL/min [20]. Azithromycin samples were analysed in an isocratic mode using a mobile phase of (80:20 v/v) methanol: 0.05 M potassium dihydrogen phosphate buffer of pH 7.5 and at a flow rate of 1.3 mL/min [21]. Each sample was assayed in triplicate over a run time of 8.0 min. Both azithromycin and clarithromycin HPLC calibration curves were made and the HPLC response was found to be liner correlated with the active pharmaceutical ingredient (API) concentration over the working concentrations range from 4.0 to 900.0 µg/mL.

2.4. Encapsulation efficiency (EE%) was determined using the dialysis membrane method

1.0 mL of the reconstituted liposomal drug formulation was loaded in the dialysis membrane and immersed in 40.0 mL of pre-chilled 10 mM Tris-HCl buffer (pH 7.4). The low temperature of 8 – 10˚C was chosen to ensure the stability of the liposome lipid membrane structure during the test [14]. After 30 min, the amount of free drug in the release medium was determined using HPLC in triplicate and compared to total amount of drug placed in the dialysis membrane. EE% of the liposomes was determined using the following equation:

$$EE\% = [(At - Af) / At] \times 100\% \qquad \text{Equation 1}$$

Where At is the total amount of API loaded in the dialysis bag and Af is the amount of free (unencapsulated) API in the medium after 30 minutes.

2.5. *in vitro* drug release from liposomes

The study of API release profiles from the spray dried liposomes was performed using the dialysis method to give an indication of rate of drug release from the liposomes when they are administered *in vivo* and to aid comparison with previously reported data in the literature [22, 23]. As these formulations are intended to be administered via the pulmonary route, their release profiles were evaluated in the simulated lung fluid (SLF, pH 7.4), although the

lung of CF patient might well contain different fluids and mucus [24]. SLF was prepared and filtered through a 0.2 µm membrane filter prior to be used as a release medium [25]. The dialysis membrane with pore size of 2.5 nm was used as previously described (section 2.4). 0.9 mL portion of reconstituted liposomes were sealed into the dialysis tube and placed inside a 100 mL laboratory (DURAN®) bottle containing 40.0 mL of fresh pre-warmed (37°C) SLF in a shaking incubator (Incubator, Gallenkamp, UK) at 37°C ± 0.5°C and 40 rpm over 24 hours (*n*=3). Samples (0.4 mL) were drawn periodically and replaced with fresh pre-warmed medium to maintain the release volume constant throughout. The similarity factor (f2) was used to compare the release profiles of the two formulations and was calculated by using Moore and Flanner equation [26].

2.6. Powder particle size analysis

The distribution of particle size for the spray-dried powder was measured by laser light diffraction analyzer using the R2 lens which allows measurements in the range of 0.1 µm to 87.5 µm (HELOS/BR; Sympatec, Clausthal-Zellerfeld, Germany). Approximately 3.0 mg of powder was suspended in chloroform in a 50-ml glass cuvette and stirred with a magnetic bar at 1000 rpm. A short period of sonication (90 s) at a power of 30 W (Cuvette, 8.5 mm diameter ultrasound tip; Sympatec, Clausthal-Zellerfeld, Germany) was used before sizing to ensure de-aggregation of particles in chloroform [27]. Based on a volume (v) distribution; results were reported as particle diameter at D_{10}, D_{50}, and D_{90} values [28]. The D_{50} (central particle diameter value) is defined as the diameter where 50% of the population is below this diameter. To express particle size distribution width, the span value is determined by the following equation:

$$\text{Span} = (d[v, {}_{90}] - d[v, {}_{10}])/d[v, {}_{50}]) \qquad \text{Equation 2}$$

A low span value is preferred and indicates a good particle size distribution [29]. The values were reported as the mean ± sd of five measurements.

2.7. Powder characterisation

Powder size was measured by laser light diffraction analyzer using HELOS/BR; Sympatec, Clausthal-Zellerfeld, Germany). Powder was suspended in chloroform [19]. D_{10}, D_{50}, D_{90} and span values are reported as mean ± sd of five measurements.

Water content was determined using a TGA (Q500; TA instruments, New Castle, PA, USA) by heating to 110°C at 10°C/minute under nitrogen. Values are reported as onset values determined from the reversing heat flow signal and are mean values of three replicate measurements. The glass transition (Tg) was determined using hermetic pans and modulated differential scanning calorimetry (MTDSC) (Q100; Differential Scanning Calorimeter, TA Instruments, New Castle, PA, USA) by heating at 3°C/min with a modulation of ± 1.0°C over 40 seconds under a nitrogen atmosphere (50 mL/min) [19].

The surface morphology was observed by SEM microscope (TM3030, Hitachi; Tokyo, Japan). Samples were gently spread on the sample holder with double-sided adhesive carbon pad and examined under high vacuum.

A stability study was performed over 12 months as per the international council for harmonisation (ICH) stability guidelines [30]. In this manner, two AZI liposomal dry products (containing 17% and 18% trehalose with 0.5% L-leucine) were stored at 20˚C for one year in the intended final packaging at either 20 °C / ambient humidity (controlled temperature room) or at 40 °C and ambient humidity (Genlab MINI/50/DIG incubator, Cheshire, UK). Liposome size, PI, ZP, EE%), drug content, water content, Tg, powder particle size and morphology were measured on freshly prepared samples and at selected time.

Next generation impactor (NGI) testing of the powders was conducted using an Aerolizer device. Results from a previous breathing study performed across T-326 inhaler device for 96 CF patients of varying age and disease severity showed that children aged 6–10 years-old (n=33) had mean peak flow of 68.7 ± 13.1 L min-1 and inhaled volumes of 1.2 ± 0.39 L while young adults (n=24) and adults (n=39) reached 79.3 ± 15.0 L min-1 and 81.1 ± 14.4 L min-1 respectively, with inhaled volumes of 1.63 ± 0.60 L and 2.06 ± 0.68 L (20). On this basis a preliminary study was conducted to determine which DPI device was most suitable (Table S2 and S3)[31]. Since the Aerolizer inhaler device exhibits very low resistance (Table 3.3) and its flow rate at 4.0 KPa pressure drop is 125.2 L min^{-1} which is greater than the upper limit of NGI operation flow rate, NGI tests were performed using the Aerolizer inhaler device at 100 L min^{-1} flow rate when the pressure drop is 3.2 KPa as USP guidelines [32, 33]. Three NGI runs were performed for each formulation to assess its pulmonary deposition pattern using inhalation parameters similar to the inhalation profile in CF patients [34]. The cut-off diameters of NGI stages with their related respiratory levels at 100 L min^{-1} flow rate are reported in Table S3 [33, 35] The FPF cut-off using these settings was defined as from stage 2 to filter (<3.42 μm). Four capsules (capsule size 3, 4.5 mm x 15.0 mm, each containing 25 mg of a formulation) were used per run. The actuation time was 0.9 second at a flow rate of 100.0 ± 1.0 L min^{-1}. Two inhalations from each capsule were sampled into the NGI with a total volume of 3.0 L of air drawn through the Aerolizer device connected to the NGI using the standard United States Pharmacopeia (USP)/European Pharmacopoeia (Ph.Eur) induction port which is roughly equivalent to two inhalation volumes of CF patients [36]. After each NGI run, the impactor was unlocked and a solvent mixture was used to separately wash and collect the powder from each stage of NGI, then filtered (0.22 µm membrane filter) and analysed using the HPLC method as described previously for quantification (n=3). Copley inhaler testing data analysis software version 2.0 (Copley Scientific, Nottingham, UK) was used to analyze NGI data. For each DPI liposomal formulation, Mass Median Aerodynamic Diameter (MMAD) and Fine Particle Fraction or Dose (FPF or FPD) values were determined.

2.8. Microbiological analysis

2.8.1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of free azithromycin were determined for *S. aureus* ATCC 29213 and CF isolates (*P. aeruginosa and S. aureus*) using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method [37]. The MIC and MBC for the optimised liposomal azithromycin formulation after reconstitution in Mueller Hinton Broth to the original volume was determined at a range of azithromycin doubling dilutions (256 – 0.5 µg/mL).

2.8.2. Biofilm studies

Bacterial cultures, grown in MHB overnight, were adjusted to an optical density (OD) of 0.15-0.18 which equated to approximately 1 x 10^8 cfu/mL. This culture was further diluted 1:100 using MHB and 160 µL added to the wells of a microtiter tray. After incubation for 24 hours at 37˚C, 80 rpm, the media was removed and the wells were gently washed twice with 250 µL of sterile PBS followed by addition of 165 µL of liposomal azithromycin reconstituted in Mueller Hinton Broth, or free azithromycin solution at different final concentrations: 256, 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 µg/mL. Following incubation for 24 h at 37˚C, 80 rpm, the media was removed, the trays were washed and biofilm formation quantified using the crystal violet assay method [38, 39].

At the same range of azithromycin concentrations, the spray dried cationic liposomal azithromycin or azithromycin solution were added to the diluted overnight culture broth in a 96-well tray and incubated for 24 hours (37˚C/80 rpm) to determine the ability of the formulation to prevent biofilm formation.

2.8.3. Determination of bacterial uptake using flow cytometry (FACS)

Formulations containing carboxyfluorescein dye (CFD) were tested as dry powders and reconstituted dispersions at concentrations corresponding to azithromycin dispersions of 2 and 256 µg/mL. These were 0.058 µg/mL carboxyfluorescein; 10.8 µg/mL lipid and 7.5 µg/mL carboxyfluorescein and 1400 µg/mL lipid. Treatments were added to 5 mL of bacterial suspensions in either log or stationary phase before incubation for one hour at 37°C/100 rpm. Samples were centrifuged and washed twice using the Eppendorf Centrifuge 5430R (Hamburg, Germany) for 5 min at 4°C and 5752 RCF (7000 rpm) and PBS, then re-suspended in 5 mL of PBS followed by ten-fold dilution and transferred to the BD flow tube. Fluorescence was measured using the BD FACSCalibur flow cytometer (BD Biosciences, USA) at 488 nm excitation wavelength. The results were calculated from histograms/dot plot based on the measurement of visible and fluorescent light emission of carboxyfluorescein in a population of 5 x 10^4 cells (n=3 per sample). Bacterial uptake value was calculated by comparison to the untreated control gated to 1% (Cyflogic software, CyFlo Ltd., Finland).

2.9. Cell culture

2.9.1. Imaging cellular uptake

Uptake in A549 cells was determined after seeding at of 2 x 10^5 cells/well onto a 4–well chamber slide in 1.0 mL of complete growth medium for 24 hours at 37°C. After replacement with fresh medium, cells were treated with 0.50 µg/mL CFD solution or liposomal CFD with lipid concentration of 9.4 µg/mL and liposomal CFD at ten-time higher concentration. After further incubation for one hour, treatments were washed three times with PBS then fixed by immersion in paraformaldehyde and mounting onto a cover slide. Cells were observed using an EVOS FL Cell Imaging System (Electron Microscopy Sciences, Hatfield, USA) with an excitation wavelength 488 nm, and images were captured and saved at 40X magnification.

2.9.2. Determination of cellular uptake using flow cytometry

A549 cells were seeded at a density of 25 x 10^3 cell/well onto 96-well in 200 µL of the complete growth RPMI medium. After twenty-four hours, cells were conditioned for one hour by replacing the medium with 100 µL of Opti-MEM reduced medium. Spray dried liposomes were reconstituted with Opti-MEM reduced medium prior to testing. After one hour, the reconstituted spray dried blank liposomes and spray dried liposomal, fresh liquid liposomal CFD and CFD solution were separately added on the cells. The tested dye/lipid concentrations were 0.12/23.4 and 8.3/1709 µg/mL. Cells were further incubated at 37°C for one hour then washed three times with PBS. The washed cells were detached from the plate by adding 1x trypsin solution in PBS. Cells were re-suspended in 400 µL of PBS and gently mixed then cells were analyzed for cell-incorporated CFD fluorescence using the flow cytometer BD FACSCalibur (BD Biosciences, USA) at 488 nm excitation wavelength. The flow cytometry results were calculated from histograms based on the measurements (n=3) of visible and fluorescent light emission of CFD in a population of 10,000 cells gated to 1% of the untreated control group using Cyflogic software.

2.9.3. Determination of spray dried liposomal azithromycin cytotoxicity

The cytotoxicity of the spray dried liposomal formulation containing 0.74% (w/v) azithromycin and 17% (w/v) trehalose with 0.5% (w/v) L-leucine was determined with A549 human lung epithelial cells [40] [40-47]. The cytotoxicity was measured using this colorimetric assay with MTS reagent which is bioreduced by cells into a coloured formazan product thus it directly proportional to the number of living cells in culture.

A549 cells were seeded at a density of ~ 2.5 × 10^4 cells/well onto 96-well tissue culture plates. After 24 hours, cells were conditioned for 2 hours in 100 µL of Opti-MEM reduced serum medium and then the reconstituted spray dried liposomal azithromycin formulation was tested at final azithromycin/lipid concentrations of 36/200, 128/700, 365/2000 and 493/2700 µg/mL on seeded A549 cells for 6 hours followed by overnight incubation in the complete growth culture medium. Then the MTS reagent was applied and the optical density was quantified at 490 nm following a shaking step for 1.0 minute (EL808 Microplate Reader, Bio-Tek Instruments, USA). The measured absorbance values are expressed as a percentage of the negative control (untreated cell - 100% viable). values are reported as means (n=3). The cell viability below 75% was defined as the cytotoxicity threshold [48]. In order to assure a

higher safety margin of the applied doses, the cell viability below 80% was considered as the cytotoxicity limit in this study.

In the same manner, the cytotoxicity of endocytosis inhibitors; including chlorpromazine, genistein and methyl-β-cyclodextrin in A549 cells were determined prior to use these endocytosis inhibitors to identify the entry pathway of this liposomal formulation into A549 cell line.

2.10. Statistical analysis

Statistical analysis was performed using student's t-test to compare two groups (Prism 5.0, GraphPad Software, CA). One-way analysis of variance (ANOVA) using Student–Newman–Keuls Post Hoc test was used for comparison of multiple groups. Significance assigned to p values < 0.05.

3. Results and discussion

3.1. Formulation optimisation

All trehalose concentrations exhibited acceptable liposomal clarithromycin properties prior to spray drying (Table 1). The size average of reconstituted liposomes after spray drying was intended to be in the range of less than 150 nm to ensure the rapid and efficient liposomes interaction with bacterial cells with the PI of less than 0.4 indicating an acceptable range of liposome size uniformity in dispersion [49, 50]. The inclusion of DDAB resulted in a surface charge of > +50 mV, which was previously found to be desirable for mucosal delivery which has been attributed to the ability to maintain repulsive forces among liposomes in a dispersion preventing aggregation and/or fusion [51]. EE% greater than 50% for inhaled liposomal antibiotic is desirable, as the unencapsulated antibiotic can also be useful in the lung [52, 53]. Increasing trehalose concentrations resulted in improvement in liposome properties after drying as evidenced by similarity in size pre and post-drying when the trehalose content increased, and when L-leucine was also included (Table 1), which was attributed to trehalose's lyoprotective properties, with the 17% w/v formulation giving the highest Tg [19, 54]. Trehalose forms an amorphous material with a high Tg [27]. Liposomes plasticize the Tg, with higher liposome concentration lowering the Tg more (Table1).; if the dryer outlet temperature surpasses *Ts* (the sticky point ~ Tg+ 10–20°C) then particle cohesion and adhesion to dryer walls increase [55] This results in unacceptable instability of the formulation as seen in the increase in size and PDI of the liposomes formulated at lower trehalose concentrations (Table 1). The liposome size increase during spray drying was ameliorated by the inclusion of 0.5% (w/v) L-leucine (Table 1), as we have previously described [19]. The size of liposomes is a key characteristic for cell uptake, with studies suggesting the size of <150 nm is crucial for rapid uptake by both bacterial and mammalian cells [56, 57] the size of 105 nm demonstrated is well below that limit with good PI. DDAB was responsible for the positive surface charge [58]. The powder particles demonstrated desirable sub-5 μm d50 of 4.54 μm and acceptable span (Table 1)[9] . These formulation concentrations were selected to take forward to manufacture comparable azithromycin formulations.

Replacing clarithromycin with azithromycin resulted in powders with similar physicochemical characteristics (Table 1). Because 0.74% (w/v) azithromycin loaded liposomal formulation containing 17% (w/v) trehalose with 0.5% L-leucine exhibited the best characteristics, it was selected for further antimicrobial and cell biology testing.

Table 1 Properties of liposomal formulations before and after spray drying at 120˚C. Each value represents the mean ± SD and *n*=3.

clarithromycin % (w/v)	0.74	0.74	0.74	-	0.74	-	-
azithromycin % (w/v)	-	-	-	-	-	0.74	0.74
L-Leucine % (w/v)	-	-	-	0.5	0.5	0.5	0.5
Trehalose % (w/v)	10	13	17	17	17	17	18
Before spray drying							
Size (nm)	98.2 ± 0.8■	102.6 ± 1.6	106.1 ± 1.6	103.2 ± 2.2	99.7 ± 0.8	104.3 ± 1.6	103.2 ± 0.1
PI	0.20 ± 0.01	0.21 ± 0.01	0.21 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.23 ± 0.01	0.21 ± 0.01
Zeta potential (mV)	72.8 ± 2.2	72.3 ± 2.8	74.7 ± 1.6	71.4 ± 2.6	73.0 ± 2.3	53.8 ± 5.6	59.5 ± 2.0
After reconstitution							
Size (nm)	352.2 ± 10.4*,■	252.2 ± 11.6*,■	159.0 ± 2.8*,■	103.6 ± 2.9	106.5 ± 0.1*	105.0 ± 2.4	104.8 ± 0.6

PI	0.62 ± 0.04*	0.67 ± 0.13*	0.42 ± 0.02*,▪	0.28 ± 0.02	0.23 ± 0.01*	0.19 ± 0.01*	0.20 ± 0.01*
Zeta potential (mV)	78.6 ± 3.0	79.0 ± 2.5*	77.7 ± 3.8	73.0 ± 1.7	75.1 ± 2.9	55.8 ± 3.3#	63.5 ± 2.6*,^
EE (%)	74.7 ± 2.6▪	80.0 ± 0.1	83.4 ± 0.8	-	76.9 ± 0.3	74.9 ± 0.3	87.3 ± 0.4
Powder properties							
Water content (%)	3.0 ± 0.1	3.4 ± 0.2▪	3.0 ± 0.1	3.3 ± 0.1	3.0 ± 0.1	2.1 ± 0.1	2.6 ± 0.1^
Tg (˚C)	43.4 ± 4.9	48.0 ± 3.0	63.7 ± 1.5▪	64.2 ± 4.0	64.4 ± 0.6	76.3 ± 4.0	68.3 ± 2.7^
PPS d[v, 50] (µm) (*n*=5)	4.39 ± 0.04	4.28± 0.01▪	4.43 ± 0.03	4.30 ± 0.01	4.08 ± 0.01#	4.54 ± 0.07#	4.73 ± 0.04^
Span units (*n*=5)	1.54 ± 0.09	1.55 ± 0.01	1.59 ± 0.01	1.53 ± 0.01	1.55 ± 0.01	1.39 ± 0.02	1.51 ± 0.01^

*$P < 0.05$ (paired t-test) comparison of liposomes prior to spray-drying and after reconstitution. ▪$P < 0.05$ (One Way ANOVA analysis with Student Newman–Keuls all pairwise multiple comparisons) comparison of all formulations without L-leucine.

#$P < 0.05$ (One Way ANOVA analysis with Student Newman–Keuls all pairwise multiple comparisons) comparison of all L-leucine formulations with 17% w/v trehalose. ^$P < 0.05$ (unpaired t-test) comparison of azithromycin formulations.

SEM images (Figure 1 and S1) showed that azithromycin and clarithromycin formulations exhibit the same typical morphological features of spray dried trehalose powders with low rugosity, smooth spherical structures and qualitatively confirmed that powder particles were within the inhalable range [59] with no evidence of crystallisation [60], together with the DSC data in Table 1 indicates that the trehalose remained in the amorphous form, optimal for protection of the paylpoad. Although the SEM images show a range of particle sizes, quantitative powder sizing using the Malvern powder sizer that uses a more representative sample size and repeat measurements, confirmed that the powder size and span were acceptable as reported in Table 1.

Figure 1 near here

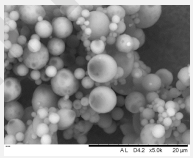
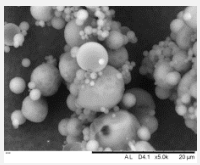
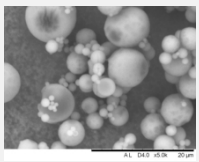
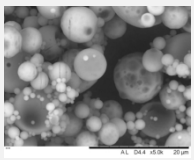
The cumulative drug release from both reconstituted azithromycin liposomes and clarithromycin liposomes were 65% and 52%, respectively over 4 hours, and increased to 78% and 58%, respectively after 24 hours (Figure 2) compared with 82% by the control. Differences in release profiles (f2=46) were attributed to difference in hydrophilicity of the drugs, with hydrophobic azithromycin located within the liposome bilayers enabling faster diffusion than the hydrophilic clarithromycin located in the aqueous core, which has to become solubilised in the lipid bilayers before diffusion. The best correlation of release kinetics was found with the Korsmeyer-Peppas model (Table S4). These results indicate excellent drug retention over time *in vitro* release setting indicating a high liposome stability (37˚C/40 rpm in SLF over 24 hours).

Figure 2 near here

The preliminary liposomes stability data confirmed that both azithromycin powder formulations showed excellent stability after storage at 20˚C for one year (Table 2).

These products are very stable in terms of their liposome properties with an accepted level of variation in water content (17% trehalose product, its container was opened several times over the 12 months for sampling) which was associated with a slight Tg decrease but still within the intended Tg range, > 50˚C (Table 2). The powder particle size distribution values of both products after one year were within the same range (Table 2) and the SEM images likewise did not reveal evidence of crystallisation [60]. Since both formulations exhibited acceptable stability, the formulation with the lower trehalose concentration contains a higher drug loading (3.4% w/w dry powder), so this product was taken forward for further evaluation.

Table 2 Stability of liposomal azithromycin formulations. Each value represents mean ± SD and *n*=3.

Liposome properties	Freshly spray dried		After 12 months at 20°C	
	17% Trehalose	**18% Trehalose**	**17% Trehalose**	**18% Trehalose**
Size (nm)	105.1 ± 2.5	104.8 ± 0.6	104.5 ± 1.0	104.1 ± 0.8
PI	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
Zeta potential (mV)	55.9 ± 3.2	63.5 ± 2.6	68.4 ± 2.6	68.1 ± 2.6
EE (%)	74.9 ± 0.3	74.5 ± 0.1	81.7 ± 0.1	79.3 ± 0.3
Powder properties				
Water content %	2.1 ± 0.1	2.6 ± 0.1	3.8 ± 0.1	3.0 ± 0.1
Tg °C	76.3 ± 4.0	68.3 ± 2.6	57.2 ± 3.2	68.8 ± 2.4
PPS d[v, 50] µm (*n*=5)	4.54 ± 0.07	4.73 ± 0.04	4.31 ± 0.01	4.32 ± 0.01
Span unit (n=5)	1.39 ± 0.02	1.52 ± 0.01	1.53 ± 0.01	1.60 ± 0.01
Representative SEM images (at X5000)				

3.2. *in vitro* lung deposition

The mean emitted dose % (*n*=12 doses) from the Aerolizer at 100 L min^{-1} inhalation flow were within the accepted limit (75 - 125%) for both inhaled powder formulations (Table 3) [34]. The clarithromycin formulation exhibited a higher FPF which was attributed to its lower MMAD. The GSD for both formulations were similar to marketed inhaled products such as tobramycin powder (TOBI® Podhaler™, Novartis), which exhibits MMAD and GSD of 3.0–4.2 µm and 1.8–2.4, respectively although these values were obtained under different testing conditions, so

caution should be used when comparing data [61]. Both liposomal clarithromycin and azithromycin exhibited similar Tg, morphology in SEM images (Table 1) and emitted dose (Table 3), so differences in antibiotic amount delivered to each stage were attributed to the smaller powder particle size and MMAD of the clarithromycin loaded powder.

Table 3 A summary of NGI data at 100 L min^{-1} flow rate for spray dried liposomal antibiotic with 17% trehalose and 0.5% L-leucine formulations using the Aerolizer with a nominal powder dose of 25 mg. Each value represents mean ± SD and *n*=3.

NGI Data	Liposomal clarithromycin	Liposomal azithromycin
Total emitted dose (mg)	21.3 ± 0.7	21.8 ± 1.7
Total emitted dose^ (% of nominal dose)	85.3 ± 2.7	87.3 ± 6.9
Induction port & Pre-separator (mg)	9.5 ± 1.8	12.1 ± 3.5
FPD (mg)	5.5 ± 0.5*	3.6 ± 0.4
FPF (% of nominal dose)	21.9 ± 2.1*	14.4 ± 1.6
FPF (% of the emitted dose)	31.5 ± 2.3*	19.8 ± 2.3
MMAD (µm)	4.20 ± 0.53*	5.23 ± 0.21
GSD	2.93 ± 0.25*	2.17 ± 0.06
Antibiotic amount in NGI stages (µg)		
Stage 1 (Pharynx)	103.8 ± 38.1	95.7 ± 15.0
Stage 2 (Trachea and Primary Bronchi)	108.6 ± 17.1	58.5 ± 10.4
Stage 3 (Secondary Bronchi)	55.2 ± 7.4	40.7 ± 5.2
Stage 4 (Terminal Bronchi)	29.3 ± 3.5	15.7 ± 2.4

Stage 5 to filter (Alveoli)	47.3 ± 4.4	11.1 ± 2.1
Drug in the intended levels (stage 3 to filter)	131.6 ± 14.9	67.7 ± 4.9

^The emitted dose% should be between 75 - 125% of the nominal dose.

*****donates $P < 0.01$ (unpaired t-test) between azithromycin and clarithromycin products.

3.3. Antimicrobial activity

In general, there was no difference in the activity of free or liposomal azithromycin against the P. *aeruginosa* isolates tested (Table S5) with the majority of isolates having MIC and MBC values >256 µg/mL, the highest concentration tested. This finding was not unexpected as *P. aeruginosa* is not usually sensitive to macrolide antibiotics including azithromycin [62-64]. Moreover, three of the *P. aeruginosa* isolates were cultured from CF patients prescribed long-term azithromycin treatment. Similarly, there was no difference in activity against *S. aureus* isolates with 2/5 clinical isolates demonstrating high level resistance to azithromycin. One of these isolates had been cultured from a patient prescribed long-term low dose azithromycin treatment or had recently received a short course of azithromycin; exposure of bacteria present in the airway microbiota to azithromycin has been shown to promote the development of resistance amongst S. aureus [65].

The activity of the liposomal formulation against biofilm forming P. aeruginosa isolates was subsequently tested. For five of the 6 isolates tested, the liposomal formulation demonstrated enhanced activity against formed biofilms with this effect most apparent at higher concentrations (Figure S2). A similar trend was observed for 3/6 isolates when the ability of the formulation to prevent biofilm formation was determined (Figure S3). Similar findings have been reported by Alhajlan et al. (2013) who demonstrated that a cationic liposomal clarithromycin formulation was more potent than free antibiotic treatment in eradicating P. aeruginosa biofilms [15]. This may be due to enhanced interaction between the cationic liposomal formulation and the negatively charged P. aeruginosa bacterial cells (Figure S4) [66]. The data presented demonstrate that neither azithromycin or liposomal azithromycin are effective in killing P. aeruginosa isolates in the planktonic mode of growth but liposomal azithromycin is more effective in preventing biofilm formation and in eradicating the formed biofilm (Fig 4). Azithromycin has been shown to alter P. aeruginosa biofilm matrix formation by inhibition of polysaccharide synthesis [67-69]. However, azithromycin has been shown to improve lung function, slow disease progression and increase time to next exacerbation in bronchiectasis, CF and COPD [70-72]. While the mechanism is still unclear, anti-inflammatory effects play a role [69, 73], and azithromycin may also have an indirect effect on P. aeruginosa due to interference with production of virulence factors and disruption of quorum sensing[74, 75].

3.6. Liposomal uptake by bacterial cells

Both fresh and spray dried liposomal CFD formulations were rapidly up taken by gram-negative and gram-positive CF clinical isolates in both the log phase and stationary phase (Figure 3) demonstrating that spray drying does not affect the liposome properties.

High uptake was attributed to charge on the liposomes as noted previously [66, 76, 77]. Unsurprisingly the formulation containing the low lipid concentration exhibited lower uptake. Maximum interaction was observed after one hour, which is considerably faster than observations with previous formulations [56, 62].

Figure 3 near here

3.7. Cell uptake and cytotoxicity

Images in Figure 4 show that both low and high dose formulations were taken up by A549 cells in one hour, with FACS flow data corroborating this. Spray drying had no effect on cell uptake (Figure 4 i and j) which is in line with the bacterial uptake findings. This was again attributed to the size and charge of the liposomes [78-80].

Figure 4 near here

Following the use of non-cytotoxic concentrations of different endocytosis inhibitors (Figure S5), the entry pathway utilized by this liposomal formulation in A549 cell line was found to be cholesterol-dependent endocytosis (Figure S6).

There was no difference in cytotoxicity observed (Figure S7) between treated and control ($P > 0.40$), which is in line with Yusuf's (2014) findings for the same liposomal composition [81]. The rapid and efficient interaction of this liposomal formulation with the respiratory epithelial cells *in vitro* demonstrated that this formulation could potentially enhance azithromycin uptake in the *in vivo* setting as well. The final product contained 3.4% w/w azithromycin, with

The *in vitro* results showed that the formulation was capable of delivering 505 µg azithromycin following the inhalation of 4 capsules. Available data for oral dosing of azithromycin shows availabilities to be 19.3 % and 0.41 % for pulmonary and oral delivery respectively in rats, with lower distribution of azithromycin into blood when delivered via inhalation [82]. More recently Hughes et al (2020) showed 10 µg/mL lung from oral delivery of 1g of azithromycin, corresponding to 200 µg azithromycin delivered to the lung from a

500 mg tablet[83]. Since this regime correlates with reduced exacerbations in long-term respiratory disease [84], it is possible that the dose achieved with the current formulation would achieve this therapeutic dose if administered as 2 inhalations twice daily. This proposed dosing schedule would be in line with those for a number of inhaled therapies including the TOBI podhaler. The dosing of tobramycin using this device corresponds to 4 capsules administered twice daily [85].

The sugar concentration is high but comparable with those used in marketed inhaled therapies. Inhaled mannitol used as an add-on maintenance treatment to improve lung function in patients with cystic fibrosis is used at a dose of 400 mg comprising 10 capsules of 40 mg, taken twice daily. Inhaled liposomal amphotericin, used as an off-label treatment for Aspergillus lung infection in people with Cystic Fibrosis or Allergic Bronchopulmonary Aspergillosis, in pediatric dosing, one dose containing 50 mg drug and 900mg of sucrose is diluted with 12 mL water and nebulised. Trehalose is used in a range of inhaled products and its safety has been demonstrated [86]. In future work we aim to manufacture the formulations using a newer more efficient spray-dryer equipped with a dehumidifier, that could further lower the water content and allow reduction in trehalose while maintain high Tg and allow further increase the drug loading.

4. Conclusions

An effective and stable respirable liposomal azithromycin powder formulation was developed that exhibited excellent physicochemical properties, including size, charge, encapsulation, and stability over one year. The *in-vitro* respiratory deposition data showed that the formulation is suitable for bronchial and alveolar delivery. The formulation enhanced activity of azithromycin against *P. aeruginosa* isolates grown in biofilm and exhibited rapid uptake by bacterial cells. These findings together with the fast and non-cytotoxic delivery of the formulation into mammalian cells suggest that this formulation might be suitable for further development, including spray drying with a more modern dryer with a dehumidifier attachment to improve the FPF, and lower the amount of trehalose required to achieve an acceptable stability. Further *in vivo* testing is warranted to understand the potential efficacy of the formulation for use by people with chronic respiratory infection.

5. Disclosure of competing interest

The authors declare no competing financial or personal interest.

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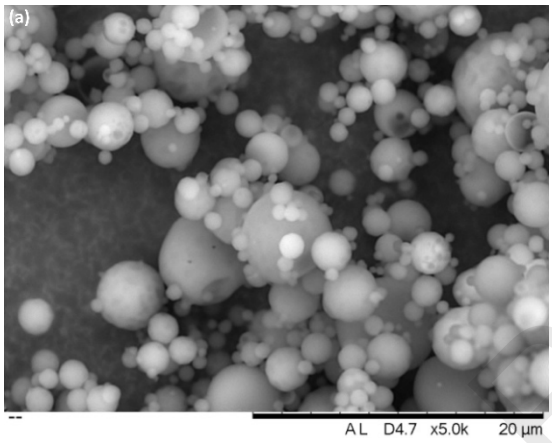

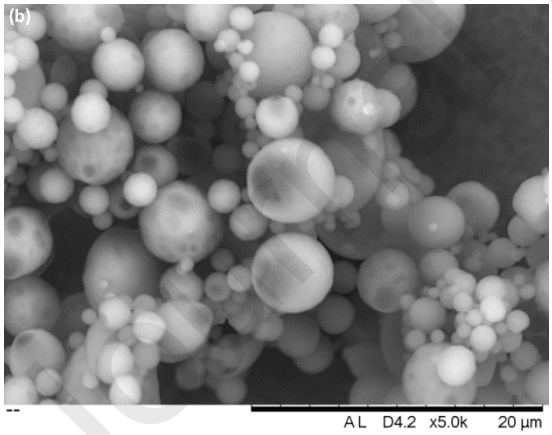

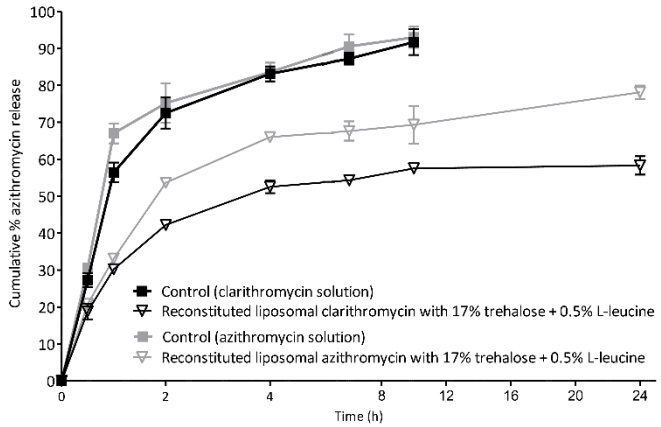

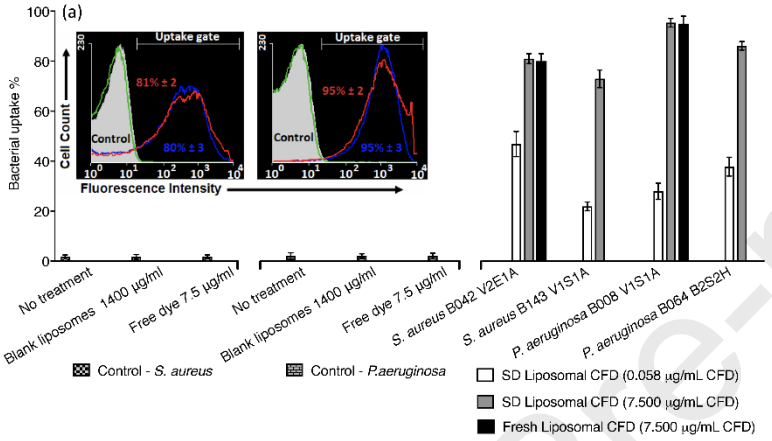

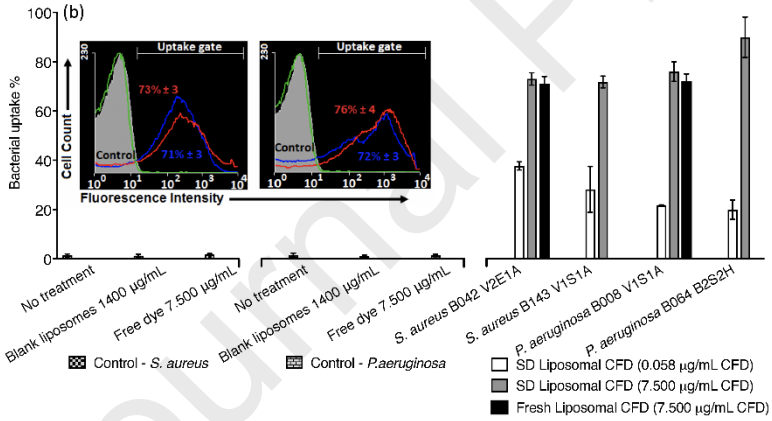

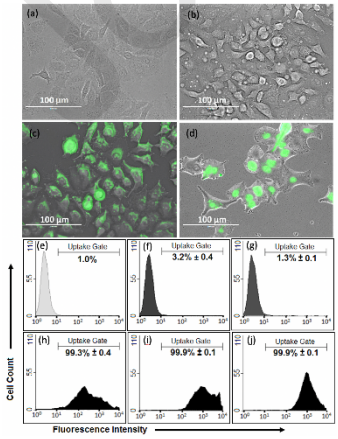

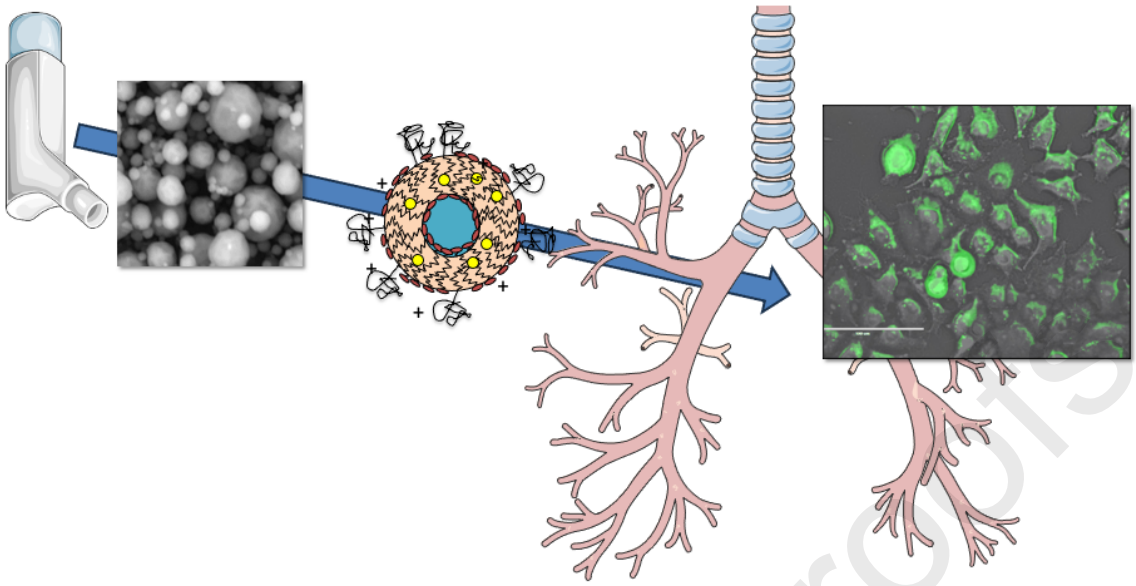

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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