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Dry powder aerosols to co-deliver antibiotics and nutrient dispersion compounds for enhanced bacterial biofilm eradication

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ABSTRACT

The purpose of this study was to formulate a dry powder for inhalation containing a combination treatment for eradication of *Pseudomonas aeruginosa* bacterial biofilms. Dry powders containing an antibiotic (ciprofloxacin hydrochloride, CH) and nutrient dispersion compound (glutamic acid, GA) at a ratio determined to eliminate the biofilms were generated by spray drying. Leucine was added to the spray dried formulation to aid powder flowability. A central composite design of experiments was performed to determine the effects of solution and processing parameters on powder yield and aerodynamic properties.

Combinations of CH and GA eradicated bacterial biofilms at lower antibiotic concentrations compared to CH alone. Spray dried powders were produced with yields up to 43% and mass mean aerodynamic diameters (MMAD) in the respirable range. Powder yield was primarily affected by variables that determine cyclone efficiency, i.e. atomizer and solution flow rates and solution concentration; while MMAD was mainly determined by solution concentration. Fine particle fractions (FPF) $< 4.46\,\mu m$ and $< 2.82\,\mu m$ of the powders ranged from 56 to 70% and 35 to 46%, respectively. This study demonstrates that dry powder aerosols containing high concentrations of a combination treatment effective against *P. aeruginosa* biofilms could be developed with high yield, aerodynamic properties appropriate for inhalation, and no loss of potency.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic bacterium that forms biofilms in response to stress in people with existing disease, such as cystic fibrosis, and those that are immunocompromised. Bacterial biofilms consist of bacterial colonies surrounded by a secreted extracellular polymeric matrix. Bacteria embedded within biofilms are difficult to eradicate as they display a 100–1000 fold increase in resistance to antibiotics compared to bacteria outside a biofilm (Taylor et al., 2014). The current treatment regimen for such infections involves long-term, aggressive administration of antibiotics. However, antibiotics alone provide only temporary relief and, in many cases, recurrence of infection is observed and chronic infections (Aaron et al., 2002; Mall and Hartl, 2014). Thus the cost of treatment is high and the search for more effective treatment options remains ongoing (Lam et al., 2013).

A variety of methods are being pursued to promote bacterial dispersion to enhance bacterial susceptibility to antibiotics. Bacterial dispersion from a biofilm is a natural process in biofilm maturation involving active release of bacteria in reaction to environmental cues (Kostakioti et al., 2013). This process can also be triggered by a variety of chemical compounds, including matrix degrading enzymes, salts, chelating agents, surfactants, and nutrients (Alkawash et al., 2006; Sauer et al., 2004; Banin et al., 2006; Chen and Stewart, 2000; Boles et al., 2005; Moulton and Montie, 1979). Nutrient compounds, such as glucose, amino acids,

Abbreviations: CH, ciprofloxacin hydrochloride; EF, emitted fraction; FPF, fine particle fraction; GA, L glutamic acid; GSD, geometric standard deviation; IOG, incidence of growth; MBEC, minimum biofilm eradication concentration; MHB, Mueller Hinton Broth; MMAD, mass median aerodynamic diameter; NGI, next generation impactor.

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and organic acids, show promise as dispersion-causing agents that could be inexpensively added to existing treatments (Sauer et al., 2004; Moulton and Montie, 1979). Nutrient compounds create a high nutrient concentration external to the biofilm, causing bacterial movement from the biofilm to the surrounding environment via chemotaxis (Kostakioti et al., 2013). The dispersed bacteria display phenotypic characteristics of planktonic bacteria (Sauer et al., 2004), suggesting they may exhibit higher susceptibility to antibiotics. Indeed, we recently demonstrated that dispersed bacteria are not only more susceptible to antibiotics, but that biofilms grown in vitro can be eradicated with lower antibiotic concentrations when combined with nutrient dispersion compounds (Sommerfeld Ross and Fiegel, 2012).

The objective of the current study was to formulate a dry powder for delivery of both antibiotics and nutrient dispersion compounds in a single aerosol via inhalation. Dry powder aerosols provide advantages over other formulations, including delivery of the therapeutic dose directly to the site of infection, which cannot be achieved with oral or IV administration, and faster administration time, portability, and simpler cleaning requirements compared to nebulization (Yang et al., 2014; Hoppentocht et al., 2014). Due to these advantages, antibiotic-containing dry powder aerosols are currently used in the treatment of infections in CF and can offer improved patient compliance (VanDevanter and Geller, 2011; Uttley and Tappenden, 2014). Here, we aimed to develop a dry powder aerosol with high yield and good aerodynamic properties for lung deposition of combination treatments containing antibiotics and dispersion compounds. We used design of experiments to optimize formulation parameters (solution pH, solution concentration, inlet temperature, atomizer flow rate, and solution flow rate) to achieve these properties.

2. Materials and methods

2.1. Materials

Ciprofloxacin hydrochloride (CH), a broad-spectrum, fluoroquinolone antibiotic used clinically against *P. aeruginosa* infections, was purchased from MP Biomedicals LLC (Solon, OH). L-glutamic acid (GA, a nutrient dispersion compound), L-leucine (an excipient to enhance powder flowability), silicone oil DC 200, and hexane mixture of isomers reagent 98.5% were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid was purchased from VWR (West Chester, PA). Sodium hydroxide was purchased from Fisher Scientific (Fair Lawn, NJ). Purified water was obtained from a NanoPure Infinity Ultrapure Water System (Barnstead Int., Dubuque, IA).

2.2. Treatment of bacterial biofilms to identify combination concentrations

P. aeruginosa mucoid lab strain PAO1 (ATCC, Manassas, VA), a common laboratory strain, was chosen as the test strain (Klockgether et al., 2010). A bacterial suspension of PAO1 containing 1.5×10^7 CFU/mL of bacteria was prepared in Mueller Hinton Broth (MHB). Biofilms were grown by adding the suspension to a Minimum Biofilm Eradication Concentration (MBEC)^{TM} assay trough (Innovotech, Edmonton, Alberta, Canada), placing the peg lid on the trough such that the 96 pegs dip in the suspension, and incubating the trough at 37 °C for 24 h (Ceri et al., 1999) on a rocker table.

Grown biofilms were then treated with combinations of CH and GA. Biofilms grown on the pegs were treated by dipping the pegs in wells of a 96-well plate containing either CH alone across antibiotic concentrations from 1 μg/mL to 1000 μg/mL, or a combination of CH at various concentrations and 20 mM GA at 37 °C for 24 h. Post-treatment, the peg lid with the residual biofilms was transferred to 96-well plate containing sterile MHB. The biofilms were dispersed in the media by sonicating the plate in a sonic water bath. The peg lid was then discarded and the 96-well plate was incubated at 37 °C for 24 h. Optical density (OD) of the bacterial suspensions was measured at 650 nm to obtain suspension turbidity, with a limit of quantification at an OD of 0.1. Greater turbidity (higher OD) was indicative of greater total bacterial count. Sterile MHB was used as the negative control. OD is reported as the average ± standard deviation. Student's *t*-test was used to determine statistically significant differences between treatments, with $p < 0.05$ signifying statistical significance.

To further quantify the variability with a single treatment across multiple biofilm samples, the incidence of growth (IOG) was determined. IOG is defined as the percentage of wells with an optical density greater than 0.1 at 650 nm (the limit of detection of the assay). Wells with OD_{650nm} below 0.1 represented 'no measurable growth', while wells with OD_{650nm} above 0.1 represented 'measurable growth'. IOG was calculated using the OD readings obtained for the post-treatment residual biofilms by taking the ratio of the number of wells for a particular treatment that gave an OD_{650nm} greater than 0.1 to the total number of wells for that treatment.

2.3. Formulation of dry powders via spray drying

A Buchi 190 spray drier (Flawil, Switzerland) was used to generate dry powder aerosols containing CH, GA, and leucine. Leucine is a commonly used excipient that reduces adhesion between particles and improves the flowability of powders (Prota et al., 2011; Fiegel et al., 2008). Previous work in our laboratory found that as little as 10 wt% leucine could be incorporated into the dry powders to improve powder dispersion and deposition (Thomas, 2009). An aqueous solution containing 10% leucine, 1.5% CH, and 88.5% GA (CH:GA ratio determined from OD experiments to eradicate biofilms) at room temperature was pumped into the spray dryer using a MasterFlex L/S peristaltic pump equipped with an Easy-Load II head and size 16 platinum-cured silicone tubing (Cole-Parmer, Vernon Hills, IL) and spray dried at an aspirator pressure of −30 mbar.

A central composite design of experiments was used to investigate the effects of formulation parameters and spray dryer processing parameters on the yield and aerodynamic properties of the generated dry powders. Solution pH (3–11), solids concentration of the spray dried solution (0.025-0.725 wt%), spray dryer inlet

Table 1
Independent variables and their respective levels in the central composite design.

Design Point	pH	Solution Concentration, wt%	Inlet Temperature, °C	Atomizer Rate, L/hr	Solution Flow Rate, mL/min
Axial	3	0.025	155	150	5
Cubic	5	0.200	165	300	7
Midpoint	7	0.375	175	450	9
Cubic	9	0.550	185	600	11
Axial	11	0.725	195	750	13