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A novel inhaled multi-pronged attack against respiratory bacteria

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ABSTRACT

Airway mucus hypersecretion is a common clinical feature of many severe respiratory diseases, and when complicated by a recalcitrant bacterial infection, the whole treatment regimen thereby becomes more challenging and protracted. The accumulation of thickened mucus secretions in the lower airways provides a nutrient-rich breeding ground for bacteria that promotes their growth and limits the ease of effective eradication. Unfortunately, no direct-inhaled dry powder formulation to treat these respiratory mucoid infections more effectively is available commercially. This work therefore seeks to develop a highly-efficacious ternary dry powder inhaler (DPI) formulation (ciprofloxacin hydrochloride (CIP), gatifloxacin hydrochloride (GAT) and ambroxol hydrochloride (AMB)) capable of delivering a novel multi-pronged attack (synergy, quorum quenching and mucociliary clearance) on *Pseudomonas aeruginosa*, a common respiratory bacteria found in mucoid infections. The powders were prepared via spray drying, evaluated on their aerosol performance via a multi-stage liquid impinger (MSLI) and tested for their efficacies in bacteria-spiked artificial sputum medium (ASM). The optimized particles were of respirable-size (d_{50} of ~1.61 ± 0.03 μm) and slightly corrugated. When dispersed via an Aerolizer® inhaler at 60 L/min, the powder showed concomitant *in vitro* deposition, minimal capsule, device and throat retention, and highly promising and uniform fine particle fractions (of the loaded dose) of ~64–69%, which was a vast improvement over the singly-delivered actives. Favourably, when tested on bacteria-spiked ASM, the optimized ternary formulation (with AMB) was more effective at killing bacteria (i.e. faster rate of killing) than just the synergistic antibiotics alone (binary formulation; without AMB). In conclusion, a ternary antibiotic–(non-antibiotic) DPI formulation involving a unique multi-pronged attack mechanism was successfully pioneered and optimized for mucoid infections.

1. Introduction

Airway mucus hypersecretion and a recalcitrant bacterial infection are the hallmarks of cystic fibrosis (CF), and as of today, this condition is the most common life-threatening inherited disease afflicting the Caucasian population (O'Sullivan and Freedman, 2009). The CF disorder is caused by mutations in a single gene on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR), a 1480 amino acid polypeptide which regulates the transport of chloride ions across epithelial membranes (Tsui, 1995). Abnormalities of CFTR function result in low volume abnormal secretions leading to mucus plugs, impaired mucociliary clearance and eventually, chronic lung inflammation and infection (Bilton, 2008).

As CF patients with *Pseudomonas aeruginosa* infections typically have more rapid declines in lung function than non-*P. aeruginosa* infected individuals (Bilton, 2008), anti-pseudomonal therapy for this group of patients should hence be initiated early and aggressively upon first isolation of the bacteria, in order to prevent its evolution into a chronic infection (Valerius et al., 1991). Early eradication of *P. aeruginosa* infection is extremely crucial as initial *P. aeruginosa* colonization typically involves only non-mucoid strains that are highly susceptible to antibiotic agents. Failure to eradicate this initial colonization would allow the non-mucoid

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strains to freely mutate into the alginate-coated mucoid strains, which are more resistant to antibiotic agents. Resistance is brought about as a consequence of poor penetration of the antibiotic agents into the anaerobic sputum plugs (Oliver et al., 2000; Riordan et al., 1989; Worlitzsch et al., 2002).

Currently, antibiotic therapy remains the mainstay of treatment as gene or protein replacement therapies have yet to become commercial realities (Ratjen and Doring, 2003). Antibiotic treatment is aimed at reducing the bacterial load and inflammatory response in the lung, so as to ameliorate infective exacerbations and prevent or delay progressive respiratory failure.

Combination drugs, as innovations in pharmacotherapy (Wertheimer and Morrison, 2002), are increasingly used to combat the spread of antibiotic-resistant bacterial pathogens (Chait et al., 2007; Mouton, 1999; Wertheimer and Morrison, 2002). A combination of antibiotics provides a much broader spectrum of coverage than any single antibiotic alone, as the probability of a bacterium developing resistance to all the antibiotics employed in the combination therapy is much lower than in the case for an antibiotic in monotherapy (Lorian, 2005). Hence, this treatment approach is considered useful in the prevention and treatment of resistant bacterial infections (Chernish and Aaron, 2003; Doring et al., 2000; Mouton, 1999; Watkins et al., 1988; Weiss and Lapointe, 1995), and whenever a favorable synergistic effect is achieved, the whole treatment regimen thereby becomes more valuable, as the same killing effect could be obtained at much reduced doses and with fewer side effects (Chernish and Aaron, 2003). Although antibiotic combinations are commonly engineered in medicine to broaden the antimicrobial spectrum and generate synergistic effects, combinations of antibiotics with non-antibiotic drugs are increasingly gaining popularity as well (Ejim et al., 2011; Heaf et al., 1983; Kalan and Wright, 2011; Lee et al., 2014). These non-antibiotic drugs could potentially augment the activity of the antibiotic/s to bring about a more favorable outcome to the patient.

Mucus hypersecretion (increased mucus secretion) is a hallmark of CF whereby the rate of mucus secretion far exceeds the rate of removal by normal ciliary action (Balsamo et al., 2010). The accumulation of thickened mucus secretions in the lower airway of patients with CF provides a nutrient-rich breeding ground for bacteria that promotes their growth and limits the ease of effective eradication.

Hence, this work aims to explore a useful inhaled antibiotic–(non-antibiotic) formulation that comprises the use of a mucoactive agent with the antibiotic/s to facilitate simultaneous treatment and clearance of respiratory bacteria and mucus respectively (mucoactive agents are drugs used to alter the viscoelastic properties of mucus and promote secretion clearance (Balsamo et al., 2010)). A dry powder inhaler (DPI) formulation is preferred over the other aerosol delivery modes (e.g. nebulizer or metered dose inhaler (MDI)) in view of the improved formulation stability associated with the powdered drug (as compared with the solution or suspension in the MDI and nebulizer), improved delivery efficiency, ease-of-use, portability and the avoidance of undesired precipitation in solutions (e.g. in the nebulizer) (Coates et al., 2001; Elmore et al., 1996; Loughlin and Eigen, 1994; Traini and Young, 2009). Though largely popular, it is worthwhile to note some potential manageable side effects associated with the use of these inhalers and they include coughing, bronchospasm and hoarseness, with coughing probably being the most common local side effect.

To ensure maximum kill and effective penetration of antibiotics through the thick mucus, a novel multi-pronged strategy involving a combined synergy, quorum quenching and mucociliary clearance approach is proposed. Quorum sensing is the mechanism employed by pathogenic bacteria to mount successful infections by allowing them to regulate expression of gene networks that co-ordinate virulence, antibiotic resistance and fitness responses. The process is mediated by small diffusible signaling molecules (Deep et al., 2011). Interference of the quorum sensing system by quorum quenching is recognized as a potential strategy for disease control (Dong et al., 2007; Tillotson and Theriault, 2013). Hence, in this study, antibiotic synergism (i.e. combined activity is more than the sum of individual activities) is expected to enhance the bactericidal activity (Chait et al., 2007; Lee et al., 2013), quorum quenching to disrupt bacterial cell-to-cell communication essential for infection and survival (Dong et al., 2007), and mucociliary clearance to remove/reduce the mucus layer for effective targeting of underlying bacteria (Balsamo et al., 2010). As airway mucus hypersecretion is a common clinical feature of many severe respiratory diseases like asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (Balsamo et al., 2010; Rogers, 2007), the proposed multi-pronged treatment strategy is thus expected to be highly applicable and beneficial to a diverse group of patients (e.g. pneumonia-COPD patients) (Lee et al., 2014).

In this work, ciprofloxacin hydrochloride (CIP) and gatifloxacin hydrochloride (GAT) were selected as the synergistic antibiotic pair (Lee et al., 2013), while ambroxol hydrochloride (AMB) was chosen for its multi-faceted properties (Paleari et al., 2011) that include quorum quenching (Dong et al., 2007; Hassett et al., 2002; Lu et al., 2010), mucoactivity (Paleari et al., 2011) and pulmonary protective effects (Li et al., 2012). In addition, ambroxol could potentially increase the bioavailability of antibiotics in the lung, particularly in the alveolar region (Paganin et al., 1995). This effect had been demonstrated for the fluoroquinolones (Paganin et al., 1995) and other drugs (Tewes et al., 2013), and similar benefits may be extended to the antibiotic–mucoactive combination.

2. Materials and methods

2.1. Materials

Ciprofloxacin hydrochloride (CIP), gatifloxacin hydrochloride (GAT) and ambroxol hydrochloride (AMB) were supplied from Junda Pharmaceutical Co. Ltd. (Changzhou, China). L-Leucine (LEU), disodium hydrogen phosphate, phosphoric acid, mucin, egg yolk emulsion, deoxyribonucleic acid (DNA) from fish sperm, casein hydrolysate, diethylenetriamine pentaacetic acid (DTPA), NaCl, KCl and mannitol were purchased from Sigma Chemical Co. (Louis, MO, USA). HPLC grade acetonitrile and molecular biology grade of 1 M Tris buffer (pH 8.0) were obtained from Merck (Darmstadt, Germany). American Type Culture Collection (ATCC) *P. aeruginosa* 90207 was used as the test microorganism.

2.2. Preparation of spray-dried powder

Powders of ciprofloxacin hydrochloride (SD-CIP), gatifloxacin hydrochloride (SD-GAT), ambroxol hydrochloride (SD-AMB), ternary combination powders of ciprofloxacin hydrochloride/gatifloxacin hydrochloride/ambroxol hydrochloride with (SD-CIP/GAT/AMB/LEU) and without leucine (SD-CIP/GAT/AMB), SD-CIP/GAT and SD-AMB/LEU were obtained by spray drying their aqueous solutions on a B-290 Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland) with the following operating parameters: inlet temperature of 190 °C (outlet temperature 100 °C), atomization rate of 670 L/h, aspiration rate of 57.6 m^3/h and feed rate of 3.5 mL/min. The feed solution concentration was maintained at 0.8% (*w*/*v*) for all the formulations. The ratio of CIP:GAT:AMB in the ternary formulations (both with and without leucine) was 1:2.5:5.5 (Lee et al., 2013). For SD-CIP/GAT/AMB/LEU and SD-AMB/LEU, the leucine content was 13% (*w*/*w*). The ratio of CIP:GAT for SD-CIP/GAT was maintained at 1:2.5. The spray-dried powders