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Azithromycin-loaded respirable microparticles for targeted pulmonary delivery for the treatment of pneumonia

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ABSTRACT

Pneumonia is a major contributor to infection-based hospitalizations and deaths in the United States. Antibiotics such as azithromycin (AZM), although effective at managing pneumonia, often suffer from off-target diffusion and poor bioavailability when administered orally or via intravenous injection. The formation of biofilms at the disease sites makes the treatment more complicated by protecting bacteria from antimicrobial agents and thus necessitating a much higher dosage of antibiotics to eradicate the biofilms. As such, targeted pulmonary delivery of antibiotics has emerged as a promising alternative by providing direct access to the lung while also allowing higher local therapeutic concentrations but minimal systemic exposure. In this study, AZM was encapsulated in N-fumaroylated diketopiperazine (FDKP) microparticles for efficient pulmonary delivery. Both *in vitro* and *in vivo* results demonstrated that AZM@FDKP-MPs administered via intratracheal insufflation achieved at least a 3.4 times higher local concentration and prolonged retention times compared to intravenous injection and oral administration, suggesting their potential to better manage bacterial pneumonia.

1. Introduction

Pneumonia is a major contributor to infection-based hospitalizations and deaths among all age groups in the United States, and is responsible for 4 million deaths worldwide every year [1,2]. Even with the introduction of pneumococcal conjugate vaccines, pneumonia has continued to be the eighth most expensive condition treated in hospitals nationwide with an estimated economic burden exceeding $\$1.62 \times 10^{10}$ in 2013 [3,4]. Pneumonia is most commonly caused by viruses or bacteria such as *S. pneumoniae*, with bacteria being the most common cause of community-acquired pneumonia (CAP) [5,6]. In the case of bacterial pneumonia, the macrolide drug azithromycin (AZM), a semi-synthetic 9-N-methylation derivative of erythromycin, is typically recommended in the United States as a first-line outpatient treatment, or as part of a combination therapy in patients who require hospitalization [7–9]. AZM is a broad-spectrum antibiotic useful for the treatment of a number of gram-positive, gram-negative, and atypical bacterial infections that lead to pneumonia, such as those caused by *S. pneumoniae*, *haemophilus influenza*, and *clamydophila pneumoniae* [10,11]. The most common routes of administration of AZM to treat pneumonia are orally, by intravenous injection, or by intravenous infusion [12]. Although generally effective and sufficient for managing bacterial pneumonia, these systemic delivery methods will inevitably lead to off-target diffusion, poor bioavailability and, consequently, higher doses to attain the necessary concentrations in the lung, especially in the epithelial lining fluid. Considering that AZM was the most prescribed antibiotic for outpatients in the US in 2010 [13], the threat of developing bacterial resistance is a concern. Moreover, bacteria such as *S. pneumoniae* tend to form biofilms that protect them from antimicrobial agents and host immune responses. These are a result of changes in metabolic processes or poor antimicrobial diffusion and could increase antibiotic resistance hundreds of times compared to planktonic bacteria [14–17]. The targeted pulmonary delivery of AZM in the form of dry powders provides direct access to the lungs,

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supplying increased local therapeutic concentrations with minimal systemic exposure. Collectively, these characteristics decrease the chance that bacteria will develop antibiotic resistance [18] [19]. As such, targeted pulmonary delivery has become an attractive method of managing lower respiratory tract infections. In addition, the advances in dry powder inhalers make this route of drug administration more efficient and more convenient for patients [20].

The large surface area, thin alveolar epithelium and lack of first-pass metabolism are all beneficial for efficient drug absorption in the lung [21]. As a major port of entry, however, the lung has evolved to protect the airways from exposure to unwanted foreign materials via phagocytic and mucociliary clearance. While this feature is effective in protecting the body from foreign invasion, it represents a significant barrier for pulmonary drug delivery [20]. Consequently, engineering particles that can achieve effective aerosolization, maximize lung deposition, and minimize macrophage uptake is of the utmost importance for effective pulmonary delivery [22]. Towards this end, optimization of the aerosolization performance of dry powders is dependent on several factors [23]. First, aerodynamic particle size and size distribution can significantly impact the delivery of the particles throughout the lung. For example, previous research has shown that optimal deep lung deposition can be achieved with particles ranging from 1 to 5 μm [24]. Second, surface morphology and hygroscopicity have been shown to influence particle flow and atomization behavior [25–31]. Previous studies have demonstrated that corrugated particles with rougher surfaces may contribute to van der Waals force changes between particles [32]. The hygroscopicity of drugs and excipients has also been shown to play a role in aerosol generation and lung deposition by altering moisture absorption capacities, which may lead to particle dissolution or aggregation [33]. Finally, excipients are frequently incorporated as carriers or extenders to improve aerosolization properties. For pulmonary drug delivery, excipients should be well-tolerated and have short retention times in the lung. Common excipients used for pulmonary drug delivery can be divided into three categories: (1) sugars, such as lactose, mannitol, and glucose, which were once the most widely used excipients with favorable safety profiles. Unfortunately, sugar-based excipients usually result in large particle sizes and can adversely affect particle lung deposition. This could lead to lower drug accumulation in the lung and eventually compromise effectiveness. (2) Lipids, such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and cholesterol, are endogenous substances in the lung and can be further modified to improve aerosolization properties. Dosage forms like liposome and solid lipid microparticles (SLM) have been shown to improve drug retention time and reduce toxicities. However, the relatively high price of lipids have hindered their wide application as pulmonary delivery excipients. (3) Biodegradable polymers such as PLGA have also been widely investigated as pulmonary delivery vehicles due to their good lung deposition ability. However, PLGA could easily degrade into acidic products such as lactic acid and glycolic acid in the lung and the accumulation of acidic degradation products may cause long-term respiratory irritations and could eventually lead to adverse immune responses in the lungs resulting in cough, edema and neutrophil infiltration.

Towards this end, fumaryl diketopiperazine (FDKP) is an FDA-approved, inert excipient that has been used as the primary component in Afrezza® to assist in the delivery of recombinant human insulin via inhalation [34,35]. FDKP possesses two carboxyl groups and undergoes acid-induced intermolecular self-assembly in acidic environments (pH < 5) to form microparticles with a negatively charged surface [36]. Protein or small molecule therapeutics with positive charges at acidic pH can therefore be adsorbed onto the surface of FDKP microparticles via electrostatic interactions. Furthermore, dry powders of drug-loaded FDKP microparticles can be obtained upon spray drying. Once deposited into the lung, FDKP microparticles can dissolve at neutral pH and release drugs rapidly. The FDKP is then distributed to other tissues and excreted from the kidney in its original form [36].

In this study, FDKP was successfully synthesized and characterized as a safe excipient to deliver AZM into the lung. FDKP microparticles (FDKP-MPs), AZM microparticles (AZM-MPs) and AZM loaded FDKP microparticles (AZM@FDKP-MPs) were formulated and compared for their physicochemical properties as well as their aerodynamic behaviors to optimize the effectiveness of pulmonary delivery. AZM@FDKP-MPs exhibited fantastic aerosolization performance, improved moisture resistance and, most importantly, exceptional deep lung deposition. *In vitro* antibacterial experiments were carried out to confirm that the addition of FDKP had no impact on the minimum inhibitory concentration (MIC) of AZM for *S. pneumonia.* Finally, pharmacokinetic and pharmacodynamic studies were conducted in a pneumonia mouse model to explore whether the use of inhalable AZM@FDKP-MPs reduced the frequency of administration and/or was more effective in reversing disease progression compared to oral administration or intravenous injection. Our findings *in vivo* clearly suggest that AZM@FDKP-MPs administered via intratracheal insufflation achieved the highest local concentration and prolonged retention time and could, thus, be a successful and novel pneumonia treatment.

2. Materials and methods

2.1. Materials and animals

N_6-trifluoroacetyl-L-lysine was purchased from Adamas-beta® (Shanghai, China); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were obtained from Aladdin® (Shanghai, China); Fumaric acid monoethyl ester was acquired from Dingchem™ (Shanghai, China); and azithromycin (AZI) was supplied by Jiangchuan pharmaceutical Co., Ltd (Chengdu, China). All other reagents, if not specified, were purchased from Sinopharm Chemical Reagent Co., Ltd and were of analytical grade.

Female normal mice and BALB/c mice ($w = 18–22$ g) were purchased from the Experimental Animal Center of Nanjing Qinglongshan. All animal experiments were conducted in accordance with the Guide for Laboratory Animal Facilities and Care and were approved by the Animal Ethics Committee of China Pharmaceutical University.

2.2. Synthesis of fumaryl diketopiperazine (FDKP)

The FDKP was synthesized using a three-step reaction. Briefly, 10 g of N_6-trifluoroacetyl-L-lysine and 1.76 g of phosphorus pentoxide were dissolved in 50 mL of N-methyl-2-pyrrolidinone (NMP) in a three-neck flask and were allowed to react at 165 °C for 1.5 h under nitrogen protection and constant stirring. The reaction mixture was cooled to room temperature and poured into 1000 mL of deionized water to allow for precipitation. The precipitates were washed twice with deionized water and were vacuum dried for 24 h to obtain trifluoroacetyl-diketopiperazine (TFA-DKP) [37]. TFA-DKP was then hydrolyzed using a 5% sodium hydroxide solution to remove the trifluoroacetyl group and then added dropwise into an active ester solution formed by EDC, NHS and fumaric acid monoethyl ester. The mixture was allowed to react for 24 h with a pH adjusted to around 7–8 and was poured into deionized water for precipitation. The precipitates were collected and were further