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Inhalable spray-dried formulation of D-LAK antimicrobial peptides targeting tuberculosis

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ABSTRACT

Tuberculosis (TB) is a global disease that is becoming more difficult to treat due to the emergence of multidrug resistant (MDR) *Mycobacterium tuberculosis*. Inhalable antimicrobial peptides (AMPs) are potentially useful alternative anti-TB agents because they can overcome resistance against classical antibiotics, reduce systemic adverse effects, and achieve local targeting. The aims of the current study were to produce inhalable dry powders containing D-enantiomeric AMPs (D-LAK120-HP13 and D-LAK120-A) and evaluate their solid state properties, aerosol performance, and structural conformation. These two peptides were spray dried with mannitol as a bulking agent at three mass ratios (peptide: mannitol 1:99, 1:49, and 1:24) from aqueous solutions. The resultant particles were spherical, with those containing D-LAK120-HP13 being more corrugated than those with D-LAK120-A. The median volumetric diameter of the particles was approximately 3 μm. The residual water content of all powders were <3% w/w and crystalline, due to the low hygroscopicity and crystallinity of mannitol, respectively. The mannitol changed from a mixture of alpha- and beta-forms to delta form with an increasing proportion of AMP in the formulation. The emitted fraction and fine particle fraction of the powders when dispersed from an Osmohaler® at 90 L/min were about 80% and 50–60% of the loaded dose, respectively, indicating good aerosol performance. Circular dichroism data showed that D-LAK120-HP13 dissolved in Tris buffer at pH 7.15 was of a disordered conformation. In contrast, D-LAK120-A showed greater α-helical conformation. Since the conformations of the AMPs were comparable to the controls (unprocessed peptides), the spray drying process did not substantially affect their secondary structures. In conclusion, spray dried powders containing D-enantiomeric AMPs with preserved secondary molecular structures and good aerosol performance could be successfully produced. They may potentially be used for treating MDR-TB when delivered by inhalation.

1. Introduction

Tuberculosis (TB) is a global health problem that caused 1.5 million deaths in 2013 (WHO, 2014). Despite the tremendous effort to fight against TB in the last few decades, it remains one of the leading causes of death among infectious diseases. TB is caused by *Mycobacterium tuberculosis* (Mtb), transmitted in airborne particles. Although the overall incidence of TB is decreasing worldwide, multidrug resistant (MDR) TB cases are on the rise, threatening global TB control (Millard et al., 2015). MDR-TB is a form of TB that is resistant to at least isoniazid (INH) and rifampicin (RIF), both are first-line anti-TB drugs. The management of MDR-TB is highly complex, requiring the use of second-line drugs that are more expensive, more toxic and less well tolerated. The duration of treatment is long (18–24 months) with severe adverse effects, leading to poor patient compliance. Over the last 40 years, only two new anti-TB drugs, bedaquiline and delamanid, were approved by the FDA (Kakkar and Dahiya, 2014; Zumla et al., 2014). There is an urgent need to identify new and effective anti-TB strategies that combat, in particular, MDR-TB.

Interest in the use of antimicrobial peptides (AMPs) to treat drug resistant bacterial infections has increased in recent years. AMPs are found naturally in living organisms as a component of the innate immune system. Naturally occurring AMPs and their

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synthetic analogues have broad-spectrum antimicrobial activities. They are often regarded as the next generation of antibiotics (Peters et al., 2010; Steckbeck et al., 2014), and have attracted considerable attention as new anti-TB agents (Abedinzadeh et al., 2015). AMPs are usually cationic, amphipathic, small peptides (15–50 amino acid residues). Although the exact mechanism of action of most of the AMPs is not fully understood, it is generally believed that their antibacterial activities are often dependent on the ability to destabilize or penetrate bacterial membranes (Brown and Hancock, 2006; Jenssen et al., 2006), although other intracellular targets including DNA, RNA and proteins are also reported to play a role (Ghosh et al., 2014; Hao et al., 2013; Nguyen et al., 2011). More importantly, development of resistance against AMPs may be less common owing to their direct and rapid bactericidal activity, as well as the ability to kill bacteria in multiple ways, and they have the potential to bypass resistance mechanisms developed towards classical antibiotics (Padhi et al., 2014; Peschel and Sahl, 2006).

In our previous study, a series of D-enantiomeric AMPs, (D-AMPs) was extensively investigated as potential new anti-TB agents (Lan et al., 2014; Vermeer et al., 2012). D-conformation peptides were used because L to D-amino acid substitution of AMPs confers resistance to the activity of proteolyic enzymes, leading to improvement of AMPs stability without impairing antimicrobial activity (Grieco et al., 2013). According to our previous findings, two D-AMPs, D-LAK120-HP13 and D-LAK120-A, were found to be effective in inhibiting the intracellular growth of Mtb, including both MDR and extensively drug-resistant (XDR) clinical isolates. Both amphipathic peptides are membrane-active and are capable of dispersing the heavily clumped Mtb colonies by altering the surface properties of mycobacteria, enhancing the efficacy of INH and RIF against MDR-TB in vitro (Lan et al., 2014).

To further develop D-LAK120-HP13 and D-LAK120-A peptides as new therapeutic agents for the treatment of TB, they need to be formulated into a suitable dosage form. Since the lung is the primary port of entry of Mtb, usually residing in the alveolar macrophages, high concentrations of anti-TB agents at the target site could be effectively achieved by pulmonary delivery (Hickey et al., 2013; Traini and Young, 2009). This non-invasive route of administration provides fast onset of action, local targeting, lower dosage and fewer systemic adverse effects. Compared with liquid aerosols, dry powders for inhalation offer convenience for storage and stability because biochemical degradation is minimized in the solid state (Chew and Chan, 2002). Moreover, dry powder inhalers, activated by patient inspiration effort, allow rapid dose administration and high local drug deposition, and has become an attractive strategy for antimicrobial formulation against lung infections (Belotti et al., 2014; Schuster et al., 2013). The major challenges of preparing inhalable formulations of peptide-based therapeutics are to maintain the physical integrity and stability of the peptides during the drying process and, at the same time, achieve good inhalability and aerodynamic properties of the powders. The present study aims to formulate D-AMPs into dry powders for inhalation by the spray drying method. Mannitol was used as the bulking excipient and various formulations containing different peptide-to-excipient mass ratios were prepared. The particle size distribution, morphology, aerodynamic performance, stability and structural integrity of the spray-dried powders were carefully evaluated in order to determine the suitability of the spray dried powders as an effective formulation for future delivery of D-AMPs.

2. Materials and methods

2.1. Materials

D-LAK120-HP13 peptide (KKALAHALKKWLPALKKLAHALAKK-NH_2) and D-LAK120-A peptide (KKLALALAKKWLALAKKLALALAKK-NH_2) at >80% purity were purchased from China Peptides (Shanghai, China) and used as provided. Mannitol (Pearlitol 160C) was purchased from Roquette (Lestrem, France). All other reagents and solvents were purchased from Sigma–Aldrich (Poole, UK) and were of analytical grade or better.

2.2. Spray drying of D-LAK peptides

Three formulations each of D-LAK120-HP13 and D-LAK120-A were prepared at different peptide to mannitol mass ratios as listed in Table 1. Both peptides and mannitol were dissolved in distilled water to give a final total mass concentration of 1% (w/v) in solution. A Büchi B-290 laboratory spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) was used to prepare the spray-dried powders of each formulation under the following conditions: inlet temperature of 75 °C, outlet temperature of 40–42 °C, aspiration at 38 m^3/min (100%), nitrogen atomization flow rate at 742 norm litre/h, and liquid feed rate at 3.6 ml/min. The spray drying conditions used were slightly modified from those for producing powders containing DNA/siRNA and pH-responsive peptides conducted earlier by the authors (Liang et al., 2015, 2014). The spray-dried powders were collected by a high efficiency cyclone and stored at room temperature with silica gel until further analysis.

2.3. Spray drying yield and recovery of D-AMPs

The spray drying yield was calculated as the mass of powder obtained after spray drying divided by the total initial amount of solid ingredients expressed as a percentage. The peptide recovery was determined as the percentage ratio of the amount of peptide measured in the spray dried powder with respect to the theoretical amount of peptide. The amount of peptides in the spray-dried powders was determined using reversed-phase high performance liquid chromatography (HPLC) (HPLC 1260 Agilent Technologies, Santa Clara, USA). A 100 μl aliquot of sample solution was injected by an auto-sampler and passed through a C18 column (140 mm × 4.6 mm, 5 μm; Vydac™ Grace™, IL, USA) at ambient

Table 1
Spray dried powders of D-LAK120-HP13 and D-LAK120-A with mannitol, their spray drying yield, peptide recovery, and water content. Data for peptide recovery presented as mean ± standard deviation ($n = 3$).

Formulation	Peptide	Peptide: mannitol (mass ratio)	Spray drying yield (% w/w)	Peptide recovery (% w/w)	Water content (% w/w)
F1	D-LAK120-HP13	1:99	46.4	94.8 ± 5.6	2.60
F2	D-LAK120-HP13	1:49	70.0	102.5 ± 2.1	1.58
F3	D-LAK120-HP13	1:24	67.7	100.2 ± 3.4	2.09
F4	D-LAK120-A	1:99	66.6	98.9 ± 3.8	1.32
F5	D-LAK120-A	1:49	74.1	102.1 ± 3.5	1.33
F6	D-LAK120-A	1:24	71.9	117.6 ± 2.8	1.19