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Phospholipid-based pyrazinamide spray-dried inhalable powders for treating tuberculosis

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ABSTRACT

Sterilization of necrotic granulomas containing *Mycobacterium tuberculosis* is difficult by oral and parenteral drug delivery of antitubercular drugs. Pulmonary delivery of these drugs should increase the concentration of drug in the granulomas and, thereby, improve the sterilization. The current study aimed to develop spray-dried (SD) powders composed of pyrazinamide, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine *N*-(carbonyl-methoxy polyethylene glycol-2000) (DSPE-PEG2k) and L-leucine to improve drug delivery to the deeper lung. Pyrazinamide SD powders with varying amounts of DPPC (5, 15 and 25% w/w) were produced using a BUCHI B-290 Mini Spray-Dryer. The powders were characterized physicochemically and for their aerosol dispersion performance using a Next Generation Impactor (NGI). All the SD powders had a narrow particle size distribution (1.29–4.26 μm) with low residual moisture (<2%). Solid state characterization confirmed that the α-polymorphic crystalline pyrazinamide transformed into the γ-polymorphic form during spray-drying. SD pyrazinamide ($PDDL_0$) without excipients showed very poor aerosolization with a fine particle fraction (FPF%) of 8.5 ± 1.0%. However, the SD powder with 25% w/w DPPC ($PDDL_3$) exhibited the best aerosolization with a FPF of 73.2 ± 4.0%. Incorporating high amounts of DPPC improved aerosolization of SD powders; however further evaluation of the developed inhalation powders is necessary to determine their therapeutic potential for treating pulmonary tuberculosis.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (M.Tb) is the second most common ancient infectious disease (Tiwari et al., 2005). According to the World Health Organization (WHO, 2014), about 9 million people were infected with tuberculosis and about 1.5 million died from this disease only in 2013. M.Tb is an aerobic, intracellular pathogen which inhabits lung tissue that is rich in oxygen. Tuberculosis is transmitted to a healthy individual when they inhale droplets carrying M.Tb. After inspiration of droplets into the alveoli of the lung, a complex immune response is triggered which activates alveolar macrophages (AM) to phagocytose the M.Tb and present internalized M.Tb to lysosomes. However, some M.Tb escape from the destructive environment of the lysosome and multiply in the alveolar macrophages. Such infected AMs become surrounded by additional macrophages and other immune cells with blood vessels and subsequently develop multicellular structures known as cellular granulomas which are the pathological hallmark of TB (Russell et al., 2009).

Later, as the granulomas mature, they become necrotized with a central caseum (cheesy liquid) portion lacking blood capillaries surrounded by a fibrotic rim. In such necrotic granulomas, infected AMs undergo cell lysis and release slowly-replicating or non-replicating M.Tb into the caseous centre. These necrotic

Abbreviations: AM, alveolar macrophages; ANOVA, analysis of variance; ATR-FTIR, attenuated total reflectance Fourier transform infrared spectroscopy; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DSC, differential scanning calorimetry; DSPE-PEG2k, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine *N*-(carbonyl-methoxypolyethylene glycol-2000); ED, emitted dose; EDX, energy dispersive X-ray; FPF, fine particle fraction; GRAS, generally recognized as safe; GSD, geometric standard deviation; HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; L_α, liquid crystalline phase; $L_{\beta'}$, planar gel; M.Tb, mycobacterium tuberculosis; MMAD, mass median aerodynamic diameter; MOC, micro-orifice contactor; NGI, next generation impactor; PDA, photodiode array; PEG, polyethylene glycol; POA, pyrazinoic acid; PXRD, powder X-ray diffraction; $P_{\beta'}$, rippled gel; RD, recovered dose; SD, spray dried; SEM, scanning electron microscopy; TB, tuberculosis; TGA, thermogravimetric analysis; WHO, World Health Organization.

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granulomas with caseous centres may create a suitable niche for slow growing M.Tb (Driver et al., 2012; Fenhalls et al., 2002; Hoff et al., 2010; Ryan et al., 2010; Seiler et al., 2003). Sterilization of such necrotic granulomas is extremely difficult using current drug therapy, even after administration of very high doses of antibiotics through oral and/or parenteral routes (Muttil et al., 2009). One of the possible reasons is the availability of sub therapeutic quantity of antibiotics for M.Tb at the granulomas that are located in peripheral regions of lungs. Hence, alternative routes, such as pulmonary route of drug administration, may be useful to deliver high quantity of anti-tubercular drugs locally in the lungs. Therapies using pulmonary drug delivery systems like dry powder inhalation therapy have several potential advantages: reduced systemic side effects; high concentrations of drug at the site of infection, potentially reducing the overall dose required for effective treatment, and thereby improving patient compliance (Bailey and Berkland, 2009; Fu et al., 2002).

Among the anti-tubercular drugs, pyrazinamide is the only drug which diffuses through the necrotic lesion caseum and is effective on non-growing persister bacteria (Mitchison, 2004; Patterson et al., 1999). From the necrotic caseum, pyrazinamide, a nicotinamide analogue, enters into the M.Tb by pH-dependent passive diffusion and is deaminated to pyrazinoic acid (POA; active form) by the bacterial pyrazinamidase/nicotinamidase. Then accumulation of the protonated POA causes acidification in the bacterial cell which disrupts the membrane potential and its transport function, leading to membrane damage (Zhang and Mitchison, 2003).

In general, phospholipids which are endogenous to lungs are generally recognized as safe (GRAS) excipients which can be rapidly metabolized and eliminated from the lungs after inhalation (Bosquillon et al., 2001; Pilcer and Amighi, 2010). Moreover, dry powder formulations composed of phospholipids either alone or in combination with other carriers have demonstrated good aerosolization behaviour (Bosquillon et al., 2001; Minne et al., 2008; Osman et al., 2013; Yang et al., 2011). The current study aimed to develop spray dried powders composed of pyrazinamide, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), distearoyl-*sn*-glycero-3-phospho ethanolamine *N*-(carbonyl-methoxypolyethylene glycol-2000) (DSPE-PEG2k) and L-leucine for effective lung delivery. DPPC is a naturally occurring phospholipid and is a major component in lung surfactant (Mansour and Zografi, 2007). DSPE-PEG2k is an amphiphilic polymer with a hydrophobic distearoyl phosphatidylethanolamine moiety which is endogenous to the lungs and has a hydrophilic, low molecular weight polyethylene glycol moiety which provides a putative mucus penetrating property to the particle (Lai et al., 2007; Muralidharan et al., 2014). Lai et al. (2007) reported that polystyrene nanoparticles coated with PEG2k are more diffusive (3× faster) through the fresh undiluted human mucus compared to non-PEGylated particles. Besides the endogenous nature of the phospholipids, both DPPC and DSPE-PEG2k have potential to decrease the drug release from the prepared particles due to their long fatty acyl chains (Carvalho et al., 2011; Croy and Kwon, 2006; Dabholkar et al., 2006; Torchilin 2005). L-leucine, an amino acid is regarded as a GRAS excipient for inhalation delivery by FDA. It is one of the most widely used amino acid in the spray dried inhalation powders which reduce the particle cohesion and improves the aerodynamic behaviour of the resultant particles. In this study, the SD powders were produced by varying the ratios of DPPC (5, 15 and 25% w/w) and pyrazinamide (70, 60 and 50% w/w), and maintaining the DSPE-PEG2k (5% w/w) and L-leucine (20% w/w) ratios constant. Further, this study investigated the effect of the powder composition on the physicochemical properties (particle size, moisture content, density, surface morphology, elemental composition, solid state nature, thermal behaviour and drug-excipient interactions) as well as the aerosolization performance.

2. Materials and methods

2.1. Materials

Pyrazinamide (Batch No. 14041/PYZ; 99.7% purity) was supplied by Amsal Chem. Pvt. Ltd. (Gujarat, India). 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC; ≥99% purity) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine *N*-(carbonyl-methoxypolyethylene glycol-2000) (DSPE-PEG2k; ≥98% purity) were purchased from Lipoid (Ludwigshafen, Germany). L-leucine (98% purity) and silicone oil (P. Code 378321; viscosity 10 cSt) were purchased from Hangzhou Dayangchem Co., Ltd. (Hangzhou, China) and Sigma-Aldrich (St. Louis, USA), respectively. Size 3 hard gelatine PEG capsules were kindly donated by Qualicaps (Osaka Japan). All other chemicals and solvents used in this study were of high performance liquid chromatography (HPLC) grade and purchased from Merck, Germany. Freshly collected and filtered (0.45 μm membrane filter) Milli-Q water was used.

2.2. HPLC analysis of pyrazinamide

A reverse-phase isocratic Shimadzu HPLC system (Shimadzu, Japan) comprising a LC-20AD solvent delivery unit, a photodiode array (PDA) detector (Shimadzu SPD-M20A), degasser (Shimadzu DGU-20A5) and an auto-sampler (Shimadzu SIL-20AC) with Classic-VP 7.4SP4 software was used to analyse pyrazinamide concentration in the samples. The mobile phase composed of 0.1 M sodium dihydrogen phosphate buffer (pH 4.4) and acetonitrile (90:10% v/v) was pumped at a flow rate of 1 mL/min through the Phenomenex® Synergi Fusion RP80A C_{18} column (4 μm, 150 mm × 4.6 mm; Phenomenex, California, USA) which was connected in series and preceded by a C_{18} security guard column (4.0 mm × 3.0 mm; Phenomenex® Fusion RP, CA, USA). Samples of 20 μL were injected using an auto sampler and analysed at a λ_{max} of 269 nm. The calibration curve was constructed over the concentration range of 0.5–80 μg/mL and was linear with a good correlation coefficient ($R^2 > 0.9997$). The intra-day and inter-day accuracy (at concentrations of 10, 50 and 70 μg/mL) and precision were within the acceptable limits (Coefficient variation %CV: ≤ 15 and % Bias: ≤ 15). The limit of detection (LOD) and limit of quantification (LOQ) of pyrazinamide were 0.04 and 0.11 μg/mL respectively.

2.3. Preparation of the spray dried pyrazinamide powders and percentage yield estimation

Table 1 shows the composition of the spray dried (SD) pyrazinamide powder formulations and their corresponding mass ratios of pyrazinamide, DPPC, DSPE-PEG2k and L-leucine. SD pyrazinamide powders were produced using a BUCHI B-290 Mini Spray Dryer (BÜCHI Labortechnik AG, Flawil, Switzerland) with a

Table 1
Composition of pyrazinamide spray-dried formulations, process yield, drug content, moisture content, particle size and powder density (mean ± SD, n = 3).

Formulations	$PDDL_0$	$PDDL_1$	$PDDL_2$	$PDDL_3$
Pyrazinamide (%)	100	70	60	50
DPPC (%)	0	5	15	25
DSPE-PEG2k (%)	0	5	5	5
L-leucine (%)	0	20	20	20
Process yield (%)	20.0	39.0	40.0	45.0
Drug content by HPLC (%)	95.3 ± 1.8	66.6 ± 0.7	57.7 ± 1.4	47.0 ± 1.7
Moisture content (%)	0.30 ± 0.10	0.03 ± 0.01	1.40 ± 0.56	1.26 ± 0.14
Particle size (μm)	4.26 ± 1.37	1.48 ± 0.60	1.37 ± 0.41	1.29 ± 0.58
Bulk density (g/cm^3)	0.22 ± 0.00	0.19 ± 0.01	0.16 ± 0.00	0.18 ± 0.02
Tapped density (g/cm^3)	0.51 ± 0.03	0.34 ± 0.02	0.32 ± 0.03	0.31 ± 0.06