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Inhaled Solid Lipid Microparticles to target alveolar macrophages for tuberculosis

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ABSTRACT

The goal of the work was to evaluate an anti-tubercular strategy based on breathable Solid Lipid Microparticles (SLM) to target alveolar macrophages and to increase the effectiveness of the conventional tuberculosis (TB) therapy. Rifampicin loaded SLM composed of stearic acid and sodium taurocholate were characterized for aerodynamic diameter, surface charge, physical state of the components, drug loading and release as well as drug biological activity on *Bacillus subtilis* strain. Moreover, SLM cytotoxicity and cell internalization ability were evaluated on murine macrophages J774 cell lines by MTT test, cytofluorimetry and confocal laser microscopy. SLM exhibited aerodynamic diameter proper to be transported up to the alveolar epithelium, negative charged surface able to promote uptake by the macrophages and preserved drug antimicrobial activity. The negligible *in vitro* release of rifampicin indicated the capacity of the microparticle matrix to entrap the drug preventing its spreading over the lung fluid. *In vitro* studies on J774 cell lines demonstrated SLM non-cytotoxicity and ability to be taken up by cell cytoplasm. The microparticulate carrier, showing features suitable for the inhaled therapy and for inducing endocytosis by alveolar macrophages, could be considered promising in a perspective of an efficacious TB inhaled therapy by means of a Dry Powder Inhaler device.

1. Introduction

Tuberculosis (TB) disease is caused by *Mycobacterium tuberculosis* which survives and replicates within human alveolar macrophages and characterized by a long chronic stage of infection and progressive pathology that mainly compromises (90% of cases) the respiratory system. In 1993, WHO declared tuberculosis a global public health emergency and today TB is still second only compared to HIV/AIDS, the greatest killer worldwide due to a single infectious agent. More than nine million people still develop active TB each year and nearly two million die. TB occurs all over the world with the highest burden in Asia and Africa (WHO, 2012).

Since 1995, over 51 million people have been successfully treated and an estimated twenty million lives saved through use of Directly Observed Treatment Short (DOTS) course. The recommended TB chemotherapy includes a multi-drug regimen with four first-line drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) administered for two months and a subsequent phase of four months with rifampicin and isoniazid (Blasi et al., 2009). However, poor adherence to administration schedules, several side effects and multi-drug resistant TB infections are chiefly responsible for chemotherapy failure. In the case of multi-drug resistant TB, the WHO recommends the use of DOTS-Plus chemotherapy that lasts 24 months with consequent severe toxicity and very high costs (Blomberg et al., 2001). In 2006, the WHO launched the Stop TB Strategy aiming to reduce the burden of TB and showing the need of new tools in prevention, diagnosis and treatment of TB, according to the Global Plan to Stop TB 2011–2015.

Current TB therapies have exploited conventional routes of administration, such as oral or intramuscular, based on high and frequent dosages to maintain the drug therapeutic concentration in infection site because of poor drug permeability, poor drug bioavailability and pre-systemic clearance. Several studies have been proposed to reduce toxicity and improve patient compliance for anti-TB drugs orally administered, such as micro- and nanoparticles with the objective of a once daily administration (Falk et al., 1997; Pandey et al., 2005). In addition, new administration routes have been explored for microparticles to be injected subcutaneously or intravenously, as well as for implants, although the discomfort related with these administration routes (Dutt and Khuller, 2001). Contrary to this, an alternative acceptable therapy to systemic treatments involves inhalation route delivering

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the drug directly to the desired site, enabling a rapid onset of the action and avoiding the long period of the current treatment and the first-pass metabolism, as well as the use of high doses of drug resulting in drug resistance onset and in severe side effects on other organs. Inhaled TB therapy presupposes the development of micro- or nanoparticles acting as drug carriers toward the alveolar region in the deepest lung so inducing the endocytosis process of alveolar macrophages being many antimicrobials difficult to be transported through cell membranes (Zhang et al., 2010). Among the breathable microparticulate systems, most of the studies have focused on biodegradable polymers such as poly (DL-lactide-co-gycolide) or alginates (Dutt and Khuller, 2001; Pandey et al., 2003; Zahoor et al., 2005; Zhang et al., 2010). Lipid-based particulate systems for TB inhaled therapy have been investigated less deeply though they were generally recognized as safe, non-swellable upon contact with the lung moisture and, consequently, able to retain the embedded drug before the target site. Among the lipid-based particulate systems, most of the studies have focused on liposomes (Chono et al., 2007; Justo and Moraes, 2003; Manca et al., 2012; Pandey et al., 2004; Rojanarat et al., 2012; Vyas et al., 2004; Zaru et al., 2009) whereas less attention has been paid to the solid lipid particles (Chuan et al., 2013; Pandey and Khuller, 2005) although their advantages over liposomes in terms of physical stability. Solid Lipid Microparticles (SLM), constituted by a solid lipid core stabilized by a surfactant at the surface, represent an advantageous approach to improve TB management. SLM exhibited several favorable properties as production without organic solvents, high drug loading levels and long-term stability. Furthermore, they could be considered proper to provide values of aerodynamic diameter essential for the particle deposition in the deep lung.

In the present study, SLM loaded with rifampicin, a first-line anti-TB drug, were designed in a perspective of an inhaled therapy for the treatment of TB infection. SLM formulation has involved the emulsification technique without the use of organic solvents that is different from those present in the literature for TB inhalation therapy and modified with respect to that used in our previous works concerning micro- and nanoparticles for the skin application (Iannuccelli et al., 2001, 2006, 2013). In this formulation, sodium taurocholate, as the anionic surfactant known to facilitate pulmonary absorption (Pilcer and Amighi, 2010), was introduced aiming to promote the uptake by the alveolar macrophages. The obtained SLM were evaluated for their aerodynamic properties (particle size and shape, bulk and tapped density), physical state of the components, surface charge, drug content and *in vitro* release. Furthermore, drug antimicrobial activity as well as microparticle cytotoxicity and ability to be taken up by murine macrophages were assayed.

2. Materials and methods

2.1. Materials

For SLM preparation, stearic acid from Fluka Chemie (Buchs, Switzerland), sodium taurocholate hydrate 97% from Alfa Aesar (Karlsruhe, Germany), rifampicin and Nile Red from Sigma-Aldrich Italia (Milan, Italy) were purchased. For the microbiological assay, tryptic soy agar from Difco Laboratories (Detroit, MI, USA) and *Bacillus subtilis* strain ATCC 6633 (10^5 CFU/ml) from American Type Cultures Collection (Manassas, VA, USA) were used. For the cytotoxicity and cell internalization investigations, murine macrophages J774 cell lines from IZSLER (Brescia, Italy), cell culture reagents DMEM (Dulbecco's Modification of Eagle's Medium) with high glucose, L-glutamin, Fetal Bovine Serum (FBS), penicillin–streptomycin (P/S), nonessential amino acids (NEAA), phosphate buffered saline (PBS) from EuroClone (Milan, Italy), and Hoechst 33258 stain from Hoechst (Frankfurt, Germany) were purchased. All the other chemicals were of analytical grade.

2.2. SLM preparation

SLM were produced by emulsifying the melted lipid through sonication. In practice, rifampicin (0.02 g) was dissolved in stearic acid (0.4 g) melted at 70 °C and emulsified with sodium taurocholate (0.06 g) in water solution (6 ml), by ultrasonic energy (Vibra-Cell, Sonics & Materials, Newtown, CT, USA) (78 W for 1 min) to obtain an oil in water (O/W) emulsion. Unloaded SLM were prepared by the same technique without rifampicin. SLM Nile Red labeled and unloaded SLM Nile Red labeled for the cell internalization studies were obtained by dissolving Nile Red (0.01%) in the melted stearic acid.

All the emulsions were rapidly cooled in ice under magnetic stirring (15 min), and freeze-dried (Lyovac GT2, Leybold-Heraues GmbH, Koln, Germany) to obtain a final dry powder. SLM were purified by a dialysis membrane (MWCO 12–14 000 Da; Medicell International Ltd, London, GB) for 3 h to remove the non-encapsulated drug before the drying process.

2.3. Morphology, size and Z-potential

Microparticle morphology, size and *Z*-potential were investigated on SLM and unloaded SLM. The morphology was evaluated by means of Environmental Scanning Electron Microscopy (ESEM, Quanta-200, Fei, Eindhoven, the Netherlands). Microparticle samples were placed on carbon stubs and imaged under low vacuum conditions (0.8 Torr) at 20 kV. Particle shape was investigated in terms of circularity parameter measured on at least 250 microparticles by means of processing software (ImageJ, CIGS, Modena, Italy) applied to ESEM photomicrographs.

Microparticle size and the size distribution were determined on ESEM photomicrographs by image processing software (Img-View, CIGS, Modena, Italy) of at least 250 microparticles for each image. *Z*-potential values, indicating particle surface charge, were measured on microparticle water suspension (0.1 g/l) by using Photon Correlation Spectroscopy (PCS) (Zetasizer version 6.12; Malvern Instruments, Worcs, U.K.) equipped with a 4 mW He–Ne laser (633 nm) and a DTS software (Version 5.0). The values were averaged on three determinations.

2.4. Density and porosity

The poured (bulk) density of SLM and unloaded SLM was determined by pouring known mass of powder under gravity into a calibrated measuring cylinder and recording the volume occupied by the powder. The tapped density of the samples was determined by volume measurement of tapped mass until no further change in the powder volume was observed. Apparent density and porosity values were determined by mercury porosimeter (Micromeritics AutoPore IV 9500; Micromeritics Instrument Corporation, Norcross, USA). All the values were averaged on three determinations.

2.5. Aerodynamic diameter

The aerodynamic diameter (d_A), defined as the diameter of a sphere of unitary density (ρ_0) that has the same sedimentation rate as the real particle, was determined for SLM and unloaded SLM using the following relationship:

$$d_A \cong d_v \left(\frac{\rho}{\chi \rho_0} \right)^{1/2}$$