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Sodium colistimethate loaded lipid nanocarriers for the treatment of *Pseudomonas aeruginosa* infections associated with cystic fibrosis

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ABSTRACT

Lung impairment is the most life-threatening factor for cystic fibrosis patients. Indeed, *Pseudomonas aeruginosa* is the main pathogen in the pulmonary infection of these patients. In this work, we developed sodium colistimethate loaded lipid nanoparticles, namely, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC), as a strategy to enhance the antimicrobial therapy against *P. aeruginosa* in cystic fibrosis patients. The nanoparticles obtained displayed a 200–400 nm size, high drug entrapment (79–94%) and a sustained drug release profile. Moreover, both SLN and NLC presented antimicrobial activity against clinically isolated *P. aeruginosa*. The integrity of the nanoparticles was not affected by nebulization through a mesh vibrating nebulizer. Moreover, lipid nanoparticles appeared to be less toxic than free sodium colistimethate in cell culture. Finally, an *in vivo* distribution experiment showed that nanoparticles spread homogenously through the lung and there was no migration of lipid nanoparticles to other organs, such as liver, spleen or kidneys.

1. Background

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the gene encoding for the CF transmembrane conductance regulator (CFTR) protein (Gibson et al., 2003). The absence of functional CFTR protein in the membrane of epithelial cells leads to chronic pulmonary disease, recidivant respiratory infections, pancreatic dysfunction, high electrolytes level in sweat and male infertility (WHO, 2002). It is estimated that 1 out of 2500 Caucasian newborns might be affected by CF, being the most common autosomal recessive disease (Sims et al., 2005). CFTR mutations cause malfunctioning of the membrane-bound cAMP regulated chloride channel, which in turn, produces plugs of mucus, obstruction and bronchial infections in the lung, constituting the main limiting factor of the disease in terms of morbidity and mortality (Heijerman et al., 2009; Ratjen et al., 2009). Among the pathogens that affect the CF patients, *Pseudomonas aeruginosa* (PA) is the most prevalent, but the treatment of its infections is often difficult due to the wide range of antimicrobial resistance of this species. This resistance to antimicrobials is a well documented phenomenon due to several molecular mechanisms such as the restricted outer membrane permeability, the presence of integron, insertion sequences, and the biosynthesis of degrading-enzymes (Fuste et al., 2013; Ruiz-Martinez et al., 2011a; Ruiz-Martinez et al., 2011b). *P. aeruginosa* infections usually start as an acute infection that finally becomes chronic. One of the main pathogenicity factors that favors *P. aeruginosa* colonization and resistance is its ability to develop a biofilm-like mucus layer in the viscous hypoxic media of CF patients' respiratory tract (Koch, 2002; Worlitzsch et al., 2002).

Currently, the preferred treatment is a high dose of inhaled antibiotic along with oral ciprofloxacin (Proesmans et al., 2013).

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The spread of multi-resistant bacteria strains together with the lack of new antibacterial agents drove the recovery of old antibiotics to treat CF patients and to apply new technologies, such as nanotechnology, to fight infections. Nowadays, both tobramycin and sodium colistimethate are the first-choice option for inhaled therapy to treat respiratory infections in CF patients (Heijerman et al., 2009). Although both the antibiotics proved to be effective against *P. aeruginosa*, they produced local side effects. Moreover, their administration is time consuming, is conditioned by an unpredictable systemic drug absorption and needs education and training. All these facts together induces poor adherence to the treatment (Heijerman et al., 2009).

Over the last decades, lipid nanoparticles have emerged as a promising drug delivery system that could overcome some limitations of the already existing drugs. Since only a few new antimicrobial entities have been discovered over the last years (Gould and Bal, 2013), nano-encapsulation of antibiotics is a good alternative for improving current treatments. Pulmonary delivery of lipid nanoparticles presents many advantages, such as, mucoadhesion, biodegradability, avoidance of first pass effect and hence the possibility to reduce the dose, good tolerability, deep lung deposition of drug and a sustained release of the API that leads to a longer dosing interval (Andrade et al., 2013; Weber et al., 2014). Many research groups have focused their efforts in developing inhalable nanoparticles to fight against bacterial resistances by encapsulating different drugs, such as amikacin (Ghaffari et al., 2011), tobramycin (Ungaro et al., 2012), ciprofloxacin (Chono et al., 2008; Wong et al., 2003), itraconazole (Alvarez et al., 2007) or amphotericin B (Gilani et al., 2011).

Taking the above into consideration, the aim of this work is to elaborate and fully characterize sodium colistimethate loaded lipid nanoparticles to be used in the treatment of infections in CF patients. Furthermore, the antimicrobial activity of the nanoparticles was assessed against a collection of *P. aeruginosa* strains isolated from CF patients. Finally, the *in vivo* pulmonary distribution was assessed.

2. Methods

2.1. Preparation of lipid nanoparticles

Two sodium colistimethate loaded formulations were elaborated, namely solid lipid nanoparticles (Colist-SLN) and nanostructured lipid carriers (Colist-NLC). An emulsion solvent evaporation technique was chosen for the preparation of Colist-SLN by modifying the procedure reported elsewhere (Soares et al., 2013). Briefly, 10 mg of antibiotic (Sigma–Aldrich, St. Louis, MO, USA) were mixed with a 5% (w/v) Precirol® ATO 5 (Gattefossé, Madrid, Spain) dichloromethane solution. Then, the organic phase and an aqueous surfactant containing solution (Poloxamer 188 at 1% w/v and Polysorbate 80 at 1% w/v) were mixed and emulsified by sonication at 20 W for 30 s (Branson Sonifier 250, Danbury, CT, US). The solvent was allowed to evaporate by magnetic stirring for 2 h at room temperature. Subsequently, the resulting SLNs were washed by centrifugation in Amicon® centrifugal filtration units (100,000 MWCO, Merck Millipore) at 2500 rpm for 15 min three times. For the Colist-NLC elaboration, a hot melt homogenization technique was selected (Beloqui et al., 2013; Obeidat et al., 2010). In brief, Precirol® ATO 5 and Miglyol® 812 (Sasol, Johannesburg, South Africa) were selected as the lipid core. Those lipids were mixed with the API and heated above the melting temperature of the solid lipid. The surfactant solution consisted of 1.3% (w/v) of Polysorbate 80 and 0.6% (w/v) of Poloxamer 188. The lipid and aqueous solutions were heated to the same temperature and then emulsified by sonication for 15 s at 20 W. Nanoparticles were stored at 4 °C overnight to allow lipid re-crystallization and particle formation. Then, a washing step was undergone by centrifugation at 2500 rpm in Amicon® centrifugal filtration units (100,000 MWCO) three times. All the nanoparticles prepared were freeze-dried with two different cryoprotectants, either D-mannitol or trehalose (15%).

For the preparation of infrared (IR) labeled NLC, the IR-783 dye was selected (Sigma–Aldrich). The use of IR-labeled NLC enables particle observation in the near infra-red (NIR) region that it is known to avoid tissue auto-fluorescence problems. NIR dye has been previously encapsulated in heparin–folic conjugates and demonstrated its ability to remain inside the nanoparticles (Yue et al., 2013). The NLCs were prepared just as mentioned previously, but by adding 50 mg of IR-783 instead of the antibiotic. The washing step was performed three times by centrifugal filtration and trehalose 15% (w/w) was added prior to the freeze drying.

2.2. Characterization of lipid nanoparticles

2.2.1. Size and zeta potential

The particle size and zeta potential (ζ) were measured in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) based on dynamic light scattering (DLS).

2.2.2. Microscopy analysis

Lipid nanoparticles were analyzed under transmission electron microscopy (TEM). Firstly, a negative staining was performed and after that the samples were observed.

In addition, lipid nanoparticles were imaged in air by using an Atomic Force Microscope (AFM) XE-70 (Park Systems, Suwon, Korea). All images were collected in a non-contact mode. Four types of images were simultaneously acquired with several scan sizes (100 μm^2, 25 μm^2 and 6.25 μm^2) at a scan rate of 0.3–0.5 Hz.

2.2.3. Encapsulation efficiency

Non-entrapped sodium colistimethate was determined from the supernatants recollected after centrifugation. The amount of non-encapsulated drug was detected by HPLC (see Section 2.2.4). For the IR-labeled nanoparticles, dye loading was calculated spectrophotometrically at 800 nm. The supernatant samples were diluted to 10 ml and compared to a calibration curve (5–80 μg/ml). In both cases, the encapsulation efficiency (EE) was calculated following this equation,

$$\mathrm{EE}\ (\%) = 100 \times \frac{(\text{initial amount of drug or dye} - \text{non} - \text{encapsulated drug or dye})}{\text{initial amount of drug or dye}}$$

2.2.4. Determination of sodium colistimethate by HPLC

The quantification of sodium colistimethate was conducted by a high performance liquid chromatographic (HPLC) technique adapted from Cancho Grande et al. (2000) and using a Waters 1525HPLC Binary Pump, UV-detector Waters 2487 and Waters 717 plus autosampler (Waters Corp., Milford, USA). The system was controlled by the Empower software. The column selected was a Novapak C18 × 150 mm with a 4 μm pore size. The mobile phase consisted of 77% of an aqueous solution and 33% of acetonitrile. The aqueous phase was prepared by dissolving (7.1 g) sodium sulphate, (0.6 g) acetic acid and (2.2 g) phosphoric acid and adjusted to pH 2.5 with tryethylamine up to 1 l. Sodium colistimethate was detected at 206 nm wavelength. The flow rate was fixed at 1.5 ml/min for isocratic elution, and 50 μl were set as an injected sample volume. This analytic technique was validated following EMA guidance for bioanalytical methods (Committee for Medicinal