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Pulmonary delivery of tobramycin-loaded nanostructured lipid carriers for *Pseudomonas aeruginosa* infections associated with cystic fibrosis

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ABSTRACT

Among the pathogens that affect cystic fibrosis (CF) patients, *Pseudomonas aeruginosa* is the most prevalent. As a way to fight against this infection, nanotechnology has emerged over the last decades as a promising alternative to overcome resistance to antibiotics in infectious diseases. The goal of this work was to elaborate and characterize lipid nanoparticles for pulmonary delivery of tobramycin.

Tobramycin-loaded nanostructured lipid carriers (Tb-NLCs) were prepared by hot melt homogenization technique. In addition, nanoparticles labeled with infrared dye (IR-NLCs) were used to investigate their *in vivo* performance after pulmonary administration.

Tb-NLCs displayed a mean diameter size around 250 nm, high drug encapsulation (93%) and sustained release profile. Tb-NLCs showed to be active against clinically isolated *P. aeruginosa*. Moreover, Tb-NLCs did not decrease cell viability and were able to overcome an artificial mucus barrier in the presence of mucolytics agents. During the *in vivo* assay, IR-NLCs were administered to several mice by the intratracheal route using a Penn Century® device. Next, the biodistribution of the nanoparticles was analyzed at different time points showing a wide nanosystem distribution in the lungs.

Altogether, tobramycin-loaded NLCs seem to us an encouraging alternative to the currently available CF therapies.

1. Introduction

Cystic fibrosis (CF) is a genetic disorder that affects nearly 70,000 patients worldwide. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a protein that form an ion channel in epithelial cell membranes whose dysfunction leads to the secretion and accumulation of a vicious mucus in the airways that become thick and sticky causing bronchial obstruction (Moreau-Marquis et al., 2008). The tenacious mucus enables chronic bacterial infection by an opportunist gram-negative bacterium, which is the most frequent pathogen identified in CF patients (Ratjen et al., 2009). Mucoid strains of *P.* aeruginosa usually develop community of microbes in an exopolysaccharide matrix called biofilm. Although CF patients routinely take antibiotics, the mucus plugs and bacterial biofilm contribute to the poor lung penetration of antimicrobial agents, leading to clinical exacerbations (Okusanya et al., 2009). Therefore the eradication of these organisms is a difficult but essential endpoint to achieve. Furthermore, over the last decades, antibiotic-resistant strains have increased due to the misuse and overuse of anti-infectious drugs (Andrade et al., 2013). As a consequence, there is a high morbidity and mortality associated to the respiratory manifestations of the CF disease hampered by the lack of effective therapies (Savla and Minko, 2013).

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In this regard, nanotechnology has emerged as a new alternative to drug encapsulation to overcome the limitations of conventional drugs. Nanoformulations such as nanostructured lipid carriers (NLCs) are made up of a solid lipid core stabilized by surfactants and have the possibility to incorporate both lipophilic and hydrophilic drugs. NLCs provide several advantages over other drug delivery systems (DDSs) such as good biocompatibility, biodegradable properties, higher drug loading, controlled drug release, long-term stability, as well as scaling-up feasibility (Weber et al., 2014).

Nanoparticles (NPs) are currently being extensively investigated for antibiotic inhalation therapy. Pulmonary drug delivery has gained much attention as a non-invasive route for the delivery of high amounts of therapeutic agents directly to the desired site of action minimizing systemic exposure and adverse effects (Patil and Sarasija, 2012). This route is a preferred route for agents such as amino-glycosides in CF patients. The inhalation of tobramycin is part of current CF therapies as it presents strong bactericidal activity against planktonic cells (Thellin et al., 2015). For instance, TOBI® or TIS (tobramycin inhalation solution) and TIP (tobramycin inhalation powder) have recently become commercially available for the treatment of chronic lung infections caused by *P. aeruginosa* (Waters and Smyth, 2015). However, the efficacy of free drug administration in CF patients is not high enough to achieve therapeutics levels at the site of infection due to its rapid clearance and poor mucus penetration (Tseng et al., 2013). These drawbacks could be overcome by nanotechnology. Encapsulation of antibiotics into nanocarriers has attracted considerable interest to improve the therapeutic index of antimicrobial drugs and for their benefits in the context of combating bacteria (Hajipour et al., 2012). Moreover, many cystic fibrosis patients present an accumulation of dehydrated and thicker mucus within the airways causing respiratory problems, therefore, it is important for therapeutic agents to penetrate into this mucus in order to distribute the drug and maximize its antibacterial effect (Yang et al., 2011). Nano-antibiotic represents a promising strategy to overcome the mucus barrier and to prolong the drug retention in the lung as other authors have also previously reported (Poyner et al., 1995).

Taking the above into account, the goal of this work was to elaborate and characterize tobramycin-loaded lipid nanocarriers (Tb-NLCs) for pulmonary delivery for the treatment of respiratory infectious diseases; in particular CF. Two different solid lipids were selected as core agents for the NLCs (Precirol® ATO 5 and Compritol® ATO 888). The antimicrobial activity against *P. aeruginosa* was investigated as well as the capability of the nanoparticles to cross the mucus barrier *per se* or after adding mucolytic agents. Finally, the NPs biodistribution was analyzed after intratracheal administration in mice.

2. Material and methods

2.1. Materials

Precirol® ATO 5 (glycerol distearate, type I) and Compritol® 888 ATO (glyceryl dibehenate) were kindly provided by Gattefossé (Madrid, Spain). Kolliphor® P188 (Poloxamer 188) was a gift from BASF (Ludwigshafen, Germany). Polysorbate, Tween® 80 was purchased from Panreac Química (Castellar del Vallès, Barcelona, Spain). Miglyol® 812 was provided by Sasol (Hamburg, Germany). Tobramycin, fluorescamine, IR-783 dye, gelatine from bovin skin type B, diethylenetriaminepentaacetic acid (DPTA), type II mucine from porcin stomach, egg yolk enrichment, amino acids and were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Coomassie Brilliant Blue was provided from Bio-Rad Laboratories (Hercules, CA, USA). PBS, DPBS, DMEM, MEM-NEAA, FBS, DMSO and Trypsin-EDTA Gibco® were supplied by Life Technologies (Thermo Fisher Scientific, Waltham, MA). Blood agar was provided by Oxoid (Microbioligical Products Thermo Fisher, Hampshire, UK). Mannitol (Pearlitol PF) and L-lysine-*S*-carboxymethyl-L-cysteine salt were provided from Roquette and Pharmazell (India), respectively. Hydroxyethylcellulose was purchased from Vencaser S.A (Bilbao, Spain). Other chemicals were all analytical grade.

2.2. Methods

2.2.1. Preparation of nanostructure lipid carriers (NLCs)

The nanostructured lipid carriers, NLCs, were elaborated by a hot melt homogenization technique (Pastor et al., 2014). In brief, Precirol® ATO 5 (NLC P) or a 50:50Compritol® 888 ATO and Precirol® ATO 5 mixture (NLC PC) together with Miglyol® 812 were selected as lipid core. These lipids were mixed with tobramycin (Tb) and heated above the melting temperature of the solid lipid until its fusion. As tobramycin is a hydrophilic drug, it was dispersed in the molten lipids. The aqueous phase was prepared by dispersing 1.3% (w/v) of Tween® 80 and 0.6% (w/v) of Poloxamer 188 in Milli-Q water and heating to the same temperature as the lipid phase. Straightaway, the hot aqueous phase was added to the oily phase, and then sonicated for 30 s at 20 W (Branson Sonifier 250, Danbury, CT, USA). The formed emulsion was stored for 12 h at 4 °C to allow the re-crystallization of the lipids and the nanoparticle formation. Then, Tb-NLCs were washed by centrifugation at 2500 rpm in Amicon® centrifugal filtration units (100,000 Da MWCO, Merck Millipore, Darmstadt, Germany) for 15 min three times. All the nanoparticles prepared were freeze-dried for 39 h (Telstar Lyobeta freeze-dryer, Terrasa, Spain) using trehalose at 15% (w/w) as cryoprotectant. Different batches of blank nanoparticles without the drug (Blank-NLCs) were also prepared for comparison.

Two types of dyes were used to label NLCs. Firstly, Coomasie blue (CB) labeled NLCs (CB-NLCs) were prepared for the artificial mucus (AM) penetration assay. NLCs were prepared just as mentioned above but instead of the drug, 1.3% (w/w) of CB was added. Secondly, to prepare labeled NLCs, an infrared dye (IR-783) was embedded into the nanoparticles by adding 50 mg of IR instead of the antibiotic. This dye is an excellent stain for the observation in the near infrared region (NIR). In both cases, the nanoparticles were washed by centrifugal filtration units and trehalose 15% (w/w) was added prior to the lyophilization step.

2.2.2. Characterization of lipid nanoparticles

2.2.2.1. Size and Zeta potential. The particle size and polydispersity index (PDI) were measured in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) based on dynamic light scattering. Zeta potential was also determined by Doppler velocimetry by means of the Zetasizer Nano ZS. Prior to the measurements NPs were dispersed in Milli-Q water at optimal intensity. All the measurements of each sample were performed in triplicate.

2.2.2.2. Microscopy analysis. NPs morphology was examined by Transmission Electron Microscope (TEM, Philips CM120) after negative staining with 2% uranyl acetate.

2.2.2.3. Encapsulation efficiency. The encapsulation efficiency (EE) of tobramycin into NLCs was determined by indirect and direct methods. By the indirect method, the supernatants obtained during the centrifugation in Amicon® devices were analyzed for Tb content by UV–vis spectrophotometer after derivatization with fluorescamine (Sampath and Robinson, 1990; Ungaro et al., 2012). Briefly, NLCs samples were diluted 1:2 (v/v) in fluorescamine solution at 0.5% (w/v) in ethanol and incubated at room