Bedaquiline loaded lipid nanoparticles: a promising candidate for TB treatment

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ABSTRACT

Four different kinds of Lipid Nanoparticles (LNP) encapsulating bedaquiline have been tested in order to evaluate the possibility to decrease adverse effects of this very efficient and recent antibiotic. The parameters that are studied among these different types of LNP are the surface charge as well as targeting of macrophages through trimannose groups grafted to the surface. It was found that these nanocarriers could encapsulate bedaquiline with a very high loading efficiency, exhibited a very good colloidal stability in storage conditions as well as in biological media, and an excellent compatibility with animal cells. Finally, first *in vivo* evaluation showed that these carriers are able to accumulate strongly in the lungs and that the almost neutral ones without targeting ligands could be the best candidate for tuberculosis treatment.

Keywords: Lipid Nanoparticles, bedaquiline, antibiotics, nanomedicine

1. INTRODUCTION

In the last years, the increase in antimicrobial resistance, together with a lack of new drugs for the treatment of bacterial infections resistant to classical antibiotics are of growing concern. In 2012 the FDA approved the use of bedaquiline, as the first new effective drug against TB in the last 40 years. Despite its effectiveness, unfortunately bedaquiline shows serious side effects including induction of life-threatening cardiac arrhythmias which can be so dangerous that at present it is to be prescribed only when no other treatment options are available.

Novel drug delivery systems based on nanocarriers are a promising strategy to overcome current therapeutic limitations thanks to nanomaterials unique physicochemical properties [1]. These include their small size, which allows them to reach the cellular level, their high surface to volume ratio, which increases interactions with target cells and their ability to be structurally and functionally modified to control their biodistribution [2].

In this context, the development of effective and safe nanotechnology-based methods to decrease side effects of bedaquiline is highly relevant. The main challenge is to obtain different properties such as high stability in storage conditions, a kinetics of drug release *in vivo* compatible with a therapeutic effect and surface properties enabling the penetration of the nanoparticles (NP) deep in the lungs. To this aim, we have assessed the behavior of neutral and positively charged lipid bedaquiline-NP in order to evaluate this kind of carrier as a novel nanotherapeutic to cure tuberculosis. Bedaquiline was encapsulated in lipid nanoparticles with high encapsulation efficiency (EE) and drug loading (DL) values. The efficacy of the drug-encapsulating nanocarriers has been demonstrated *in vitro* against *Mycobacterium tuberculosis*, combined with an excellent compatibility of the carriers with human cells. The accumulation of these LNP in the lungs of *M. tuberculosis* infected mice treated by repetitive injections of fluorescent bedaquiline–LNP has also been demonstrated as well as their efficacy to decrease batcerial burden in the lungs. The obtained results open the way for further studies on multi-drug resistant strains of *M. tuberculosis* and for *in vivo* studies of the optimized nanocarriers.

2. MATERIALS AND METHODS

2.1 Materials

Bedaquiline was obtained from AURUM Pharmatech LLC (Franklin Park, NJ, USA). Myrj™ S40 (PEG 40 stearate, 1980 Da) and Super Refined Soybean Oil were obtained from Croda Uniquema (Chocques, France). Suppocire NB™ was purchased from Gattefosse S.A. (Saint-Priest, France). Lipoid® S75-3 (soybean lecithin at 69% of phosphatidylcholine) and hydrogenated S75 were provided by Lipoid GmbH (Ludwigshafen, Germany). DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane (chloride salt)) was purchased from Avanti® Polar Lipids, Inc. (Alabaster, Alabama, USA). The fluorophore DiI Stain, D282) was purchased from ThermoFisher Scientific (Illkirch, France).

Plasma sample from healthy donor (Etablissement Français du Sang (EFS), Grenoble, FRANCE) collected by apheresis was used. According to the ethical and legal

standards of our blood supplier (EFS), informed consent was given by blood donors. Blood usage was allowed by Health Department of Research Ministry as described in the delivered French directive #DC-2008-334. *M. tuberculosis* cultures, reference strain H37Rv, were maintained in a BSL-3 laboratory. Difco™ Middlebrook 7H9 broth and BBLTM Middlebrook ADC Enrichment were purchased from BD (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Resazurin sodium salt was from Sigma.

Cell lines THP-1 were and HepG2 were obtained from the ECACC: (cat. N° 88,081,201 and 85,011,430 respectively). Cell line A549 was available for this work at the University of Zaragoza. Cell culture media, Roswell Park Memorial Institute medium (RPMI) and Dulbecco's Modified Eagle Medium (DMEM), used for cytotoxicity assays, cell culture maintenance and release profile assays, were obtained from Lonza (Basel, Switzerland). The Fetal Bovine Serum was from GIBCO. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromid) and Neutral Red were obtained from Sigma-Aldrich, as well as Phorbol 12-myristate 13-acetate (PMA).

2.2 Preparation of bedaquiline-loaded LNP

Lipid nanoparticles (LNP) were prepared by blending lipid phase, composed of wax, oil, soya lecithin and DOTAP for LNP(-) only, and aqueous phase containing MyrjS40 solubilized in PBS, using ultrasonic process to provide stable nanodroplets. After homogenization at 55 °C, both phases are crudely mixed and sonication cycles are performed at 55 °C during 5 min. Non-encapsulated components are separated from Lipid Nanoparticles (LNPs) by dialysis with PBS, changing twice the equilibrating PBS buffer (with a volume equals 200× the LNP dispersion volume) and during 24 h.

For LNP(-), the lecithin/PEG surfactant weight ratio was of 0.17 and the surfactant/core weight ratio was of 0.85. For cationic LNPs, the lecithin/ PEG surfactant weight ratio was of 0.051 and surfactant/core weight ratio was of 0.67.

2.3 Determination of bedaquiline EE and DL

To determine the EE of bedaquiline in LNPs, the drug concentration in the purified dispersion, after intense dialysis, was measured by destabilization of LNPs in hydro-organic medium leading to liquid extraction of bedaquiline in organic solvent. Bedaquiline-loaded LNPs were diluted to 1:4 with acetonitrile, then vortexed to destroy the LNPs and release bedaquiline. A centrifugation of samples was then performed at 13,000 G for 10 min. The supernatant was gently withdrawn and transferred in convenient vial for HPLC-UV analysis.

2.4 Characterization of bedaquiline-loaded LNP

The hydrodynamic diameter, polydispersity index and ζ potential of the lipid nanoparticles were measured with a Zeta Sizer Nano instrument (NanoZS, Malvern). The hydrodynamic diameter and PDI were measured with a 0.6 mg/mL LNP dispersion in PBS at 25 °C. ζ Potential was measured with a 0.4 mg/mL LNP dispersion in 1mM NaCl, at pH 7.4 and at 25 °C.

2.5 MIC and Cytotoxicity assays

M. tuberculosis (reference strain, H37Rv) cultures were routinely grown at 37 °C in Middlebrook 7H9 medium supplemented with 10% ADC and 0.05% Tween® 80. The Minimum Inhibitory Concentration (MIC) was determined using the Resazurin Microtiter Assay Plate (REMA).

Cell lines A549 and HepG2 cells were cultured in T25 vented flasks with DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin, incubated at 37 °C/5% CO2. For the assays cells were seeded at a cell density of 10^4 cell/mL for A549, and 2.5×10^4 cell/mL for HepG2. THP-1 cells were cultured in T25 vented flasks with RPMI-1640 supplemented with 10% fetal bovine serum, penicillin and streptomycin. For the assays, cells were seeded at a density of 5×10^4 cell/mL, and they differentiated into adherent macrophage-like cells by incubating with PMA at 5 ng/mL for 48 h at 37 °C/5% CO2 [24]. After this period, the medium was changed to RPMI without PMA for 24 h more before they were exposed to the nanocarriers [24].

For the MTT viability assay, cells were seeded into a 96-well plate and maintained in culture for 24 h at 37 °C/5% CO_2 to form a semiconfluent monolayer. They were then exposed to the nanocarriers using for this purpose DMEM or RPMI medium without phenol red, to avoid interference with the results. LNPs were tested at 8 different concentrations prepared as twofold serial dilutions. The range of final concentrations tested was from 1 mg/mL to 7.81 µg/mL. After 24 h of exposure, the culture medium was removed and 50 µL of 1 mg/mL MTT was added to each well and incubated for 2 h at 37 °C/5% CO_2. After this incubation, supernatant was removed and the formed formazan crystals were solubilized in isopropanol. The colour intensity of the dissolved formazan is determined by photometric measurements at 570 nm and the percentage of viable cells was obtained by correlation to the intensity of the untreated control (100% viability). For Neutral Red Uptake (NRU) assay, cells were seeded into a 96- well plate and maintained during 24 h at 37 °C/5% CO_2 to form a semiconfluent monolayer. They were then exposed to the nanocarriers using for this purpose DMEM or RPMI without phenol red, to avoid interference with the results. After 24 h exposure, the culture medium was removed and 100 µL of 0.005% neutral red solution were added to each well and further incubated at 37 °C/5% CO2 for 3 h. The plates were then washed with PBS and the dye was extracted with ethanol/acetic acid solution before reading fluorescence of each well (530/645 nm). The number of

viable cells was obtained by correlation to the intensity of the untreated control (100% viability) [25].

2.6 *In vivo* LNP Biodistribution and efficacy

Mice C3HeB/FeJ were infected at day 0 by *M.tb*. H37Rv through an aerosol exposure. After 30 days, mice were treated by repeated injections every 2 days of bedaquiline-loaded LNP through IV injections in the tail. Fluorescence imaging using the IVIS®Kinetic system (Perkin Elmer Inc.) was performed at day 7 and 13 after first injection of LNPs. Images were taken using the following settings: excitation filter 605 nm, emission filter 680 nm, f-stop 1.2, exposure time 0.1 s. Fluorescence intensity is presented as efficiency and depicted as false colour, where dark red corresponds to the lowest intensity and yellow to the highest intensity.

At day 13 after first injection of LNPs, animals were sacrificed by cervical dislocation whilst under anesthesia. Lungs and spleens were removed aseptically and homogenized by mechanical disruption in sterile WTA buffer consisting of 1% (w/v) BSA (Serva, Heidelberg, Germany), 1% (v/v) Tween 80 (Sigma-Aldrich) in sterile water. A cfu assay was performed by plating a series of 10-fold dilutions of tissue homogenates onto 7H11 agar plates with 10% OADC supplement and 0.5% glycerol. Bacteria were grown on Middlebrook 7H11 agar plates. Mycobacterial colonies were counted after 3-4 weeks incubation at 37°C.

3. RESULTS

3.1 LNP characterization

The physicochemical properties of LNPs have been characterized. Both types of LNPs exhibited as expected [3, 4] a small size, below 100nm, in order to exacerbate interactions at the cellular level and to penetrate in tissues, especially in the lungs. They are weakly polydisperse as PDI values are below 0.15 and zeta potential were in agreement with the differences on nanocarrier, indicating the presence of a strong positive charge in the case of LNPs(+). Finally, as expected, the LNPs are very stable in storage conditions, without significant drug release during 6 months.

	DL, %	EE, %	Hydrodynamic diameter, nm	PDI	Z-potential, mV	DL (6 months)/DL (t0),%
LNP(−)	2.8 ± 0.15	15 93 ± 6	86 ± 1.3	0.148	−10 ± 1.1	97 ± 5
LNP(+)	2.8 ± 0.15	93 ± 7	83 ± 3.1	0.123	+28 ± 3	98 ± 4

Table 1: Nanocarriers characterization after bedaquiline encapsulation

The profile of the release of bedaquiline from nanocarriers was determined in media selected on the basis of conditions used in the in vitro assays. LNPs loaded with bedaquiline were found to be very stable in PBS, RPMI and Middlebrook 7H9 medium (in all cases less than 10% drug was released after one week) indicating again a very good colloidal stability of these carriers.

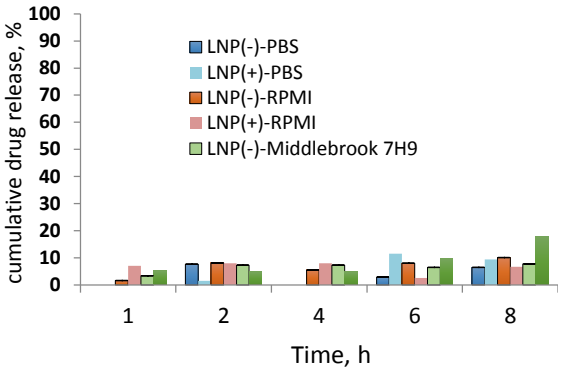

Figure 1: Cumulative drug release at 37°C of LNP(-) and LNP(+) in storage buffer, PBS, and in vitro culture media.

Release in human plasma was also studied for future intravenous injection applications and data are reported in Figure 2. Results indicated that the kinetics of the release is fully compatible with a potential therapeutic effect as a circulating time of a few hours should allow distribution of the LNPs to the lungs.

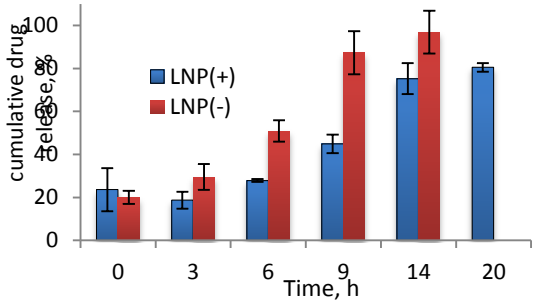

Figure 2: Cumulative drug release at 37°C of LNP(-) and LNP(+) in human plasma.

3.2 *In vitro* antimicrobial activity and cytotoxicity studies

The in vitro antimicrobial activity of the nanocarriers against M. *tuberculosis* H37Rv was determined using the well-established REMA assay [23], and compared to that of free bedaquiline (Table 2). The MIC of empty carriers was determined as well, to ensure that, within the range of concentrations used in the assay, the antibacterial effect was only due to the antimicrobial drug and not to the nanocarrier itself (data not shown). As shown in Table 2, bedaquiline was still very active against *M. tuberculosis* after encapsulation in the carriers. No difference have been

found, showing that the drug is still active after encapsulation.

Nanocarrier	MIC (µg/mL of BQ)	Corresponding concentration of nanocarrier (µg/mL)
Free BQ	0.03	—
LNP(−)	0.03	1–2
LNP(+)	0.03	1–2

Table 2: MIC values of bedaquiline-loaded nanocarrier

Both methods MTT and NRU were assayed in three different cell lines: lung epithelial human cells (A549) because *M. tuberculosis* infection is located in the lungs, liver epithelial human cells (HepG2) as the nanoparticles would circulate through the liver, and human monocytes from peripheral blood (THP-1), chosen because *M. tuberculosis* is an intracellular pathogen that resides inside the macrophages. Results are reported in Table 3. The cytotoxicity of the particles was detected only at very high concentrations, over 1 mg/mL. The tolerated concentrations are thus far superior to those needed to reach the minimum inhibitory concentration of the drug, i.e. 1–2 µg/mL.

	Nanocarrier concentration corresponding to 70% of viability (µg/mL of nanocarrier)					
	MTT			NRU		
	A549	HepG2	THP1	A549	HepG2	THP1
LNP(−)	850	>1000	>1000	>1000	>1000	>1000
LNP(+)	600	>1000	>1000	>1000	250	>1000

Table 3: MTT and NRU results in different cell lines.

3.3 *In vivo* LNP Biodistribution and efficacy

In addition, in vivo drug LNP biodistribution has been performed with TB infected mice, in order to evaluate the ability of these carriers to accumulate in the lungs. Results showed that both kinds of LNPs accumulated strongly in the lungs during a 2 weeks administration of the bedaquiline loaded carriers, indicating that these carriers are well designed for the intended application thanks to their size, below 100nm, as well as their good colloidal stability in complex biological media like blood. For the two different surface charges of LNP(+) and LNP(-), no significant difference in accumulation in the lungs could be observed related to trimannose groups beared or not at the surface of the particles for macrophages targeting. This could be due to the protein corona formation in blood which can hinder targeting ligands. On the contrary, higher accumulation seemed to occur with slightly anionic LNPs compared to cationic ones. This indicates that regardless of the size, the surface charge of the nanocarriers is an essential feature for lungs targeting and that almost neutral LNPs are more able to enter deep in this organ colmpared to strongly positively charged LNPs.

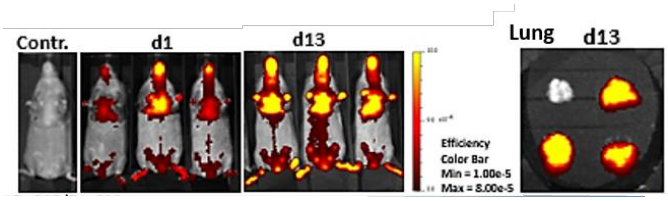

Figure 3: LNP accumulation in mice

Finally bacterial burden in the lungs has been determined in order to evaluate the activity of these bedaquiline-loaded nanocarriers. After 13 days of repeated injections every 2 days of the different types of LNPs, first results indicated that bedaquiline-loaded nanocarriers were able to diminish bacterial infection of the lungs as the bacterial burden was significantly decreased compared to the control without treatment. Moreover it seemed that slightly anionic LNPs are more efficient than cationic ones and that trimannose groups at the surface of the LNPs did not improve efficiency of the treatment. These results, in good correlation with fluorescence imaging of the accumulation of LNPs in the lungs, have been obtained with a limited number of mice/group and will be confirmed in order to chose definitely the best candidate for future in vivo toxicity study.

4. CONCLUSION

In conclusion, this work enabled us to demonstrate that nanoencapsulation of bedaquiline in lipid nanocarrier is a very promising way to improve bedaquiline-based treatments of *M. tuberculosis* infections. Up to now, we could manage to identify the best LNP carrier among 4 types, with different surface charges and with or without active targeting against macrophages, which appear very promising due to its capacity to enter inflamed tissues. Further studies would imply to better characterize whether this new bedaquiline formulation can decrease adverse effects, like cardiac ones thanks to a lower accumulation in heart when the drug is delivered after nanoencapsulation. The other perspective is to test this new nanotherapeutics against resistant strains both *in vitro* and *in vivo* with the aim to improve treatment against MDR-TB.

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