1 **INHALABLE CLARITHROMYCIN MICROPARTICLES FOR TREATMENT OF**
2 **RESPIRATORY INFECTIONS**
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26 **ABSTRACT**
27 **Purpose** The aim of this work was to develop clarithromycin microparticles (CLARI-
28 MP) and evaluate the aerodynamic behavior, safety in bronchial cells and anti-bacterial
29 efficacy.
30 **Methods** Microparticles containing clarithromycin were prepared as dry powder carrier
31 for inhalation, using leucine and chitosan. CLARI-MPs were deposited on Calu-3 grown
32 at air-interface condition, using the pharmaceutical aerosol deposition device on cell

cultures (PADD OCC). Deposition efficacy, transport across the cells and cytotoxicity were determined. Anti-antibacterial effect was evaluated against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*.

Results Microparticles were of spherical shape, smooth surface and size of about 765 nm. Aerosolization performance showed a fine particle fraction (FPF) of 73.3%, and a mass median aerodynamic diameter (MMAD) of 1.8 μm . Deposition on Calu-3 cells using the PADD OCC showed that 8.7 $\mu\text{g}/\text{cm}^2$ of deposited powder were transported to the basolateral compartment after 24 h. The safety of this formulation is supported by the integrity of the cellular epithelial barrier and absence of toxicity. Clarithromycin microparticles maintained their antimicrobial efficacy against Gram positive and Gram negative bacteria.

Conclusions The appropriate aerodynamic properties and the excellent deposition on Calu-3 cells indicate that clarithromycin microparticles are suitable for administration via pulmonary route and are efficient to inhibit bacteria proliferation.

KEY WORDS

Aerosol; Dry Powder Inhaler; Lung infection; Safety

RUNNING HEAD

Clarithromycin microparticles for respiratory infections

ABBREVIATIONS

CLARI - Clarithromycin

CLARI-MPs – Clarithromycin microparticles

DPI - dry powder inhalers

DSC - differential scanning calorimetry

ED – emitted dose

FPF - fine particle fraction

57 IS – internal standard
58 LC-MS/MS - liquid chromatography - tandem mass spectrometry
59 MIC - minimal inhibitory concentration
60 MMAD - mass median aerodynamic diameter
61 NGI - next-generation pharmaceutical impactor
62 PADD OCC - pharmaceutical aerosol deposition device on cell cultures
63 PM – physical mixture
64 RI – respiratory infection
65 SEM - scanning electron microscopy
66 TEER - transepithelial electrical resistance

68 INTRODUCTION

69 Respiratory infections (RI) have been treated since long time with antibiotics. Despite
70 of the great progress of antimicrobial agents in the last century, it is still a challenge to
71 treat bacterial infection, mainly due to the development of resistance. Traditionally, the
72 treatment of RI consists on high doses of single or combined antibiotics administered
73 by oral or intravenous route, which induce undesirable side effects mainly due to high
74 systemic bioavailability. To overcome this and improve the efficacy of RI therapy, the
75 inhalation route has been used with pressurized metered dose inhalers (pMDIs),
76 nebulizers and dry powder inhalers (DPI) (1-4). Inhalation route has several
77 advantages including: rapid onset of action (2), high drug amount targeted to the lungs
78 (1, 5), lower systemic bioavailability leading to a decrease of both, side effects (6) and
79 drug resistance build-up (7). However, DPI devices have some advantages over other
80 methods of pulmonary drug delivery, such as the direct delivery of drug into the deep
81 lungs utilizing the patient's breathing.
82 Among several methods available to produce inhalable particles as DPI, spray drying is
83 one of the most used. Spray drying is performed in a one-step process, and according
84 to the materials and conditions used (8) it is possible to produce dry powder particles

with specific and controlled characteristics (such as particle size, size distribution and particle surface morphology). The particles properties might have direct influence on the aerosol properties of DPI formulation and consequently on the DPI deposition in the respiratory tract (9).

Nevertheless, the safety and efficacy of new DPI formulations must always be tested to predict their effects on the body before clinical use. Moreover, deposition *in vitro* should considerer the physiological condition *in vivo*. Thereby Hein et al. developed a device called Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADD OCC), which allows studying the DPI deposition and absorption in pulmonary epithelial cells (10). This system avoids the cell damage caused by turbulences of air streams as shown for example with electrostatic precipitation (11). Moreover, the PADD OCC use the sedimentation process, that is the mainly deposition mechanism in the deep lung, while twin stage impinger (TSI), multistage liquid impinger (MSLI) and anderson cascade impactor (ACI) rely on deposition by impaction (4, 12-14).

Tobramycin is the only antibiotic available in the market as DPI (15), but others antibiotics are under investigation to be also used as DPI for the treatment of RI; these include aminoglycosides, fluoroquinolones, glycopeptides, macrolides, monolactams and polypeptides (16).

Clarithromycin (CLARI) is a hydrophobic macrolide, which is known by its bacteriostatic action and the anti-inflammatory effect (17, 18). Macrolides bind reversibly to domain V of 23S ribosomal RNA (rRNA) in the 50s subunit of the bacterial ribosome, inhibiting RNA-dependent protein synthesis (18). CLARI together with its active metabolite, 14-hydroxycarithromycin, are responsible for enhanced antimicrobial activity against respiratory pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Escherichia coli*, *Mycobacteria smegmatis* and *Haemophilus influenza* (18). Despite been used to that broad pathogen spectrum, several problems are reported with CLARI treatment, mainly due to its low oral bioavailability (55 %), side effects (gastrointestinal disturbs, hepatotoxicity and renal

tubular degeneration) and pathogen resistance caused by the high antibiotic doses administered (19, 20). The administration of clarithromycin as DPI could improve the antimicrobial activity and overcome adverse effect, reaching thus a better patient compliance. Some works have been done towards to develop DPI-CLARI. Park and coworkers already developed a DPI-CLARI formulation with excellent aerosol performance (1). Moreover, Moghaddam increased the yield and fine powder fraction (FPF) with the addition of leucine in a new PLGA-clarithromycin formulation (21). However, these papers did not test the efficacy of the formulations against respiratory pathogens and the safety in pulmonary cells. Recently, Haghi et al. developed a clarithromycin as pMDIs intended for anti-inflammatory therapy, with beneficial effects on cell barrier integrity, regulation of anti-inflammatory cytokine (IL-8) and inhibition of mucus production after deposition of Clarithromycin at low concentration on Calu-3 cells (4). The administration of clarithromycin, a hydrophobic drug, as pMDIs or nebulizers needs to use solvents to solubilize it, while DPI will avoid solvent exposition. Therefore the aim of this work was to explore clarithromycin-microparticles as DPI, using chitosan as mucoadhesive and L-leucine as carrier in order to extend the lung residence time and improve the yield and FPF, respectively. For this purpose, we evaluated microparticle characteristics, *in vitro* aerosolization performance, *in vitro* deposition on Calu-3 cells using the Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADD OCC) and their effect on the growth of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Materials

Clarithromycin, L-leucine, ammonium acetate, amitriptyline, dimethyl sulfoxide (DMSO), sigmacote, sodium pyruvate and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis,

USA). Ultrapure chitosan chloride Protasan UP CL113 with a molecular weight of 50–150 kDa and a degree of deacetylation between 75% and 90% was purchased from NovaMatrix (FMC Bio-Polymer, Drammen, Norway). Calu-3 cells (HTB-55) were obtained from the DSMZ GmbH (Braunschweig, Germany). Minimum essential medium (MEM) containing Earl's Salts and L-glutamine was bought from Gibco (Life Technologies, Paisley, UK). Fetal calf serum (FCS) was bought from Lonza (Vervieers, Belgium), and Nonessential amino acid (NEAA) solution from GE Healthcare (PAA laboratories, Pasching, Austria). Methanol, ethanol and all other chemicals and solvents used were analytical grade. Purified water was produced by Milli-Q water purification system (Merk Millipore, Billerica, USA).

CLARI loaded microparticles

Initially, 0.05 g of Leucine and 0.05 g of chitosan were solubilized in 25 mL of water at room temperature by controlled magnetic stirring. Separately, 0.05 g of CLARI was dissolved in 5 mL of ethanol. After both phases were completely dissolved, they were mixed under moderate magnetic stirring at room temperature during 15 minutes. The solution was atomized with the Nano Spray Dryer B-90 (Büchi Labortechnik AG, Switzerland). The equipment was maintained in open mode configuration using the following parameters: pump mode 2 with 80 % of spray rate, airflow of 120 ± 5 L/min, inlet temperature of 80 °C and spray mesh with aperture size of 4.0 µm. The separation of Clarithromycin microparticles (CLARI-MPs) from particle-collecting electrode were carried-out with a soft brush.

Spray-dried powder characterization

Yield and Drug content

The yield was determined through the related amount of powder obtained in the initial concentration of all materials used in the solution. The clarithromycin content was

quantified by liquid chromatography - tandem mass spectrometry (LC-MS/MS) method as described above.

LC-MS/MS Method

Clarithromycin quantification was carried out using a LC-MS/MS technique based on a previously validated method (22). This system consists of a TSQ Quantum[®] Access MAX Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, San Jose, USA) equipped with an Accela 1250 pump, Accela Autosampler, and the TSQ Quantum. CLARI separation was made with an Accucore RP-MS column (150 mm x 2.1 mm, 2.6 μ m, Thermo Fisher Scientific, San Jose, USA) kept at 30 °C. The isocratic mobile phase consisted of 70% of methanol containing 0.1 % of formic acid (v/v) and 30% of ammonium acetate buffer (5 mM) containing 0.1 % of formic acid (v/v) was eluted at a flow rate of 0.25 mL/min during 4 min. We used the Mass Spectrometry conditions: heated electrospray ionization source (HESI-II), positive mode; sheath gas, nitrogen at a flow rate of 35 arbitrary units; collision gas, argon; vaporizer temperature, 300 °C; ion transfer capillary temperature, 250 °C; skimmer offset, 0 V; spray voltage, 4000 V. The mass spectrometer was operated in the high-resolution selected reaction monitoring (H-SRM) mode. The precursor ion transition of CLARI at m/z 748.343 to ion at m/z 158.145 was selected with collision energy of 28 V; scan time, 0.1 s; scan width, m/z 0.02; and tube lens offset, 111 V. While, for amitriptyline, used as internal standard (IS), the precursor ion transition at m/z 278.5 to ion at m/z 233.150 was selected with collision energy of 14 V; scan time, 0.1 s; scan width, m/z 0.02; and tube lens offset, 91 V. The peak areas were integrated automatically using the software Thermo Xcalibur (v. 2.2, San Jose, USA) with the settings as follows: peak detection algorithm, ICIS; smoothing points, 5; baseline window, 80; area noise factor, 5; peak noise factor, 10. Briefly, 100 μ L of samples were extracted with 0.9 mL of methanol:water (1:1, v/v) containing the IS (500 ng/mL) under ultra sonication for 10 minutes. The samples were centrifuged (10 000 g, 10 min) and the supernatant (5 μ L) was injected using partial

loop injection mode in the LC-MS/MS system. The developed method was linear on the concentration range of 5–500 ng/mL ($R^2 = 0.99$).

Scanning Electron Microscopy

Morphology and particle size analysis of CLARI-MPs were evaluated with scanning electron microscopy (SEM, Zeiss EVO HD15, Oberkochen, Germany). Samples of CLARI-MPs were added in aluminum stubs with carbon conductive double-sided tape and sputter-coated with a 10 nm layer of gold (Quorum Q150R ES, Ashford, UK). Analyses were conducted under high vacuum conditions at an acceleration voltage of 10 kV. Particle size distribution was analyzed using the software ImageJ (version 1.47v, National Institute of Health, USA) and it is expressed as the mean diameter $\pm$ standard deviation.

Differential Scanning Calorimetry (DSC)

Thermal analysis of pure raw materials, their physical mixture and the microparticle developed were performed using differential scanning calorimetry (DSC) TA Q100 (TA Instruments, New Castle, USA). The physical mixture was prepared using the same amounts of each component and it was mixed with a pestle and a mortar. Approximately 5 mg of samples were placed on aluminum pans, hermetic sealed and heated from 20 to 350 °C with a scan rate of 10 °C/min under a nitrogen atmosphere of 10 mL/min. DSC heating curves were analyzed using Universal Analysis 2000 software (Version 4.5a, TA Instruments, New Castle, USA).

Dissolution

The dissolution of CLARI-MP was performed in a dissolutor Sotax AT6 (Sotax AG, Basel, Switzerland). Approximately 6 mg of the powder CLARI-MP (containing about 2 mg of CLARI) were filled in hypromellose capsule (size n° 3). In order to compare the dissolution of CLARI, it was also determined the dissolution of CLARI raw material (2

mg) with a paddle speed of 100 rpm, The sink conditions was maintained employing 1000 mL of phosphate buffer medium with pH = 7.4 and temperature of 37 ± 1 °C. Samples (1 mL) were withdrawn at predetermined time points at 15, 30, 45, 60, 90 and 120 min. The samples (0.1 mL) were diluted with 0.9 mL of methanol:water (1:1) containing the IS and filtered through 0.45 µm and analyzed in the LC-MS/MS, as described before. The dissolution of 50% of the drug for both formulations was obtained from the release profiles fitted according to the first-order ($C = C_0 \cdot e^{-kt}$) mathematical model. Where k and C_0 are the apparent kinetic rate constant and the initial drug concentration at time t (h), respectively.

Aerodynamic particle analysis

The aerodynamic particle size distribution of CLARI-MP was determined with the next-generation pharmaceutical impactor (NGI) (Copley Scientific, Nottingham, UK), set at a flow rate of 60 L/min from a pump (HCP5 High Capacity Pump, Copley Scientific, Nottingham, UK) with the critical flow controller TPK (Copley Scientific, UK). The equipment was previously calibrated with a flow meter DFM3 (Copley Scientific, Nottingham, UK). The NGI was fitted with the USP throat and its appropriate pre-separator. The effective cut-off diameters of stages 1, 2, 3, 4, 5, 6 and 7 at 60 L/min flow rate were 8.06, 4.46, 2.82, 1.66, 0.94, 0.55 and 0.34 µm, respectively. Before the experiment, the NGI collection cups were coated with Sigmacote in order to avoid bias from particle bounce and re-entrainment within the impactor. CLARI-MP powder was accurately weighed (about 6 mg) into size n° 3 hypromellose capsules and loaded into a HandiHaler® dry powder inhaler (Boehringer, Ingelheim, Germany). After the capsule was perforated, the powder was released into the NGI for 4 sec at 60 L/min. The particles retained in the inhalation device, mouthpiece adaptor, throat device, pre-separator and each stage from one to eight were removed with 1:1 (methanol-water) solution and collected into volumetric flasks. The samples (0.1 mL) were diluted with 0.9 mL of methanol:water (1:1) containing the IS, filtered through 0.45 µm and injected

in the LC-MS/MS for analysis. The emitted dose (ED) was calculated from the proportion of drug that entered in the NGI compared to the total drug present in each capsule. The fine particle fraction (FPF), which is the percentage of particles with aerodynamic diameter lower than 5 μm , was determined from the sum of the stages 2 of the filter of the NGI.

Particle Size Distribution

The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were determined with the NGI data based on the cumulative particle size distribution functions. MMAD is defined as the diameter above and below which lies 50% of the mass of the particles. While GSD, a dimensionless number, is equal to the ratio between the MMAD and either 84% or 16% of the diameter size distribution. The GSD, together with the MMAD, describe the particle size distribution of the formulation.

In Vitro Deposition Study

Cell Culture

Calu-3 cells (HTB-55) were used between passage 30 and 42. The cells were cultivated in 75 cm^2 flasks in MEM supplemented with Earl's Salts and L-glutamine supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acid (NEAA), 50 mM sodium pyruvate, 100 units/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. The cells were incubated at 37 $^{\circ}\text{C}$, 5% CO_2 and 95% humidity. Once reached confluence Calu-3 were detached with Trypsin and seeded in a 12 well Snapwells polyester inserts (1.12 cm^2 , 0.4 μm pore size, Corning Costar, Lowell, MA, USA) at 1.33×10^5 cells/ cm^2 concentration. After 48h, the cells were cultivated at air-liquid interfaced (ALI) by removing the apical medium and feeding only the basolateral part with 1.0 mL of complete MEM. ALI cultures were grown for 12-14 days, in order to differentiate and perform ideal epithelial barriers (23).

279

280 *Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADDOCC)*

281 Deposition experiments were performed with PADDOCC, as previously described by
282 Hein et al. (10, 24). Briefly, it consists of an airflow control unit (Akita[®], Activaero,
283 Gemünden/Wohra, Germany), an aerosolization unit and a deposition unit, which are
284 all connected by silicon tubes. The PADDOCC (without the air flow control unit) was
285 accommodated in an incubator at 37 °C and the following airflow parameters were
286 used: dispersion impulse of 60 L/min for 0.2 s and a ventilation flow of 6 L/min for 2.0 s.

287

288 *CLARI deposition*

289 To evaluate the total deposition of clarithromycin, a dry powder capsule with
290 approximately 6 mg of CLARI-MPs was aerosolized with the PADDOCC and deposited
291 in sampling wells (i.e. without cells) with 0.5 mL of methanol:water (1:1). After three
292 aerosolization–deposition cycles of 10 minutes, each well of the sampling unit was
293 washed with 0.5 mL of methanol:water (1:1) and quantified by LC-MS/MS analytic
294 method, as previously described.

295

296 *Permeability*

297 Firstly, the Snapwells[®] were placed into the sampling wells of the PADDOCC with 0.5
298 mL of MEM at 37 °C in the basolateral compartment and were pre-equilibrated for 15
299 minutes. Then, the system was set up for three deposition cycles resulting in 30 min for
300 aerosolization and deposition process. After this period, Snapwells[®] containing the cells
301 were placed in a cell culture plate, and 1.5 mL of cell culture medium was added in the
302 basolateral compartment. The cells were incubated at 37 °C, 5% CO₂ and 95%
303 humidity with a rotational shaker at 150 rpm. Samples of 100 µL were acquired each
304 30 minutes during 4 hours and at 24h from the basolateral compartment to evaluate
305 the formulation permeability across Calu-3. To analyze drug concentration, samples
306 were extracted with methanol:water (1:1) containing the IS, centrifuged at 10.000g for

10 min at 12 °C and the supernatants quantified by LC-MS/MS analysis. After the permeability experiments, TEER values were measured to verify the integrity of cell monolayer as mentioned below.

TEER Values

Transepithelial electrical resistance (TEER) was measured before and after the permeability experiment to monitor cellular barrier integrity using a Volt-Ohm Meter (EVOM with STX-2 chopstick electrodes, World Precision Instruments, Berlin, Germany). Briefly, pre-warmed cell culture medium was added in the apical compartment (0.5 mL) and in the basolateral compartment (1.5 mL) before TEER measurement and incubated for 30 min. The electrical resistance of insert membranes without cells (120 Ω) was subtracted from all samples, and the resistance values were multiplied by the surface area of the inserts (1.12 cm²).

Cell viability

Cell viability of CLARI-MPs was determined by MTT [(3-(4,5- dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] assay. After 24 hours of the aerosolization and deposition process with the PADD OCC system, the cells were washed with PBS and 5 mg/mL MTT solution in PBS pH 7.4 were added to each well. After 4h incubation, the cells were lysed with DMSO and the formation of blue formazan crystals evaluated by measuring absorbance at 550 nm with the plate reader Infinite M200 PRO (Tecan, Grödig, Austria). Untreated cells and cells incubated with Triton X-100 1% were used as negative and positive controls, respectively.

Antibacterial studies

Minimal Inhibitory Concentration (MIC) assays

MIC50 values were determined in *Pseudomonas aeruginosa* PA14, *Escherichia coli* K12 and *Staphylococcus aureus subsp. aureus* (Newman strain) and were performed as described recently (25), with slight modifications. Stock test formulations of 2 mg/mL were prepared, while CLARI was dissolved in DMSO (maximal DMSO concentration in the experiment: 1%), CLARI-MP was dispersed in water. Final concentrations of the test formulations were obtained ranging from 0.02 to 20 µg/mL. The optical density (OD) was determined after addition of formulations and again after incubation for 18 h at 37 °C and 50 rpm (200 rpm for *P. aeruginosa*) in 96 well plates (Sarstedt, Nümbrecht, Germany) using a FLUOStar Omega (BMG labtech, Ortenberg, Germany). Given MIC50 values are means of three independent determinations and are defined as the concentration of compounds that reduced the OD600 by 50%.

Bacteria Scanning Electron Microscopy

The interactions between the bacteria (*S. aureus* or *P. aeruginosa*) with CLARI-MPs or CLARI were visualized with SEM. For that the samples were previously fixed with 1.0% glutaraldehyde in 0.2 M hepes buffer (pH 7.4), dehydrated in a graded ethanol series (from 30% to 100%) and finally dried by evaporation with hexamethyldisilazane. The specimens were mounted on stubs and sputtered with gold to a layer thickness of 10 nm (Quorum Q150R ES, Ashford, UK) and observed at 5 kV under high vacuum conditions on a Zeiss EVO HD15 SEM (Zeiss, Oberkochen, Germany).

Data Analysis

The experiments values were expressed as means ± standard deviation (S.D.). Data were subjected to statistical analysis using the software package Prism 6 (GraphPad Prism, San Diego, USA). The significance were analyzed by *t*-test. Difference was considered statistically significant when $p < 0.05$.

RESULTS

Microparticles characterization

CLARI-loaded microparticles as DPI were developed and evaluated regarding cell interaction and anti-microbial activity. A DPI formulation containing clarithromycin microparticles was produced by spray drying technique using the Nano Spray Dryer Buchi B-90®, with a yield of $60.7 \pm 2.3\%$. The obtained powder had a drug loading of $36.0 \pm 1.4\%$ (m/m). Microparticles were nearly spherical, showed a smooth surface with some holes and were not agglomerated (Fig 1). Particle size of the powders ranged from approximately 0.3 to $2.5 \mu\text{m}$ with an average of $765 \pm 358 \text{ nm}$ (data not shown) which is suitable for deposition in the deeper regions of the lungs (26).

Fig. 1 Scanning electron micrographs of Clarithromycin microparticles.

The interactions between the different components of the microparticle were evaluated by DSC. Analysis was carried out for CLARI-MP, its physical mixture (PM) and raw materials. As showed in Fig. 2, clarithromycin melting and decomposition temperature were respectively, 227°C and 280°C . When the drug was mixed with chitosan and leucine (PM), an early onset of endothermic peak (214.8°C) was observed. Furthermore, the onset of endothermic peak of CLARI-MP sample was even lower (184.5°C).

Fig. 2 DSC thermograms for the raw materials (leucine, chitosan and clarithromycin), physical mixture and clarithromycin microparticle formulation (CLARI-MP).

Dissolution study

The dissolution rate of CLARI-MP obtained by Nano Spray Dryer B-90[®] was compared with CLARI raw material (Fig. 3). The release profiles were expressed as the percentage of drug release versus the time. After 30 min, more than 90 % of CLARI was released in the dissolution medium, while for such release of CLARI-MP this was observed only after 60 min.

Fig. 3 Dissolution profile of clarithromycin as raw material (CLARI) and clarithromycin microparticle formulation (CLARI-MP). Each point represents the mean $\pm$ S.D. (n = 3, from 3 independent experiments).

Aerodynamic behavior

The aerodynamic performance of microparticles is an important parameter for particle deposition in the lung. Aerosolization studies using NGI were conducted at a flow of 60 L/min during 4s (Fig. 4). The ED obtained from hypromellose capsules and dry powder inhaler was $87.5 \pm 4.4 \%$. Moreover, the percentage of particles that could be deposited in the deep lung was measured by the FPF. For particles deposited between the stages 2 and the filter (lower than $4.46 \mu\text{m}$) the FPF was $73.3 \pm 2.3 \%$. In addition, the MMAD and the GSD of CLARI-MP were $1.81 \pm 0.14 \mu\text{m}$ and 2.59 ± 0.08 , respectively.

Fig. 4 Aerosol performance of clarithromycin microparticles using the Next Generator Impactor (NGI) at a flow of 60 L/min for 4s. C, T, PS and F represent the percentage of particles retained in the capsule, throat, pre-separator and filter stages, respectively. Each point represents the mean $\pm$ S.D. ($n = 4$, from 2 independent experiments).

***In vitro* CLARI-MP deposition**

CLARI-MPs deposition was carried out in the PADD OCC with capsules of CLARI-MPs, deposited in three cycles of 10 min each, using aerosolization chamber without cells. CLAR-MPs were uniform and reproducible deposited at concentration of $16.2 \pm 6.9 \mu\text{g}/\text{cm}^2$ of the aerosolized dose. Moreover, the total amount collected in all the three

wells per capsule corresponded to 2.37 ± 0.84 % of the aerosolized dose CLARI-MPs deposition. Thereafter, CLARI-MPs deposition was evaluated on Calu-3 monolayers previously grown on Snapwell filter inserts. For that, aliquots of basolateral medium were collected during 24h (Fig. 5A). The results show that CLARI-MPs transport across the monolayer increased progressively reaching 8.7 ± 1.7 $\mu\text{g}/\text{cm}^2$. Epithelial barrier property was also evaluated by TEER measurements before and after 24 h of the deposition of CLARI-MPs (Fig. 5B). Before exposition in the PADD OCC, the TEER of Calu-3 cells remained around $300 \Omega.\text{cm}^2$, dropped slightly to $266.67 \pm 28.46 \Omega.\text{cm}^2$ and were not significant different from the untreated monolayers ($248 \pm 7 \Omega.\text{cm}^2$). CLARI-MPs incubated for 24h in Calu-3 did not induce toxic effect, as assessed with MTT assay (Fig. 5C).

Fig. 5 Deposition of clarithromycin microparticles (CLARI-MPs) on Calu-3 monolayers using the Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADD OCC) system. (A) Drug Transport ($n = 15$, from 2 independent experiments); (B) TEER values of Calu-3 monolayers before and after 24h of deposition of CLARI-MP; (C) Cell viability of Calu-3 using MTT assay after 24h of deposition of CLARI-MP in the PADD OCC; Cells without any treatment were used as CONTROL. No significant difference was observed between the groups (t -test , $p > 0.05$; $n = 9$ from 2 independent experiments, mean $\pm$ S.D.)

Antimicrobial activity of CLARI-MP

The antimicrobial activity of CLARI-MPs was tested in relevant respiratory pathogens, either Gram negative (*Pseudomonas aeruginosa* and *Escherichia coli*) or Gram positive (*Staphylococcus aureus*). The results showed that the concentration of CLARI-MPs needed to reduce 50% of bacterial growth was 20 $\mu\text{g ml}^{-1}$ for *P. aeruginosa*, 6 $\mu\text{g ml}^{-1}$ for *E. coli* and 1 $\mu\text{g ml}^{-1}$ for *S. aureus*. These concentrations were similar to those observed in the MIC50 for the free drug (Fig 6A). As a control, drug-free microparticles did not show any antibacterial activity against all strains tested (data not shown). To further characterize the interaction of bacteria with MPs, the samples were processed by SEM (Fig. 6B-G). Untreated *P. aeruginosa* and *S. aureus* (Fig. 6B, E, respectively) showed a normal size and typical morphologies for Gram positive and negative strains. After the treatment with free drug (CLARI) as well as CLARI-MPs, we observed altered morphology in the bacterial population, with a melting-like network (Fig 6C-D, F-G). For *S. aureus* we observed apoptosis-like structures, e.g. cytoplasmic blebs (Fig. 6F, G).

Fig. 6 (A) Minimum inhibitory concentration 50% (MIC50) of clarithromycin solution (CLARI) and clarithromycin microparticles (CLARI-MP) against *P. aeruginosa*, *E. coli* and *S. aureus*; *t*-test did not show any significant differences between the two formulations for either bacteria ($p > 0.05$; $n=3$ from 3 independent experiments). Scanning electron micrographs (SEM) of *P. aeruginosa* (B-D) and *S. aureus* (E-G) untreated (B,E), or treated with free drug (C,F) or CLARI-MP (D,G). Examples of melting-like network and apoptosis-like structures are shown with black and white arrows, respectively.

DISCUSSION

Drug administration via the oral route may suffer from reduced bioavailability due to the pre-systemic metabolism during the passage through gut wall and liver. Therefore delivering antibiotic to the lungs by inhalation could decrease adverse effects since lower systemic drug concentrations are reached compared to oral delivery. However after the deposition in the lungs, the drug may be eliminated by clearance systems, such as mucociliary clearance. The use of carriers systems loaded with an active compound show as promise strategy to overcome clearance in the lungs. Moreover such approach could also have a better anti-infective efficacy, since the particle shall reach directly the infection site, and release the active compound. Here we developed dry powder microparticles intended for pulmonary application, constituted by chitosan and leucine, loaded with the hydrophobic antibiotic clarithromycin. With this formulation we aim to improve the lung residence time, yield and FPF of the particles. Thus inhalable CLARI-MPs were prepared by means of spray drying, and safety on lung cells and their efficacy against gram positive and gram negative bacteria were determined.

Chitosan, a cationic polysaccharide, has the advantage to be non-toxic and biodegradable, and has already been used for the pulmonary route (27). The use of chitosan avoided particle agglomeration and increase particle deposition in the electrostatic particles collector. In assisting to the dry process of the particles, leucine can improve powder dispersibility and aerodynamic behavior due to its ability to form hollow particles with low particle density (26, 28). Higher concentrations of this amino acid were previously reported to increase the FPF of formulations comparing to lactose, the most-used excipient in antibiotic DPI formulation, without induction of apparent cytotoxicity (21, 28, 29).

Clarithromycin, a hydrophobic antibiotic with broad spectrum, is widely used against respiratory infections (18). However as CLARI has poor solubility in water, the

formation of CLAR-MPs as dry powder could improve the antibiotic activity, since it enables the inhalation with maximum bioavailability and target delivery to the cells. New clarithromycin microparticles or nanoparticles have already been described to achieve improved delivery to the lungs, however in most of the cases only exploring the pharmaceutical development (1, 3, 5, 21), without demonstrating some biological properties of such formulations, which is essential to predict both efficacy and safety regarding their clinical application *in vivo*. While antimicrobial activity has been demonstrated for other CLARI formulations (30, 31), no tests regarding potential toxicity on mammalian cells were performed.

After optimizing the processing parameters and excipients, a CLARI-MPs powder formulation was obtained in high yields with excellent aerodynamic properties and without suffering from the problem of incrustations at the piezoelectric nozzle as reported in other studies using the Nano Spray Dryer B-90® (32). We obtained a higher yield (more than 60%) in the process compared with other spray drying processes (lower than 50 %) (33), mainly grace to the electrostatic particle collector. The effect of the electrostatic potential difference causes an increase in the particle attraction compared to the more common cyclone collector when cationic ions are presented in the formulation (33).

Thermal analysis of the particles performed with DSC is a useful tool to verify interactions between formulation components. We observed clarithromycin melting temperature of 227 °C, as reported also by others authors (34). Importantly, we observed a shift of the onset temperature for the physical mixture, and even to a greater extend for CLARI-MPs, compared to the free drug, suggesting some thermodynamic interaction between the components of this formulation.

The dissolution test showed some slight delay of clarithromycin release from microparticles compared to the raw material, similar to other aerosol powder formulations containing different hydrophobic drugs (35, 36) and apparently suitable for pulmonary administration. The fast dissolution rate after 60 min could be an advantage to overcome clearance mechanism and optimize drug local activity.

Before deposition of CLARI-MPs to pulmonary epithelial cells, we evaluated the aerodynamic behavior of aerosol particles that could be generated using hypromellose capsules and a HandiHaler® by collecting them in a NGI device. The ED of the formulation (about 87%) accomplished the European pharmacopoeia specification, which should be higher than 75% of the loaded dose. In addition, more than 75 % of the particles were deposited in stages 2 – 7 (FPF). This high FPF value of DPI formulation is a valuable indication of good aerosol performance when applied *in vivo* since it is greater than 40%, as recommended for antibiotic products (26, 37) and it is 55 % higher than the FPF obtained for clarithromycin pMDI (3). Moreover, the MMAD (about 1.9 µm) is in the range required for the deposition in deep lung (26).

The PADD OCC had been previously developed for reproducible metered deposition of aerosol powders on pulmonary epithelial cell cultures under physiological conditions, allowing a simulation of the complex processes of aerosol deposition and absorption *in vivo* under controlled conditions *in vitro*. In this study, we used the bronchial epithelial cell line Calu-3 grown at air-liquid interface conditions due to their known barrier properties and easy accessibility (23, 38). Hein et al. 2011 showed that the deposited amount of drug is directly proportional of the capsule dose using the PADD OCC (10). Previous studies showed a low deposition fraction (about 1.5%) for budesonide and salbutamol sulfate using the PADD OCC and modified twin stage impinger, respectively (10, 14). After deposition CLARI-MPs, we obtained a uniform deposition of approximately 2.4% of total amount in the capsule. This value may appear to be low

compared to the FPF ($73.3 \pm 2.3\%$) obtained by NGI for these particles. However, our deposition result is higher than other deposition studies (4, 10, 14). In addition, the recovery of drug obtained after administered ≈ 2 mg of clarithromycin was about $16.2 \mu\text{g}/\text{cm}^2$. Considering that the cells are seeded in an area of 3.36 cm^2 we could speculate that the drug recover in our system correspond to $0.168 \text{ ng}/\text{cm}^2$ in the lungs (100 m^2), assuming the same rate of deposition (2.4%).

After 24h of CLARI-MP deposition, about 50% of CLARI were transported across the Calu-3 epithelial barrier, suggesting that there might be also a substantial systemic absorption after aerosol delivery of such microparticles. This might have advantages as CLARI also has some anti-inflammatory and immune modulatory effects, according to a recent paper where the compound was applied as solution-based pressurized metered dose inhalers (pMDIs) (4). However the administration of clarithromycin as pMDIs or nebulizers requires the use of solvents in order to solubilize it; in case of CLARI-MPs as DPI no solvent exposition is required. Our results also show that the epithelial barrier function was not influenced by the deposition of particles, as indicated by the absence of any significant effect on TEER over 24h. Additionally CLARI-NP did not induce toxic effect on Calu-3 cells, which further endorses the safety of these particles.

Finally the efficacy of CLARI-MPs was tested on extracellular Gram positive (*S. aureus*) and Gram negative (*P. aeruginosa* and *E. coli*) bacteria. From the *in vitro* evaluation our formulation maintained the same antimicrobial efficacy (MIC) as observed for the free drug solubilized in DMSO. SEM results suggest that there were even a higher proportion of bacteria fragments compared to the treatment with free drug. A similar pattern was found for *P. aeruginosa* and *E. coli* strains, which could make these extracellular bacteria more susceptible to phagocytosis and degradation by macrophages.

CONCLUSION

In this paper we described a new dry powder inhaler formulation containing clarithromycin with aerodynamic particle properties suitable to reach the deep lung with safety and efficacy to kill bacteria. The formulation was easily achieved by spray drying technique in merely one step. Regarding the treatment of respiratory infections we could show a new drug delivery system efficient against both gram positive and negative bacteria, as *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. Importantly, we also showed an efficient deposition of clarithromycin particles on bronchial cells (Calu-3) without inducing apparent toxicity. This technology could pave the way to better DPI formulations of CLARI and other anti-infective drugs in the future.

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Legend to Figures

Fig. 1 Scanning electron micrographs of Clarithromycin microparticles.

Fig. 2 DSC thermograms for the raw materials (leucine, chitosan and clarithromycin), physical mixture and clarithromycin microparticle formulation (CLARI-MP).

Fig. 3 Dissolution profile of clarithromycin as raw material (CLARI) and clarithromycin microparticle formulation (CLARI-MP). Each point represents the mean $\pm$ S.D. ($n = 3$, from 3 independent experiments).

Fig. 4 Aerosol performance of clarithromycin microparticles using the Next Generator Impactor (NGI) at a flow of 60 L/min for 4s. C, T, PS and F represent the percentage of particles retained in the capsule, throat, pre-separator and filter stages, respectively. Each point represents the mean $\pm$ S.D. ($n = 4$, from 2 independent experiments).

Fig. 5 Deposition of clarithromycin microparticles (CLARI-MPs) on Calu-3 monolayers using the Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADD OCC) system. (A) Drug Transport ($n = 15$, from 2 independent experiments); (B) TEER values of Calu-3 monolayers before and after 24h of deposition of CLARI-MP; (C) Cell viability of Calu-3 using MTT assay after 24h of deposition of CLARI-MP in the PADD OCC; Cells without any treatment were used as CONTROL. *t*-test did not show any significant differences ($p > 0.05$; $n = 9$ in 2 independent experiments, mean $\pm$ S.D.)

Fig. 6 (A) Minimum inhibitory concentration 50% (MIC₅₀) of clarithromycin solution (CLARI) and clarithromycin microparticles (CLARI-MP) against *P. aeruginosa*, *E. coli* and *S. aureus*; *t*-test did not show any significant differences between the two formulations for either bacteria ($p > 0.05$; $n=3$ independent experiments). Scanning electron micrographs (SEM) of *P. aeruginosa* (B-D) and *S. aureus* (E-G) untreated (B,E), or treated with free drug (C,F) or MP-CLARI (D,G). Examples of melting-like network and apoptosis-like structures are shown with black and white arrows, respectively.

Figure 1
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EVO HD15

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10.00 K X

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