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(54) Title: INHALABLE MICROPARTICLES LOADED WITH A FLUOROQUINOLONE/METAL CATION COMPLEX FOR THE TREATMENT OF RESPIRATORY DISEASES

(57) Abstract: This invention relates to inhalable microparticles loaded with a fluoroquinolone/metal cation complex for the treatment of respiratory diseases.



INHALABLE MICROPARTICLES LOADED WITH A FLUOROQUINOLONE/METAL CATION COMPLEX FOR THE TREATMENT OF RESPIRATORY DISEASES

5 FIELD OF THE INVENTION

This invention relates to inhalable microparticles loaded with a fluoroquinolone/metal cation complex, and applications thereof.

BACKGROUND OF THE INVENTION

10 *P. aeruginosa* is responsible for chronic lung infections in patient with lung diseases such as cystic fibrosis, bronchiectasis or chronic obstructive pulmonary disease (COPD).

This type of bacterium that grows principally in the mucus and pulmonary epithelial lining fluid (ELF) of the patients is treated by fluoroquinolones that display a good *in vitro* activity against *P. aeruginosa*. Thus, the ELF is considered the target site for the treatment of
15 pneumonia caused by extracellular pathogens such as *P. aeruginosa*. Several studies have shown that maximizing fluoroquinolone exposure is essential to reduce the increasing rate of antibiotic resistance or to eradicate biofilm. Therefore obtaining pharmacologically relevant concentrations of fluoroquinolone in the ELF is capital to ensure optimal treatment of lung infections.

20 Fluoroquinolones are typically administered orally and intravenously. However, such administration routes require high doses of antibiotics and have undesirable side effects.

The search for more efficient therapeutic approaches has driven to the development of inhaled fluoroquinolones. Pulmonary delivery of fluoroquinolones is an interesting approach to treat lung infections as it may lead to high local concentrations while minimizing systemic
25 exposure. However, fluoroquinolones have a rapid diffusion through the lung epithelium giving the pulmonary route no advantage compared to the oral route.

SUMMARY AND DETAILED DESCRIPTION OF THE INVENTION

In order to solve the above-mentioned technical problems, the inventors of the present
30 invention have developed inhalable microparticles loaded with a fluoroquinolone/metal cation complex which enable sustained fluoroquinolone lung exposure.

Advantageously these inhalable microparticles sustain the fluoroquinolone in the pulmonary epithelial lining fluid (ELF) by decreasing the fluoroquinolone apparent permeability across the lung epithelium.

- 5 The present invention relates to an inhalable microparticle loaded with a fluoroquinolone/metal cation complex.

Fluoroquinolones constitute a family of antibiotics which exert antibacterial effect by acting on the bacterial DNA. In particular, fluoroquinolones inhibit the DNA gyrase and DNA
10 topoisomerase IV (Karl Drlica. *Mechanism of fluoroquinolone action*. Current Opinion in Microbiology, Volume 2, Issue 5, 1 October 1999, Pages 504–508).

Examples of fluoroquinolones are, but not limited to, ciprofloxacin, levofloxacin, ofloxacin, gatifloxacin, enoxacin, norfloxacin, moxifloxacin, gemifloxacin, pefloxacin, sparfloxacin, garenoxacin, sitafloxacin, DX-619 and lomefloxacin.

15

In a preferred embodiment, the fluoroquinolone is ciprofloxacin.

Examples of metal cations which can be used to form the fluoroquinolone/metal cation complex are, but not limited to, Cu^{2+} , Al^{3+} , Zn^{2+} , Mg^{2+} , Ca^{2+} and mixture thereof.

20

In a preferred embodiment, the metal cation is Cu^{2+} , Al^{3+} , Zn^{2+} , or a mixture thereof.

In a more preferred embodiment, the metal cation is Cu^{2+} .

In a preferred embodiment, the fluoroquinolone/metal cation complex is a ciprofloxacin/ Cu^{2+}
25 complex.

The size of the inhalable microparticles loaded with a fluoroquinolone/metal cation complex is chosen so as to enable a pulmonary administration in aerosol form.

Typically, the geometric diameter of the microparticle is comprised between 1 and 20 μm , preferably between 1 and 10 μm , more preferably between 1 and 7 μm and even more preferably between 1 and 5 μm .
30

Typically, the mass median aerodynamic diameter (MMAD) is comprised between 1 and 5 μm .

Typically, the fluoroquinolone content is comprised between 10 and 60 wt. % in relation to the total weight of the loaded microparticle, preferably between 25 and 60 wt. %, and even more preferably between 30 and 60 wt. % in relation to the total weight of the loaded microparticle.

- 5 Typically, at least 80 wt. %, preferably at least 90%, 95%, 99%, more preferably 100% of the fluoroquinolone in the microparticle is complexed with the metal cation.

10 The present invention also relates to a dry powder formulation comprising the microparticles according to the invention.

Inhalable particules for aerosol administration to the lung are well known, see for review for example Healy et al., Adv Drug Deliv Rev. 2014 Aug;75:32-52, Loira-Pastoriza et al., Adv Drug Deliv Rev. 2014 Aug;75:81-91 or Zhou et al., Adv Drug Deliv Rev. 2015 May;85:83-15 99.

Thus, the person skilled in the art might choose the inhalable microparticles in which the fluoroquinolone/metal cation complex will be loaded according to the desired sustained release.

Typically, the microparticles according to the invention may be obtained by spray drying
20 Typically, the microparticle may be an inorganic, an organic or an inorganic-organic microparticle.

In a particular embodiment the microparticle is a polymeric microparticle, such as a poly(lactic-co-glycolic acid) PLGA microparticle.

In a preferred embodiment the microparticle is a microparticle comprising calcium and a
25 biopolymer. Example of suitable biopolymer is a Hyaluronic acid biopolymer.

Therapeutic uses

Fluoroquinolones constitute a family of antibacterial agents. Fluoroquinolones are indicated for the treatment of several bacterial infections. Several bacterial infections include
30 respiratory infections such as bacterial bronchitis, bronchiolitis, pneumonia, tuberculosis, tonsillitis pharyngitis, otitis and sinusitis,. More particularly, fluoroquinolones are known to have an activity against a wide range of gram-positive and gram-negative organisms.

The present invention relates to a microparticle according to the invention for use as a medicament.

5 The present invention also provides a pharmaceutical composition comprising as active principle, the microparticle according to the invention and a pharmaceutically acceptable excipient.

10 The term “pharmaceutically acceptable carrier or excipient” of the present invention refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredient(s) and which is not excessively toxic to the host at the concentration at which it is administered. Said carriers and excipients are selected from the usual excipients known by a person skilled in the art.

15 Another object of the present invention relates to microparticle according to the invention for use in the treatment of a bacterial lung infection.

The present invention relates to a method for treating a bacterial lung infection, comprising administering to a patient by aerosol administration an effective amount of the microparticles according to the invention.

20 Typically, the microparticles are administered to the patient via a dry powder inhaler.

In a particular embodiment, the bacterial lung infection is bacterial bronchitis, bronchiolitis or pneumonia.

In a particular embodiment, the bacterial lung infection is a chronic bacterial lung infection.

25 In a particular embodiment the bacterial lung infection is a *Pseudomonas aeruginosa* infection.

Suitable dosage ranges depend upon numerous factors such as the severity of the infection to be treated, the age and health of the subject. Furthermore, the dosage ranges depend on the the fluoroquinolone content of the microparticles of the invention.

30

The term “treatment” of the present invention refers to a method or process that is aimed at (1) slowing down or stopping the progression, aggravation, or deterioration of the symptoms of the disease; (2) bringing about amelioration of the symptoms of the disease; or (3) curing

the disease. A treatment may thus be administered after initiation of the disease, for a therapeutic action.

The term “effective amount” of the present invention refers to any amount of fluoroquinolone that is sufficient to fulfil its intended purpose(s), *e.g.* a desired biological or medicinal response in a cell, tissue, system or patient.

The term “patient” of the present invention refers to a human or another mammal (e.g., primate, mouse, rat, rabbit, dog, cat, horse, cow, pig, camel, and the like). Preferably, the patient is a human.

In a particular embodiment, the patient is a patient with a lung disease. Typically the patient is a patient with cystic fibrosis, bronchiectasis or chronic obstructive pulmonary disease (COPD).

Although the exemplary embodiments of the present invention have been disclosed for illustrative purposes, a person skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

The present invention will now be illustrated using the following examples and figures, which are given by way of illustration, and are in no way limiting.

BRIEF DESCRIPTION OF FIGURES

Figure 1: CIP concentrations in ELF (plain dot) and plasma unbound (open square) versus time after (A) IT nebulization of a CIP solution, (B) IT administration of CIP-Ca microparticles, or (C) IT administration of CIP-Cu microparticles. The CIP dose was 3 mg/kg. Each time points are averaged values $\pm$ SD of 5-6 individual measurements.

Figure 2: Total copper and calcium concentration versus time profiles. Calcium (right panel) and Copper (left panel) concentrations in ELF (plain dot) and plasma (open square) after IT administration of CIP-Cu microparticles.

Figure 3: Copper and CIP molar ELF concentration versus time profiles.

Figure 4: Number of bacteria (*P. aeruginosa*) per lung of infected rat (CFU/lung) measured 8 days after pulmonary infection and after inhalation of particles made of ciprofloxacin hydrochloride at 0.3mg/kg (CIP-HCl) or ciprofloxacin-copper loaded CaCO₃-based particles at 0.3mg of CIP /kg (SD CIP-Cu) or in non-treated animals (control)

5

EXAMPLES

Example 1: In vitro biopharmaceutical evaluation of ciprofloxacin/metal cations complexes for pulmonary administration

10 In this study, ciprofloxacin (CIP) was chosen as a representative fluoroquinolone (FQ) and the 5 five following cations were used to study the complexation with CIP: Ca²⁺, Mg²⁺, Zn²⁺, Al³⁺ and Cu²⁺. Assays were carried out in medium and conditions that mimic the biological characteristics of the lung: Interaction between FQs and cations was assayed in a physiological saline buffer, the permeability of CIP in the presence of cations was assayed
15 using the Calu-3 cell line and the antimicrobial efficacy of CIP in the presence of cations was evaluated against *Pseudomonas aeruginosa*, a frequent opportunistic infectious agent in the lung.

Materials & Methods

20 1. Chemicals

Sodium fluorescein, CIP, CaCl₂, 2H₂O; MgCl₂, 4.5H₂O; ZnSO₄, 7H₂O; CuSO₄, 5H₂O; Al(OH)₃; MOPS, TRIS and HEPES were obtained from Sigma-Aldrich. HPLC-grade acetonitrile was purchased from VWR International (Fontenay sous Bois, France). Mueller Hinton II agar and Mueller Hinton II Broth (cation adjusted) were from Becton Dickinson (Le
25 Pont-de-Claix, France). All other reagents were of analytical grade.

2. Apparent constant of association (K)

To study the interaction between FQs and metal cations in medium having ion composition and pH close to biological samples such as the epithelial lining fluid (ELF) of the lung
30 alveoli, CIP was incubated in saline solution (NaCl and KCl) at pH = 7.4. To assess the possible interaction between CIP and buffers, HEPES, MOPS or TRIS buffer was added to a 30 μM CIP solution in water at different concentrations (0, 1, 2.5, 7.5 and 10 mM) and pH was adjusted to 7.4 with 1 M sodium hydroxide. One ml of each solution was placed in 24

well plates and fluorescence intensity emission spectrum of CIP was recorded at room temperature with a plate reader ($\lambda_{exc} = 274$ nm and λ_{em} ranging from 360 to 500 nm; VarioskanFlash, Thermo Scientific, Villebon sur Yvette, France). MOPS and Tris buffers shifted and lowered the spectrum respectively, HEPES buffer did not affect the CIP

5 fluorescence from 360 to 500 nm with a concentration up to 10 mM (data not shown). Moreover, HEPES is generally considered a non-complexing buffer and is suitable for use in solutions with metal ions. This buffer was then chosen for all the following experiments. Fluorescence emission spectrum of CIP in the presence of increasing concentrations of the different cations did not show a shift of the maximum emission wavelength. The set of 274

10 and 408 nm for the excitation and emission wavelengths was chosen for all the titration experiments. Fluorescence titration profiles were obtained by adding cation solutions with concentrations ranging from 500 to 10^5 μ M for calcium and magnesium ions, from 15 to 500 μ M for zinc ions and from 15 to 150 μ M for copper and aluminum ions to a 3 μ M CIP solution. Solutions contained 5.3 mM KCl and were maintained at constant pH 7.4 with 10

15 mM HEPES and adjusted with NaCl to a constant ionic strength (155 mM for Mg^{2+} , Zn^{2+} , Al^{3+} , Cu^{2+} and 1205 mM for Ca^{2+}). The final volume of each sample was 1 ml and fluorescence was recorded using the VarioskanFlash plate reader. Binding isotherm equations for 1:1 and 2:1 complexes were built to describe the data according to Hargrove et al., (Hargrove et al. 2010. New journal of chemistry 34, 348-354) (for the Equations and

20 mathematical development see below). The experimental data were fitted with the two models (1:1 and 2:1) and the comparison was evaluated with the Akaike's information criteria (AIC).

Equations and mathematical development

For 1:1 complex (CIP:I) between ciprofloxacin (CIP) and a metal ion (I), the equilibrium,

25 apparent binding constant K and mass balance equations are expressed in eqn (1)-(4).



$$K = \frac{[CIP:I]}{[CIP][I]} \quad (2)$$

$$[CIP]_t = [CIP] + [CIP:I] \quad (3)$$

$$[I]_t = [I] + [CIP:I] \quad (4)$$

Where $[CIP]_t$ and $[I]_t$ are the concentrations of the total CIP and metal ions and $[CIP]$ and $[I]$ are the concentrations of the free CIP and metal ions. $[CIP:I]$ is the concentration of the complex. Rearranging eqn (2) with eqn (3) and (4) yields to the quadratic equation (5) from which the eqn (6) is a solution.

$$5 \quad [CIP]^2 + \left([I]_t - [CIP]_t + \frac{1}{K}\right)[CIP] - \frac{[CIP]_t}{K} = 0 \quad (5)$$

$$[CIP] = \frac{-\left([I]_t - [CIP]_t + \frac{1}{K}\right) + \sqrt{\left([I]_t - [CIP]_t + \frac{1}{K}\right)^2 + \frac{4[CIP]_t}{K}}}{2} \quad (6)$$

The observed fluorescence (F_t) is the sum of the CIP and CIP:I complex fluorescences ($FCIP$ and $FCIP:I$), considering the ions are not fluorescent:

$$F_t = FCIP + FCIP:I = \delta_{CIP}[CIP] + \delta_{CIP:I}[CIP:I] \quad (7)$$

- 10 where δ_{CIP} and $\delta_{CIP:I}$ are the proportionality coefficients between concentration and fluorescence for the CIP and the complex respectively. Rearranging eqn (7) with eqn (3) and into eqn (6) gives the final 1:1 binding isotherm (eqn 8).

$$F_t = (\delta_{CIP} - \delta_{CIP:I}) \left\{ \frac{\left([CIP]_t - [I]_t - \frac{1}{K}\right) + \sqrt{\left([I]_t - [CIP]_t + \frac{1}{K}\right)^2 + \frac{4[CIP]_t}{K}}}{2} \right\} + \delta_{CIP:I}[CIP]_t \quad (8)$$

- 15 For 2:1 complex ($CIP_2:I$) between ciprofloxacin (CIP) and a metal ion (I), the equilibriums, binding constants K_1 and K_2 and mass balance equations are expressed in eqn (9)-(14). For eqn (14), the assumption that the initial concentration of ions ($[I]_t$) is much higher than the initial concentration of CIP ($[CIP]_t$) leads to a simplified equation. Experimentally, ion concentrations were always at least 5 times higher than CIP.



$$K_1 = \frac{[CIP:I]}{[CIP][I]} \quad (11)$$

$$K_2 = \frac{[CIP_2:I]}{[CIP:I][CIP]} \quad (12)$$

$$[CIP]_t = [CIP] + [CIP:I] + 2[CIP2:I] \quad (13)$$

$$[I]_t = [I] + [CIP:I] + [CIP2:I] \approx [I] \quad (14)$$

Rearranging eqn (11) and (12) with eqn (13) and (14) yields to the quadratic equation (15) from which the eqn (16) is a solution.

$$5 \quad [CIP]^2 + \left(\frac{1+K_1[I]_t}{2K_1K_2[I]_t} \right) [CIP] - \frac{[CIP]_t}{2K_1K_2[I]_t} = 0 \quad (15)$$

$$[CIP] = \frac{-\left(\frac{1+K_1[I]_t}{2K_1K_2[I]_t} \right) + \sqrt{\left(\frac{1+K_1[I]_t}{2K_1K_2[I]_t} \right)^2 + \frac{2[CIP]_t}{K_1K_2[I]_t}}}{2} \quad (16)$$

The observed fluorescence (F_t) is the sum of the CIP, CIP:I and CIP2:I complex fluorescences ($FCIP$, $FCIP:I$ and $FCIP2:I$), considering that the ions are not fluorescent:

$$F_t = FCIP + FCIP:I + FCIP2:I = \delta_{CIP}[CIP] + \delta_{CIP:I}[CIP:I] + \delta_{CIP2:I}[CIP2:I] \quad (17)$$

- 10 where δ_{CIP} , $\delta_{CIP:I}$ and $\delta_{CIP2:I}$ are the proportionality coefficients between concentration and fluorescence for the CIP and the complexes. Rearranging eqn (17) with eqn (11) and (12) gives the final 2:1 binding isotherm (eqn 18) together with eqn (16).

$$F_t = (\delta_{CIP} + \delta_{CIP:I} K_1 [I]_t) [CIP] + \delta_{CIP2:I} K_1 K_2 [I]_t [CIP]^2 \quad (18)$$

15 3. Apparent permeability (P_{app}) of CIP in the presence of metal cations

3.1. Calu-3 cell culture

- Calu-3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM/Ham's F12 (1/1) supplemented with L-glutamine (2
20 mM) and 10% foetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany) and incubated at 37°C under 90-95% of relative humidity and 5% v/v of CO₂ in air. The Calu-3 cells at passages 50-60 were seeded at a density of 15×10^4 cells/cm² onto 12-well plate Transwell inserts (Corning Transwell Clear PET membrane 0.4µm, Thermofischer Scientific). The cells were cultured under air-interface conditions for 15 days. The growth medium in the
25 basolateral compartment (1.5 ml) was replaced by fresh medium every other day.

3.2. Transport experiments

Transport experiments were conducted in apical-to-basolateral directions as described elsewhere (Brillault et al. 2010. Antimicrobial agents and chemotherapy 54, 543-545). Briefly, on study day the Calu-3 monolayers were quickly rinsed with the transport medium (TM: NaCl 134 mM, KCl 5.3 mM, glucose 5.5 mM, CaCl₂ 1.3 mM, MgCl₂ 1 mM, buffered with 10 mM HEPES and adjusted to pH 7.4) and then incubated for 30 min in TM. This transport medium is a modified formula of the classical HBSS medium without bicarbonate or phosphate known to form complexes with the metal ions. Following the equilibration period, the TM in the donor compartment (apical side) was replaced by fresh TM containing 50 µM of CIP with 0, 5, 10, 50 or 100 mM Mg²⁺. After 60 min of incubation at 37°C, sample aliquots were taken from the acceptor compartment (basolateral side). Apparent permeability (P_{app}) was calculated using eqn (1) where Q is the amount of CIP in the acceptor compartment after a time Δt, S is the insert membrane surface (1.12 cm²), [CIP]₀ is the initial CIP concentration in the donor compartment.

$$P_{app} = \frac{Q}{[CIP]_0 \cdot \Delta t \cdot S} \quad (1)$$

P_{app} versus Mg²⁺ concentration data were analysed with the following equation (eqn 2).

$$\% \text{ of } P_{app} = 100 - \frac{(100 - P_{app \text{ Min}}) \cdot [X]^{nH}}{EC50^{nH} + [X]^{nH}} \quad (2)$$

Where P_{app Min} is the low plateau of P_{app}, [X] the Mg²⁺ concentration, EC50 the Mg²⁺ concentration necessary to get 50% of the maximum effect (100- P_{app Min}) and nH the Hill number. In a second experiment, P_{app} were determined after cells were incubated in TM with 50 µM of CIP and 40 mM of Ca²⁺, 5.5 mM of Mg²⁺, 1.5 mM of Zn²⁺, 0.1 mM of Al³⁺ or 0.04 mM of Cu²⁺ for a 60 min incubation period before sampling in the acceptor compartment. Following the transport studies, routine controls of the monolayer integrity were performed using sodium fluorescein. Briefly, the monolayers were rinsed with TM, fresh TM was added in the basolateral side and a solution of sodium fluorescein in TM (10 µg/ml) was poured in the apical side. The inserts were incubated and samples were taken after 60 min from the acceptor compartment. A threshold P_{app} value of 0.7 x 10⁻⁶ cm.s⁻¹ for fluorescein was retained for the tight junction integrity rejection parameter for all experiments. This corresponds to the transfer of less than 0.5% of the initial amount in the apical compartment (Brillault et al., 2010).

3.3. CIP and sodium fluorescein assay

CIP was assayed using a HPLC method with fluorometric detection (λ_{exc} =280 nm; λ_{em} =460 nm) using a Jasco FP-920 fluorescence detector (Jasco France, Lisses, France). The

stationary phase was an XTerra MS C18 column, 5 μ m, 100 $\times$ 2.1 mm (Waters, Milford, MA). The mobile phase (flow rate: 0.25 ml/min using a Hitachi L-2130 pump, Hitachi High technologies Co., Berkshire, UK) consisted of a 20:80 (v:v) mixture of acetonitrile and water containing 0.1% formic acid and 0.2% heptane sulfonic acid. Samples, standards (7 levels with concentrations ranging from 1.56 to 50 ng/ml) and quality controls (3.12, 12.5 and 37.5 ng/ml) prepared in the same solvent were injected (75 μ l using a Hitachi L-2200 autosampler) and eluted over a run time of 6.5 min. The precision and accuracy were less than 15% for the 3 quality control concentrations. Controls with various concentrations of CIP and cations were assayed to ensure that the presence of cations did not interfere with the analysis of CIP.

Fluorescein concentrations in TM were measured using the VarioskanFlash plate reader with the excitation and emission wavelengths set at 490 nm and 530 nm, respectively.

4. Cytotoxicity assay

Calu-3 cells were seeded in 96 well plates at the density of 5000 cells/well. Incubation medium (100 μ l) was Gibco MEM (Thermo Fisher Scientific) supplemented with 5% (v/v) foetal calf serum. CIP and ions solutions were prepared at concentrations necessary to achieve 80 % of complex. Namely, 150 μ M of CIP were mixed with 40.1 mM of Ca^{2+} , 5.6 mM of Mg^{2+} , 1.6 mM of Zn^{2+} , 0.17 mM of Al^{3+} or 0.125 mM of Cu^{2+} . The cells were then incubated with a serial dilution of these solutions or the CIP alone with concentrations ranging from 0.3 to 150 μ M and the plates were returned to the incubator. Control cells were incubated with ions alone with serial dilution from 100 to 0.05 mM for Ca^{2+} and Mg^{2+} and from 5 to 0.0025 mM for Zn^{2+} , Al^{3+} and Cu^{2+} . Following a 24 h incubation time, the incubation medium was removed and replaced with HBSS medium and cell viability was evaluated with a MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega, Charbonnières-les-Bains, France) according to the manufacturer protocol. The linearity of the response was verified thanks to preliminary experiments. Data were analysed with the following equation (eqn 3).

$$\% \text{ of viability} = \text{Top} - \frac{(\text{Top} - \text{Bottom}) \cdot [\text{X}]^{nH}}{\text{EC}_{50}^{nH} + [\text{X}]^{nH}} \quad (3)$$

where [X] is the concentration of CIP or ions, Top and Bottom are plateaus in the units of the Y axis, EC50 is the concentration that gives half of the total effect (Top-Bottom) and nH is the Hill number.

5. MIC assay

The effect of cation (Mg^{2+} , Ca^{2+} , Al^{3+} , Zn^{2+} , Cu^{2+}) concentrations on the CIP antibacterial activity was assessed by measuring the apparent CIP minimum inhibitory concentration (MIC) against *Pseudomonas aeruginosa* PAO1 (CIP 104116, Institut Pasteur, Paris, France).

5 Bacteria were stored at -80°C . One day before the experiment, they were grown on Mueller-Hinton II agar for 24 h at 37°C . Prior to MIC test, the strains were grown to logarithmic phase in fresh Mueller-Hinton broth II (MHB) for 2-3 hours and then adjusted to 0.5 McFarland standards. The MIC test was performed using the broth microdilution method: The adjusted cultures were diluted 100-times in MHB containing cations, CIP or both and 100 μl of these
 10 bacterial suspensions were seeded in 96 well plates. CIP concentrations were 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0 $\mu\text{g/ml}$. Mg^{2+} concentrations were 20, 10, 5, 2, 1, 0.5, 0 mM. Ca^{2+} concentrations were 100, 50, 20, 10, 5, 2, 1, 0.5, 0 mM. Zn^{2+} concentrations were 1, 0.5, 0.1, 0.05, 0.02, 0.01, 0 mM. Al^{3+} and Cu^{2+} concentrations were 0.1, 0.05, 0.02, 0.01, 0.005, 0 mM. The growth of bacterial cultures at 37°C was determined after 18 hours by monitoring the
 15 optical density of the culture at 600 nm using the VarioskanFlash plate reader. Controls without bacteria were made to check the absence of contamination of MHB and to evaluate the effect of the cations on the absorbance reading at 600 nm. Controls without CIP were made to assess the effect of the cations on bacterial growth.

20 6. Statistical analysis

Data analysis and modeling were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results

25 1. Apparent constants of association

Titration curves of CIP with Mg^{2+} , Ca^{2+} , Zn^{2+} , Al^{3+} and Cu^{2+} ions were made in HEPES buffered saline solutions. Increasing the concentration of Ca^{2+} , Mg^{2+} and Zn^{2+} resulted in an increase in the total fluorescence, while with Al^{3+} and Cu^{2+} the total fluorescence was quenched. The equation corresponding to a 1:1 complex model fitted the data well for all the
 30 ions with a satisfactory accuracy for the estimated apparent constant of association (Table 1). On the other hand, the equation from the 2:1 complex model resulted in a very broad estimation of the parameters and higher AIC values (data not shown). This suggested a 1:1 (CIP:metal cation) complex stoichiometry. The estimated apparent association constants

ranked with the descending order: $\text{Cu}^{2+} > \text{Al}^{3+} > \text{Zn}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+}$, with almost a 9000 fold ratio between the minimum and maximum values (Table 1).

2. Influence of complexation on Calu-3 cell layer permeability

5 In a preliminary experiment, Mg^{2+} was chosen to test the influence of the ion concentration on the apical to basolateral permeability of CIP through the Calu-3 cell model. In the control conditions where no magnesium was added to the transport medium, the Papp was $0.34 \pm 0.02 \times 10^{-6}$ cm/s. The results showed a decrease in the CIP Papp dependent on the concentration of Mg^{2+} . As estimated by the modeling, 6.7 mM of added magnesium reduced
10 the Papp to 50% of the control and a plateau was reached at 11.5 % of the control Papp (0.04×10^{-6} cm/s). According to the mass balance equations from 1:1 model and to the apparent constant of association determined in table 1, the percentage of complexation of CIP by Mg^{2+} was calculated and plotted against the Papp. These data were modeled with eqn 2 and showed that it is necessary to form 86% of complexation to reduce the Papp of 50%. Since the
15 complexation apparently correlated with the decrease in permeability, the transport of CIP was assayed at concentrations of metal cations necessary to reach the same level of complexation. As seen with the Mg^{2+} , a high level of complexation is needed to induce a significant decrease in permeability, but high concentrations of ions, as for Ca^{2+} would lead to hypertonic and deleterious conditions for the cells. For these reasons, a target of 80%
20 complexation was chosen for the next experiment and theoretical ion concentrations were calculated for Ca^{2+} , Mg^{2+} , Zn^{2+} , Cu^{2+} and Al^{3+} . Thus the cells were incubated in the presence of 50 μM of CIP and 40 mM of Ca^{2+} , 5.5 mM of Mg^{2+} , 1.5 mM of Zn^{2+} , 0.1 mM of Al^{3+} or 0.04 mM of Cu^{2+} . Results confirmed that such cation concentrations led to a decrease in CIP permeability close to 50%.

25

3. Cytotoxicity assay

As shown in table 2, ions may be classified into two groups according to their cytotoxicity: the first group (low toxicity) includes Mg^{2+} and Ca^{2+} with an estimated EC_{50} at 36×10^3 and 131×10^3 μM , respectively; the second group (higher toxicity) includes Zn^{2+} , Al^{3+} and Cu^{2+}
30 with EC_{50} close to 1×10^3 μM . Cytotoxicity of the CIP was evaluated in the presence of cations. The highest CIP concentration was 150 μM and ion concentrations were chosen to reach 80 % complexation. For Cu^{2+} , Mg^{2+} , Al^{3+} and Ca^{2+} , the concentrations were several times lower than their respective EC_{50} and for Zn^{2+} equal to its EC_{50} (Table 2). Thus, the presence of ions at concentrations forming 80% complexes with CIP should not increase the

cell toxicity of the CIP. As shown in table 2, the EC₅₀ of the CIP over the Calu-3, which was evaluated to be 7 μ M, was not apparently affected by the presence of these ions.

4. MIC assay

5 The influence of the cations was also tested on the antibacterial effect of CIP against *P. aeruginosa*. In the presence of the ions alone at all concentrations tested, the bacterial growth was similar to the control (data not shown). The MIC of CIP was 0.25 μ g/ml. In the presence of Ca^{2+} or Mg^{2+} the MIC of CIP was increased from an ion concentration of 5 mM (33% of complexation) and 2 mM (50% of complexation) onwards, respectively. In the presence of
10 Zn^{2+} , the MIC was unchanged up to a concentration of 500 μ M (50% of complexation) where it decreased of one level. The MIC remained unchanged for Al^{3+} and Cu^{2+} , except at 50 μ M for copper (98% of complexation).

Conclusions

15 Fluoroquinolones (FQs) have a rapid diffusion through the lung epithelium giving the pulmonary route no advantage compared to the oral route. Interactions between FQs and metal cations form complexes which limit the diffusion through the epithelial barrier and would reduce the absorption of FQs and maintain high concentrations in the lung. In this study, Ciprofloxacin (CIP) was chosen as a representative FQ and 5 cations (Ca^{2+} , Mg^{2+} , Zn^{2+} ,
20 Al^{3+} , Cu^{2+}) were selected to study the complexation and its effects on permeability, antimicrobial efficacy and cell toxicity. The results showed that the apparent association constants between CIP and cations ranked with the descending order: $\text{Cu}^{2+} > \text{Al}^{3+} > \text{Zn}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+}$. When a target of 80% complexation was reached with the adequate concentrations of cations, the CIP permeability through the Calu-3 lung epithelial
25 cells was decreased of 50%. Toxicity of the CIP on the Calu-3 cells, with an EC₅₀ evaluated at 7 μ M, was not significantly affected by the presence of the cations. The minimum inhibitory concentration of CIP for *Pseudomonas aeruginosa* was not affected or slightly increased in the range of cation concentrations tested, except for Mg^{2+} .

In conclusion, permeability was the main parameter that was affected by the metal cation
30 complexation while cell toxicity and antimicrobial activity were not or slightly modified. Cu^{2+} , with the highest apparent constant of association and with no effect on cell toxicity and antimicrobial activity of the CIP, appeared as a promising cation for the development of a controlled-permeability formulation of FQs for lung treatment.

Apparent association constant K (M ⁻¹)		
Ca ²⁺	1 x 10 ²	[13-188]
Mg ²⁺	7.2 x 10 ²	[176-1272]
Zn ²⁺	27 x 10 ²	[1792-3614]
Al ³⁺	880 x 10 ²	[56540-119406]
Cu ²⁺	9069 x 10 ²	[849417-964359]

Table 1. Apparent association constant between CIP and ions (with the 95% confidence interval) in saline solution at pH 7.4.

Calu-3 cell toxicity EC50 (μM)			
		CIP	7 [5-11]
Ca ²⁺	36 x 10 ³ [22-61 x 10 ³]	CIP:Ca²⁺	5 [4-6]
Mg ²⁺	131 x 10 ³ [111-152 x 10 ³]	CIP:Mg²⁺	11 [8-14]
Zn ²⁺	0.9 x 10 ³ [0.7-1.1 x 10 ³]	CIP:Zn²⁺	3 [3-4]
Al ³⁺	1.0 x 10 ³ [0.7-1.4 x 10 ³]	CIP:Al³⁺	7 [5-9]
Cu ²⁺	1.1 x 10 ³ [0.7-1.5 x 10 ³]	CIP:Cu²⁺	14 [12-16]

Table 2. Cell toxicity EC50 of cations alone or CIP:cations mix in 80% complexation conditions over a 24 h incubation period. Values are given with their 95% confidence interval.

Example 2: In vivo Control of the Ciprofloxacin Pulmonary Concentration by Control of the Blood-Lung Barrier Permeability

10 Materials and Methods.

Chemicals. Ciprofloxacin (CIP) powder (purity ≥ 98,0 %), copper hydroxide Cu(OH)₂, hyaluronic acid (HA) sodium salt from *Streptococcus equi*, calcium hydroxide Ca(OH)₂, formic acid, ammonium carbonate (NH₄)₂CO₃, magnesium chloride Mg(Cl₂), sodium chloride Na(Cl₂), potassium chloride K(Cl₂), sodium phosphate dibasic Na₂HPO₄, sodium sulfate Na₂SO₄, calcium chloride Ca(Cl₂), sodium acetate trihydrate C₂H₃O₂Na.3H₂O, sodium carbonate Na₂CO₃, sodium citrate monohydrate C₆H₅Na₃O₇.H₂O, sodium hydroxide NaOH, citric acid C₆H₈O₇.H₂O, glycine C₂H₅NO₂, sodium tartrate C₄H₄Na₂O₆.2H₂O, sodium lactate C₃H₅NaO₃ and sodium pyruvate C₃H₃NaO₃ were purchase from Sigma. All chemicals used were of analytical grade, and solvents were of high-performance liquid chromatography (HPLC) grade.

Animals. This work was carried out in compliance with EC Directive 2010/63/EU after agreement by the Ethic Committee and registration by the French Ministry of Higher Education and Research (COMETHEA, n° 2015042116017243). Male Sprague-Dawley rats (n=78) from Janvier Labs (Le Genest-St.-Isle, France), weighing between 300 and 350g, were

used for the *in vivo* pharmacokinetic investigations. All animals were acclimatized in wire cages in a 12h light-dark cycle for 5 days after their arrival and before experiments. During this period, they had free access to food and water.

Preparation of CIP microparticles. Microparticles composed of calcium carbonate, sodium hyaluronate and CIP-Ca or CIP-Cu complex were prepared by spray-drying as previously described (F. Tewes, et al. ACS applied materials & interfaces, 8 (2016) 1164-1175)). Briefly, two aqueous solutions were prepared. One solution was made of CIP, calcium hydroxide, hyaluronate and formic acid, and when specified, copper hydroxide. The other one was made of ammonium carbonate (Table 3). These solutions were prepared separately and mixed during the microparticle preparation process using a Y-tube connected to the feeding tube of the Büchi B-290 mini spray dryer. The spray dryer was operated in the sucking open mode and settings were: 30% peristaltic pump rate, 15 L/min spraying air flow rate, 630 L/h drying air flow rate, 120°C inlet temperature.

Table 3: Concentrations of the solutions used for the preparation of microparticles by spray-drying

Formula code	CIP (g/L)	Cu(OH) ₂ (g/L)	HA (g/L)	(NH ₄) ₂ CO ₃ (g/L)	Ca(OH) ₂ (g/L)	HCOOH % (v/v)
F1a	0.8	0	0.4	2.4	0.8	0.1
F1b	0.8	0.12	0.4	2.4	0.8	0.1
F1c	0.8	0.168	0.4	2.4	0.8	0.1
F1d	0.8	0.24	0.4	2.4	0.8	0.1
F2a	1.6	0	0.4	2.4	0.8	0.1
F2b	1.6	0.448	0.4	2.4	0.8	0.1

Powder X-ray diffraction (XRD). X-Ray powder diffraction was measured using a Rigaku Miniflex II desktop X-Ray diffractometer (Rigaku, Japan) with Ni-filtered Cu K α radiation (λ = 1.54 Å).

Attenuated total reflectance (ATR)-FTIR measurements were performed on powders placed on a wedged diamond crystal using a Spectrum 400FT spectrometer (PerkinElmer, Ireland). For each spectrum, 32-scan interferograms were collected with 4 cm^{-1} resolution. Clean crystal surface was used as reference. Background spectra were subtracted from sample spectra.

Scanning electron microscopy (SEM). SEM micrographs were made with a TescanMIRA Variable Pressure Field Emission Scanning Electron Microscope (Czech Republic). Samples were fixed on aluminium stubs using double-sided adhesive tape and sputter-coated with gold. Visualization was performed at 5kV and micrographs were taken at different magnifications in more than one region of the sample.

Volume-weighted geometric particle size distribution (PSD). The PSD was determined using a Malvern Mastersizer 2000 laser diffraction instrument (Malvern Instruments Ltd. Worcestershire, UK). The Sirocco 2000 dry powder feeder was set with 2 bar air pressure and a vibration feed rate of 50% to disperse the particles. Analyses were undertaken using areal part refractive index of 1.572 and an absorption part of 0.01. Results presented are the average of three determinations.

Specific surface area (SSA) and pore size determination. SSA and porosity were investigated by gas adsorption-desorption isotherms at 77 K using a Micromeritics Gemini 2835c (SMS Ltd., London, UK). Adsorption measurements were performed with nitrogen gas as the analytical (adsorptive) gas and helium as the reference gas for free space measurements. Prior to analysis the samples were degassed under nitrogen gas for 24h at 30°C to remove residual solvent content. The evacuation was carried out at a rate of 500 mmHg/min, for 1 min. Equilibration time for adsorption was 10s. The amounts of nitrogen gas adsorbed under six relative pressure values ($0.05 < P/P_0 < 0.30$) were determined in order to calculate SSA according to the Brunauer, Emmett and Teller (B.E.T.) method. Analyses were performed in triplicate for each sample.

Particle true density. The particles true density was measured by an AccuPyc 1330 Pycnometer (Micromeritics®) using helium (99.995% purity) to determine the volume of samples, dried for 24 h prior to analysis, at 30°C under nitrogen flow ($n = 2$).

Release studies. Release studies were performed under sink conditions (i.e. the maximum concentration of dissolved CIP never exceeded 20 % of the CIP solubility) in an artificial

lysosomal fluid (ALF) or in a simulated lung fluid (SLF) prepared according to Marques *et al.* Dissolution technologies, 18 (2011).

Powders containing around 3 mg of CIP were dispersed in a sealed bottle containing 200 mL of ALF or SLF at 37°C and orbitally shaken at 300 rpm. At predetermined time points over 96h, samples (1 mL) were collected and centrifuged and supernatants were assayed for CIP using the method described below. All samples were stored at 4°C prior to HPLC assay (n=3).

In vivo experiments. Target CIP dose for intratracheal (IT) administration of aerosolized powder or nebulized solution was 3 mg.kg⁻¹. The intratracheal administrations were performed under isoflurane anesthesia. The dry powders were administered using a PennCentury™ Dry Powder Insufflator, Model DP-4 (Penn-Century Inc., Philadelphia, USA) and the CIP solution was administered using a MicroSprayer IA-1B apparatus (Penn-Century Inc, Philadelphia, USA) (Gontijo *et al.* Antimicrobial agents and chemotherapy, 58 (2014) 3942-3949; Marchand *et al.* 54 (2010) 3702-3707). The delivery tube was directly inserted between the rat's vocal chords as previously described (Marchand *et al.* 54 (2010)). The broncho-alveolar lavage (BAL) sampling was carried out as previously described (Gontijo *et al.* Antimicrobial agents and chemotherapy, 58 (2014) 3942-3949; Marchand *et al.* 54 (2010) 3702-3707) at determined time points over 18h after administration of CIP-Cu and CIP-Ca microparticles or over 4h after administration of CIP solution. Briefly, rats (5 to 7 per time point) were anesthetized with inhaled isoflurane and immobilized in a supine position with cervical hyperextension. One mL of saline at 37°C in a pre-filled syringe was injected into the airways via a polyethylene catheter (0.58 mm i.d. and 0.96 mm o.d.; Harvard, Les Ulis, France) inserted into the trachea (50 mm deep). The BAL samples (300 to 800 µL) were immediately collected by aspiration, centrifuged at 2000 rpm for 5 min and the supernatants were stored at -20°C until CIP and urea assays. Blood samples were collected by intracardiac puncture soon after the BAL sample collection and centrifuged at 3000 t/min for 10 minutes. The plasma samples were collected and stored at -20°C until CIP assay.

Analytical assays.

CIP concentrations were determined by reversed-phase HPLC coupled to a fluorometer for detection (λ_{exit} : 280 nm, λ_{em} : 460 nm). Reversed-phase chromatography was performed by using a security guard cartridge (Gemini C₁₈, Phenomenex) and a C₁₈ X Terra MS column (5 µm pore size, 100 x 2.1 mm) for BAL, ALF and SLF samples assay or a C₁₈ Phenomex column (5 µm pore size, 150 x 2.1 mm) for plasma samples assay. The mobile phase flowing

at a rate of 0.25 mL.min⁻¹ was made of 0.1% formic acid in water, acetonitrile and sodium heptanesulfonate (PIC[®] B7, Waters) mixed in a volume ratio of 80:20:1 (v:v:v) for the BAL, ALF or SLF samples or 86:14:1 (v:v:v) for the plasma samples. For assay in ALF and SLF, seven calibration standards (from 0.1 to 10 µg.mL⁻¹) and 3 levels of control (0.2, 1, 7.5 µg.mL⁻¹) were prepared in blank ALF or SLF. For assay in BAL, seven calibration standards (from 1.56 to 10 ng.mL⁻¹) and 3 levels of control (3.125, 2.5, 7.5 ng.mL⁻¹) were prepared in blank BAL. For assay in plasma, six calibration standards (from 5 to 100 ng.mL⁻¹) and 3 levels of control (10, 25, 75 ng.mL⁻¹) were prepared in blank plasma. Intra- and interday variabilities were characterized at four and three levels of drug concentrations respectively, with a precision and accuracy always lower than 15%. For the calculation of the slope and the intercept of the calibration curve, a 1/X²-weighted linear regression was applied. The BAL, ALF and SLF samples were directly injected, with or without dilution. For the preparation of CIP standards, controls and samples in plasma, 50 µL of perchloric acid 7%:methanol (50:50 (v:v)) was added to 100 µL of plasma samples and then centrifuged at 14000 rpm during 15 min at 4°C. The supernatants were collected and injected directly.

The urea concentration in BAL was assayed by using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Gontijo et al. Antimicrobial agents and chemotherapy, 58 (2014) 3942-3949; Marchand et al. 54 (2010) 3702-3707).

Urea, transaminases (ALAT, ASAT), C reactive protein (CRP) and lactate dehydrogenase (LDH) concentrations in plasma samples and LDH and alkaline phosphatase (AP) and the total protein (TP) concentrations in BAL samples were measured using a Cobas[®] 8000 modular analyzer (Roche Laboratories, CHU of Poitiers, France).

Cu and Ca concentrations in BAL were assayed using an Inductively Coupled-Plasma Optical Emission Spectrometer (ICP-OES, Perkin Elmer Optima 2000DV). Five-point calibration standards (from 50 to 200 ppb for Cu and from 50 to 2000 ppb for Ca) were prepared. Four measurements were made at 327.393 nm and 317.933 nm for Cu and Ca, respectively.

Conversion of the BAL concentrations to ELF concentrations. In order to estimate ELF concentrations, concentrations measured in BAL were corrected by a dilution factor calculated using the BAL/plasma urea concentration ratios as previously described (Gontijo et al. Antimicrobial agents and chemotherapy, 58 (2014) 3942-3949; Marchand et al. 54 (2010) 3702-3707).

Data analysis. CIP concentrations in ALF, SLF, ELF and plasma were presented as means $\pm$ SD. ELF versus unbound plasma CIP concentration ratios between formulations were analyzed by using Two-way ANOVA test followed by Bonferroni post-test for each time of sampling. The unbound plasma fraction was fixed to 59% (Roosendaal et al., Antimicrob Agents Chemother, 31 (1987) 1809-1815). Mean areas under the unbound plasma and ELF concentrations versus time curves (AUC) from zero and the time corresponding to the end of sampling were estimated from mean plasma and ELF concentration by using trapezoidal method and extrapolations to infinity were performed by the slope (WinNonLin 6.2, Pharsight, US) ($AUC_{u, plasma}$ and AUC_{ELF}).

10 Results.

Microparticle characterization.

The microparticles were formulated based on the $CaCO_3$ mineralization bio-inspired process previously described (F. Tewes, et al. ACS applied materials & interfaces, 8 (2016) 1164-1175). The present innovation results in the addition of copper with the aim to form a CIP-Cu complex.

Only a few differences appear between formulations prepared with various copper or CIP concentrations (Table 4). The increase in Cu^{2+} or CIP concentration tested in the spray dried solutions had little impact on the microparticle morphology observed by SEM and light shell-like microparticles were obtained in all the formulation conditions. Likewise, similar geometric PSD were observed between the formulations (Table 4). Still, excepted for the formulation F1c, an increase in Cu^{2+} concentration for a similar CIP concentration in the sprayed solution had several minor effects. First, it increased the particle density from 2.27 ± 0.03 to 2.56 ± 0.04 g/cm³ for the F1 formulations and from 1.76 ± 0.10 to 2.00 ± 0.04 g/cm³ for the F2. Second, it decreased the CIP loading (Table 4). This effect was observed for the formulations F1 and F2 prepared by using solutions having CIP concentrations of 0.8 and 1.6 g/L, respectively. Third, it decreased the specific surface area (SAA) measured from the N₂ sorption (Table 4). These SSA values calculated using the BET method were 15 to 30 times higher than the SSA calculated using the particles size distribution (Table 4). High SSA is a good metric indicative of a good aerodynamic performance of particles [23, 24]. The difference between SSA values may be explained by the porosity of the particles [24]. The particles shell porosity (Table 4) can be calculated using Equation 1:

$$porosity = \frac{void\ volume}{solid\ volume + void\ volume} = \frac{TPV}{\frac{1}{\rho_s} + TPV} \quad \text{Equation 1}$$

Where TPV was the total pore volume (cm³/g) measured from the N₂ sorption-desorption isotherm (supplement S4) and ρ_s was the apparent particle density (g/cm³) measured by the pycnometer. The particle porosity decreased when CIP or Cu²⁺ concentration increased in the sprayed solutions (Table 2). Porosity decreased from 0.21 to 0.12 when the CIP loading was doubled (F1a to F2a). For the formulations F1, porosity decreased from 0.21 to 0.15 when Cu(OH)₂ concentration in the sprayed solution increased from 0 to 0.24 g/L.

Table 4: Physical properties of the microparticles.

CIP Loading (w/w)	%	True density (g/cm ³)	N ₂ sorption		Particle shell porosity	Geometric PSD			Specific surface area (m ² /g)*
			BET	SSA		d(0.1) (μm)	d(0.5) (μm)	d(0.9) (μm)	
F1a	34.3 ± 0.2	2.27 ± 0.03	28.8 ± 0.4		0,21	2.0 ± 0.1	8.7 ± 0.4	20.2 ± 1.4	1.3 ± 0.1
F1b	28.0 ± 5.3	2.38 ± 0.03	25.3 ± 0.4		0,20	1.7 ± 0.1	7.5 ± 0.3	19.2 ± 0.8	1.6 ± 0.1
F1c	28.8 ± 2.9	1.87 ± 0.21	16.5 ± 0.7		0,18	1.9 ± 0.1	6.7 ± 1.1	15.8 ± 2.1	1.5 ± 0.2
F1d	27.7 ± 4.0	2.56 ± 0.04	23.9 ± 0.5		0,15	2.6 ± 0.2	8.9 ± 0.1	19.9 ± 1.5	1.2 ± 0.1
F2a	52.15 ± 19.8	1.76 ± 0.10	22.5 ± 0.5		0,12	2.0 ± 0.0	8.7 ± 0.6	18.9 ± 2.4	1.4 ± 0.4
F2b	41.94 ± 10.4	2.00 ± 0.04	14.8 ± 0.6		0,09	2.2 ± 0.2	7.6 ± 0.2	17.7 ± 1.3	1.5 ± 0.2

*Calculated from particle size distribution

The solid state characteristics of the powders were analysed by powder XRD and ATR-FTIR. No diffraction peaks were observed on all the XRD scans meaning that the formulations were XRD amorphous. ATR-FTIR spectra recorded for the spray dried powders presented absorption bands typical of carbonate, suggesting the presence of calcium carbonate. The unloaded particles were characterized by a broad band of the carbonate bending at 866 cm⁻¹ and a split in the asymmetric stretch of the carbonate ion at 1412 and 1488 cm⁻¹, which is specific to amorphous calcium carbonate (ACC). The absence of a band around 1710 cm⁻¹ in the spectra of the CIP-loaded particle indicated that the CIP carboxylate group was deprotonated and thus potentially available to interact with copper or calcium ions.

In the presence of Cu^{2+} in the powders, a band at 822 cm^{-1} present in the CIP spectrum and in the formulation F1a, containing a CIP-Ca complex disappeared and a new band at 812 cm^{-1} was observed. These bands were attributed to the vibration of the aromatic proton in the quinolone ring of the CIP that is shifted due to the formation of CIP-Cu complex. Also, new bands were observed at 748 cm^{-1} , 947 cm^{-1} , (1258 cm^{-1} , 1268 cm^{-1} , split band) and (1302 cm^{-1} , 1313 cm^{-1} , split band) for the CIP-loaded particles. These bands were not present in the spectrum of the unloaded particles or those of the CIP raw material and are also absent from the spectra of CIP solid form such as CIP hydrochloride salt and CIP hexahydrate. The split bands at 1258 , 1268 cm^{-1} and 1302 , 1313 cm^{-1} suggested C–O stretching vibration in the carboxyl group. These bands were previously identified in the FTIR spectrum of a 1:1 CIP:copper complex and could be due to the formation of the same complex or to the formation of a 1:1 CIP:calcium complex.

CIP release from the particles was evaluated at physiological pH (SLF medium) and pH 5 (ALF medium), representing the lung ELF pH and phagolysosome pH, respectively. Interestingly, the CIP release profiles were the same in the presence of Ca^{2+} alone or Cu^{2+} in the formulations. CIP release was total after 0.25h (data not shown), for each formulation in both simulated media.

Since the formulation properties were similar, the formulations with the highest CIP loading, i.e. formulation F2b for the CIP-Cu microparticles and formulation F2a for the CIP-Ca microparticles (Table 4) were selected for the pharmacokinetic studies in rats.

The CIP plasma and ELF concentrations versus time profiles are presented in Figure 1. The CIP concentrations were maximal at the first sampling time then decreased according to a first order kinetic. The CIP concentrations were higher in ELF than in plasma with the three formulations tested. However, the complexation with the cations allowed reaching higher CIP concentration in the ELF, and decreased the lung ELF CIP elimination rate. Beyond four hours after nebulization of the CIP solution, CIP concentrations in BAL and plasma samples were under the limits of quantification (0.0016 and $0.005\text{ }\mu\text{g.mL}^{-1}$, respectively) and could not be plotted on Figure 1A. In comparison, eighteen hours after the administration of the same dose of CIP as CIP-Cu microparticles, the CIP concentrations were 0.440 ± 0.260 and lower than the limit of quantification in ELF and plasma, respectively. This CIP ELF concentration was higher than the CIP MIC against *P. aeruginosa*.

The copper was more enabling than the calcium to change the CIP lung PK. For example, IT administrations of CIP-Cu microparticles (Figure 1C) allowed reaching and maintaining higher CIP concentrations in ELF than after IT administration of CIP-Ca microparticles (Figure 1B). Thus, the CIP ELF/plasma_(unbound) concentration ratios were above 1000 up to 4 hours after IT administration of CIP-Cu microparticles, above 100 up to 2 hours after IT administration of CIP-Ca microparticles and above 10 up to 0.5 hour after the nebulisation of CIP solution. This concentration and time effect was quantified by calculating the area under the CIP ELF concentration versus time curve divided by the area under the CIP plasma concentration versus time curve ($AUC_{ELF}/AUC_{u,plasma}$) ratios from 30 min to the last time point of measurable concentration, which were equal to 1152, 183 and 8.9 after CIP-Cu microparticle, CIP-Ca microparticle and CIP solution pulmonary administration, respectively.

The total copper and calcium plasma and ELF concentrations versus time profiles obtained after IT administration of CIP-Cu microparticles are presented in Figure 2. The total copper and calcium plasma concentrations measured after IT administration of CIP-Cu microparticles were stable (Figure 2) and did not change compare with physiological values. In the ELF, the calcium concentration increased to reach a maximal concentration of $251 \pm 289 \mu\text{g/mL}$ 2 hours after the microparticle IT administration. Then, the calcium concentration in ELF quickly decreased to reach a stable concentration, equal to the physiological calcium plasma concentration, 4 hours after IT administration. Similar data were obtained by using the CIP-Ca loaded microparticles (data not shown). This increase in calcium concentration in the ELF might be related to the CaCO_3 particles dissolution in the small volume of the lung ELF. Total copper concentrations in ELF were maximal for the initial sampling time $37 \pm 12 \mu\text{g/mL}$, then decreased following a first order kinetic to reach a concentration of $2.5 \pm 3.7 \mu\text{g/mL}$ 18h after the microparticle IT administration. This last Cu concentration was similar to the plasma physiological value ($0.93 \pm 0.1 \mu\text{g/mL}$). The Copper and CIP ELF kinetic analysed using the molar concentrations (Figure 3) showed that the Copper elimination rate was slower than the CIP one. The IT administration of the pure CIP solution did not induce a change in calcium and copper concentrations in ELF or plasma.

Toxicity studies.

In order to evaluate the potential toxicity of the formulations *in vivo*, markers of the lung integrity, i.e. the Lactate dehydrogenase (LDH) and the alkaline phosphatase (ALP) generally used to monitor pulmonary damage, were assayed in BAL fluids and activity values were

adjusted using the urea dilution factor to obtain the ELF values . On a time scale of 18 hours, ALP activity in the ELF was not significantly altered after administration of the 3 formulations compared to the activity measured in blank ELF. The CIP-Cu loaded microparticles did not induce the release of LDH compared to the blank. However, LDH activity was increased after administration of the CIP solution or of the CIP-Ca loaded microparticles. The increases in BAL fluid protein concentrations are generally consistent with an increased permeability of plasma proteins in the alveolar regions. Compared to the blank BAL, no alteration of the protein concentration values were observed after the administration of the microspheres. CRP was negative in plasma and ELF for all the formulations. The level of ALAT, ASAT or LDH in plasma did not vary significantly compared to blank samples.

Discussion.

In the present study we demonstrated that it was possible to obtain high and stable CIP concentrations in the rat pulmonary ELF by controlling the CIP elimination rate. Example 1 showed that the CIP apparent permeability across a pulmonary epithelium model can be controlled by the strength of its complexation with a metallic divalent cation. The higher was the CIP-cation complex affinity, the lower was the CIP apparent permeability. *In vitro*, Ca^{2+} was the less effective cation at reducing CIP apparent permeability. Copper (Cu^{2+}), which had an apparent association constant with CIP 10 000-times higher than that measured for Ca^{2+} , was the cation the most potent in reducing the CIP transport across the epithelial monolayer model. The same trend was observed in the present *in vivo* study. The IT administration of CIP-Ca complex-loaded microparticles enabled to increase 22-times the $\text{AUC}_{\text{ELF}}/\text{AUC}_{\text{u,plasma}}$ ratio compared to the ratio measured after nebulization of a pure CIP solution. In comparison, the IT administration of CIP-Cu complex-loaded microparticles increased 130-times this ratio compare to the values obtained with a CIP solution. The difference of CIP PK profiles in ELF obtained after IT administration of the two types of microparticles could have been due to a difference in the CIP release profiles. In fact, a slow release from microparticles can lead to high antibiotic concentration. However, no significant difference was observed between the release profile of CIP from CIP-Ca and CIP-Cu loaded microparticles. Therefore, the difference observed *in vivo* between the two formulations was attributed to the effect of the CIP-cation complexation on the diminution of CIP apparent permeability. These results support our hypothesis that the control of the CIP apparent permeability allows to control the CIP pulmonary-to-blood absorption rate and its pulmonary concentration.

The CIP dose used in our experiments (3 mg/kg) was around 3-times lower than the actual recommended dose used in oral treatment for lung infection with *P. aeruginosa* (500 mg/12h). However, the AUC/MIC ratio was largely above 125 with the 2 microparticle formulations but not with the CIP solution. Therefore, lower CIP dose may be envisaged with the microparticle formulations that would still be able to reach pharmacological optimal CIP concentration. Such an adaptation of the dosage regimen may improve the benefit-risk factor.

This study shows that CIP inhalation using advanced formulation can be beneficial in reaching high CIP pulmonary concentration, while using lower or less frequent dosing.

This study shows that the presence of copper did not induce acute toxicity in the lung.

The CIP/Cu ratio that was used in this study was calculated using the association constant previously measured in example 1 to obtain 100% of the CIP complexed and maximizes the chance to obtain significantly different results. However, lower percentage of complexation could have been used to obtain a result on the CIP PK that should be intermediate to these obtained with CIP-Cu and CIP-Ca loaded microparticles.

Toxicity in humans due to copper can occur at high concentrations, but exposure to copper is considered safe, as is evidenced by the widespread use of copper intrauterine devices and the documented low risk of adverse reactions due to contact with copper. The low sensitivity of human tissue to copper can be contrasted with micro-organisms which are extremely sensitive to its toxic effects that could be benefic to eliminate *P. aeruginosa* from the lung.

Conclusion

The results of example 1 showing that it was possible to control the fluoroquinolone apparent permeability on an epithelium model by selecting the affinity of fluoroquinolone complexation with different cation were confirmed *in vivo* in rat to control of the pulmonary epithelial lining fluid concentration.

References

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

CLAIMS

1. An inhalable microparticle loaded with a fluoroquinolone/metal cation complex.
- 5 2. The microparticle of claim 1, wherein the fluoroquinolone is selected from the group consisting of ciprofloxacin, levofloxacin, ofloxacin, gatifloxacin, enoxacin, norfloxacin, moxifloxacin, gemifloxacin, pefloxacin, sparfloxacin, garenoxacin, sitafloxacin, DX-619 and lomefloxacin.
- 10 3. The microparticle of claim 1, wherein the fluoroquinolone is ciprofloxacin.
4. The microparticle of any of claims 1-3, wherein the metal cation is selected from the group consisting of Cu^{2+} , Al^{3+} , Zn^{2+} , Mg^{2+} , Ca^{2+} and mixture thereof.
- 15 5. The microparticle of claim 4, wherein the metal cation is selected from the group consisting of Cu^{2+} , Al^{3+} , Zn^{2+} , or a mixture thereof.
6. The microparticle of claim 4, wherein the metal cation is Cu^{2+} .
- 20 7. The microparticle of claim 1, wherein the fluoroquinolone/metal cation complex is a ciprofloxacin/ Cu^{2+} complex.
8. A dry powder formulation comprising the microparticles as defined in any of claims 1-7.
- 25 9. The microparticle of any of claims 1-7 for use as a medicament.
10. The microparticle of any of claims 1-7 for use in the treatment of a bacterial lung infection.

FIGURE 1

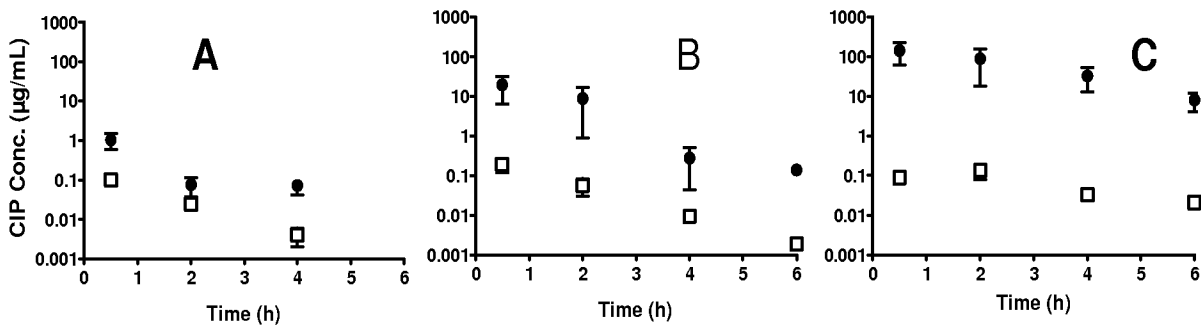


FIGURE 2

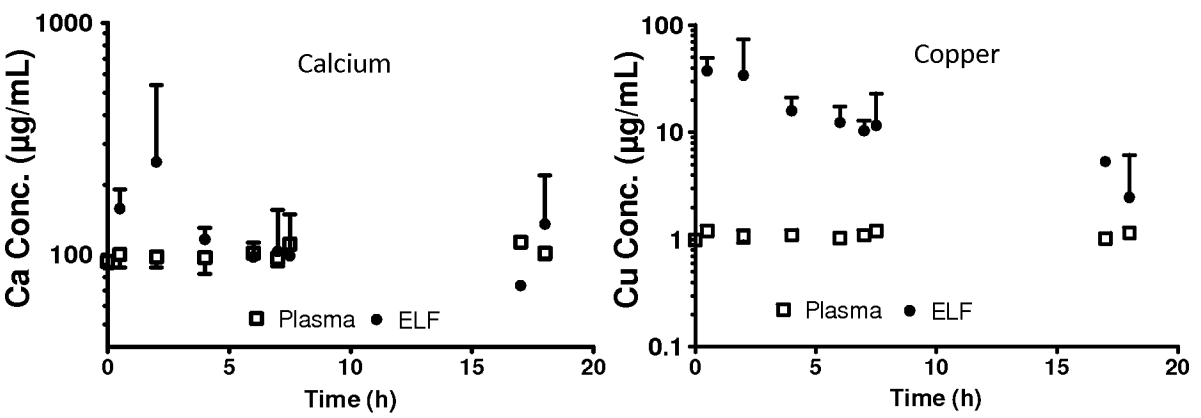


FIGURE 3

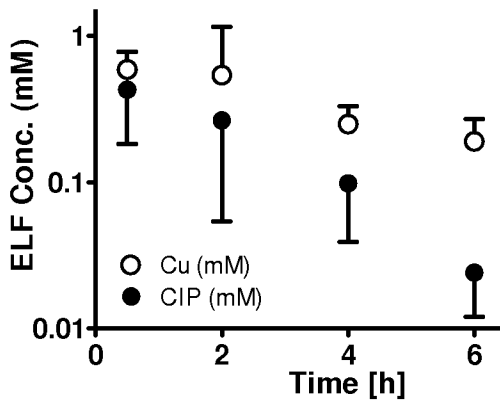
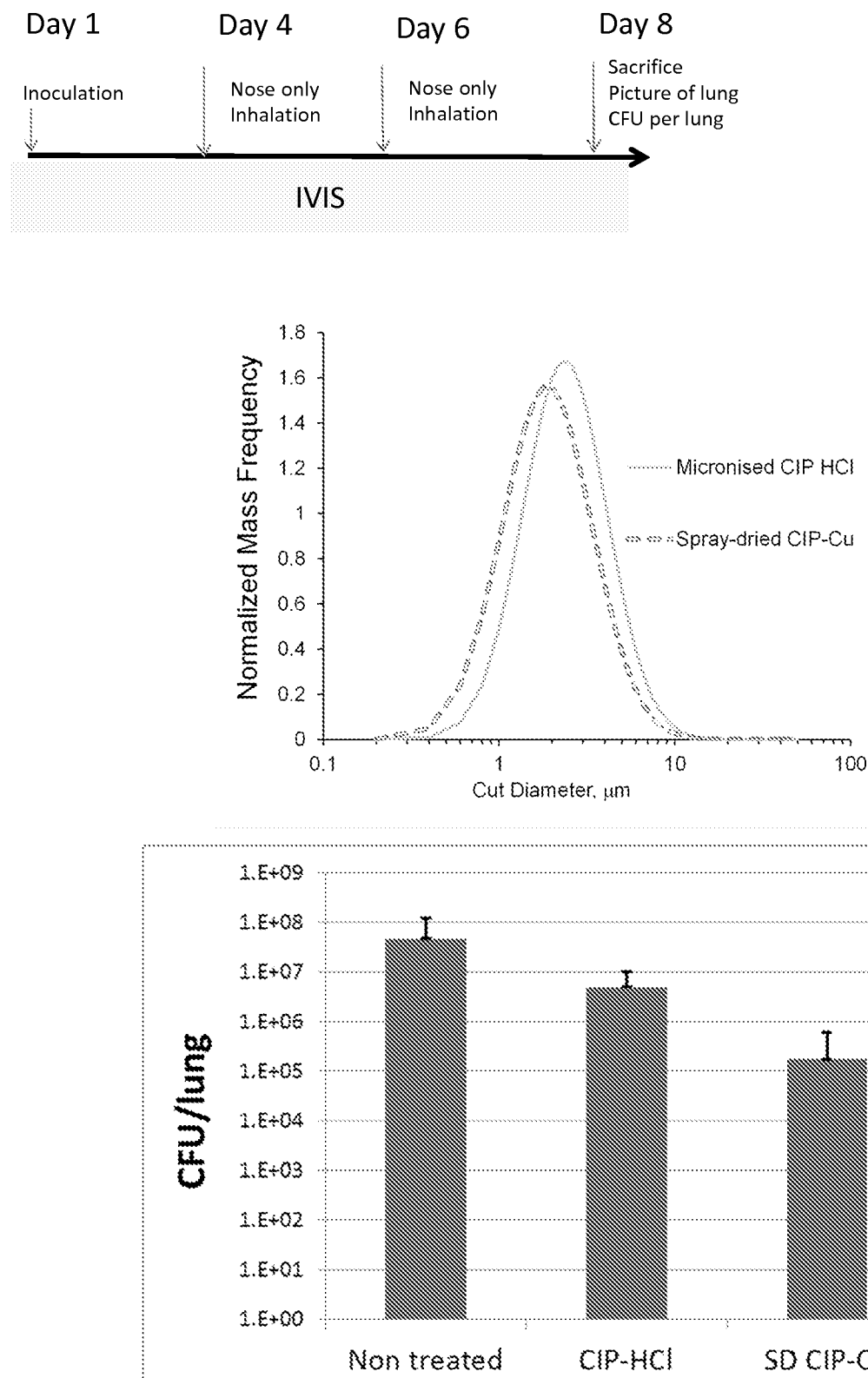


FIGURE 4



INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/001866

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/00 A61K9/50 A61K31/496
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FREDERIC TEWES ET AL: "Ciprofloxacin-Loaded Inorganic-Organic Composite Microparticles To Treat Bacterial Lung Infection", MOLECULAR PHARMACEUTICS, vol. 13, no. 1, 4 January 2016 (2016-01-04), pages 100-112, XP055347608, US ISSN: 1543-8384, DOI: 10.1021/acs.molpharmaceut.5b00543 abstract point 2.2 figure 7 page 101, left-hand column, last two paragraphs page 106, left-hand column, paragraph first reference number 50 -/--	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

20 February 2017

Date of mailing of the international search report

28/02/2017

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Authorized officer

Vázquez Lantes, M

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2016/001866

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	----- GUANGGUO WU ET AL: "Synthesis, Crystal Structure, Stacking Effect and Antibacterial Studies of a Novel Quaternary Copper (II) Complex with Quinolone", MOLECULES, vol. 8, no. 2, 28 February 2003 (2003-02-28), pages 287-296, XP055347774, DOI: 10.3390/80200287 abstract -----	6,7