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Formulation of Rifampicin – cyclodextrin complexes for lung nebulization

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Abstract

Lung administrations of antibiotics by nebulization are promising for improving treatment efficiency against pulmonary infections, as they increase drug concentration at sites of infection while minimizing systemic side effects. For low soluble molecules like rifampicin, cyclodextrins (CD) may improve lung delivery by permitting higher dosing. For this purpose, we investigated rifampicin-CD complexes in terms of rifampicin apparent solubility enhancement, effect on *in vitro* permeability on a Calu-3 broncho-alveolar model and of *in vitro* antibacterial activity against *Acinetobacter baumannii*. Complexation efficiency between rifampicin and hydroxypropyl- β -Cyclodextrin (HP β CD) or methylated β -cyclodextrin (RAMEB) was pH-dependent, involving the piperazin group. Rifampicin phase solubility diagrams constructed at pH 9 showed an A_L-type curve for RAMEB and a B_S-type for HP β CD. Stability constants calculated for a 1:1 molar ratio of CD/ rifampicin were $73.4 \pm 8.2 \text{ M}^{-1}$ for RAMEB and $68.5 \pm 5.2 \text{ M}^{-1}$ for HP β CD. Complexes with HP β CD or RAMEB increased 7.6 times and 22 times respectively the apparent solubility of rifampicin and were found to be satisfactorily stable for 2 days when diluted in a solution at physiological pH. Activity of RAMEB and HP β CD complexes measured by the total rifampicin MIC against *A. baumannii* was similar or lower to free rifampicin MIC respectively. Complexation did not alter the rifampicin permeability in the timescale of 1 hour as evaluated with a Calu-3 epithelial cell model, but acted as a reservoir for rifampicin. In conclusion, this work reports that CDs can be used as vectors for pulmonary nebulization to increase the amount of active rifampicin and optimize its lung pharmacokinetic profile.

Introduction

Antibiotic overuse and misuse have led to the positive selection of bacteria which are resistant to most antibiotics. These multidrug-resistant (MDR) bacteria, mainly isolated in hospital units, are responsible for nosocomial infections that represent a physician's challenge throughout the world. Recently, *Acinetobacter baumannii*, responsible for hospital-acquired pneumonia, has emerged as one of the most ubiquitous antibiotic-resistant gram-negative nosocomial pathogens among critically ill patients [1-3]. Although typically used carbapenems, ampicillin-sulbactam, and amikacin have retained excellent *in vitro* and clinical activities against susceptible strains of *A. baumannii*, a growing number of reports indicates resistance to these antibacterials [4-8]. This gives rise to uncertainty about which antimicrobials should be chosen for treatment, resulting in increased patient mortality [9].

As a result, older antibiotics, including colistin and rifampicin (Fig. 1) are administered by intravenous route to treat patients infected with MDR *A. baumannii* [4, 9-11]. However, pulmonary infections do not always respond well to such systemic therapy, due to insufficient drug diffusion into pulmonary tissue and lumen [12, 13]. Bacteria that have persisted over treatment may develop resistance, but higher drug doses to compensate for poor diffusion may lead to systemic toxicity. Furthermore systemic antibiotic administration may not be justified for *A. baumannii* nosocomial pneumonia as these infections are generally limited to the pulmonary area. Additionally, most patients find difficult to adhere to treatments that necessitate several administrations a day to maintain efficient antibiotics concentration. Therefore, it is imperative to develop new ways to deliver and use antibiotics to avoid the selection and spread of resistant *A. baumannii* and to improve patients' compliance by decreasing dosing frequency.

Antibiotic delivery systems administered as aerosols via the pulmonary route aim to deliver high drug concentrations directly at the site of infection while minimizing systemic biodistribution and toxicity. Rifampicin is a so-called concentration-dependent antibiotic [14]. The rate and extent of bacterial kill is related to the attainment of sufficiently high maximum concentration (C_{max}) relative to the minimal inhibitory concentration (MIC). Higher its concentration in targeted sites higher its bactericide activity. However, rifampicin has a rather low aqueous solubility (from 1.58 to 3.35 mg/mL at pH 7.4 [15, 16]), limiting the possibility of achieving high concentration in the broncho-alveolar epithelium-lining fluid (ELF) when administered as aerosolized solution.

One way to enhance rifampicin (apparent) solubility is the use of cyclodextrins (CDs), as complexing and solubilizing agent. Cyclodextrins enhance aqueous apparent solubility due to the formation of water-soluble inclusion complexes in which apolar interior of the hollow, truncated cone-like CD structure encapsulates hydrophobic parts of drug molecules. CDs have already been proposed for lung administration [17]. Aqueous solutions of CD derivatives (hydroxypropyl- β -cyclodextrin (HP β CD) and randomly methylated β -cyclodextrin (RAMEB) could be aerosolized with nebulizers commonly used in clinical practice, resulting in droplet size range compatible with pulmonary deposition. Additionally, the short-term exposure to inhaled HP β CD and RAMEB solutions was found to be non-toxic [18].

The purpose of the present work was to study the complexation behavior of rifampicin with HP β CD or RAMEB in order to optimize the formulation of inclusion complexes and to demonstrate their usefulness in terms of rifampicin solubility enhancement. Then lung transport of rifampicin as complex with cyclodextrins was evaluated in an *in vitro* Calu-3 broncho-alveolar epithelial cell model [19]. Finally the antibacterial activity was assessed *in vitro* against *A. Baumannii*.

Materials and Methods

Materials

Rifampicin ($\geq 97.0\%$ (HPLC)) and fluorescein (sodium salt) were purchased from Sigma-Aldrich. 2-hydroxypropyl- β -cyclodextrin (HP β CD) with a degree of substitution per glucose unit of 0.6 – 0.9 and a MW of 1510 g/mol (Kleptose[®] HP) was obtained from Roquette. Cavalol[®] W7 M pharma, a randomly methylated β -cyclodextrin derivative (RAMEB) with a degree of substitution per glucose unit of 1.6 and with a MW of 1310 g/mol was obtained from Wacker. Cell culture media and supplements were from Hyclone. Phosphate buffered saline (PBS) tablets and all other chemicals of reagent grade were purchased from Sigma-Aldrich. Ultrapure water was produced by a Direct-Q[™] water purification system (Millipore).

Rifampicin – CD complexation studies

Preliminary experiments were carried out in order to determine the optimal pH for the formation of complexes. An excess amount of rifampicin (until saturation) was added to 1 mL of 5% w/v of CD (0.033 M of HP β CD – 0.038 M RAMEB) solutions buffered either at pH 4 with 0.05 M of citric acid/ sodium citrate, pH 7.4 with 0.05 M of PBS or pH 9 with 0.05 M of sodium tetraborate. Suspensions were stirred in a 37°C water bath for 7 days in order to reach equilibrium. Samples were filtered through a 0.2 μ m syringe filter (Acrodisc[®]). The first 500 μ L was discarded to avoid unspecific rifampicin membrane filter adsorption and the total rifampicin concentration was assayed in the remaining volume by HPLC. Each experiment was carried out in triplicate. Phase solubility diagrams were performed similarly by using various cyclodextrin concentration solutions buffered at pH 9. Total cyclodextrin concentration ranges were 0, 2.5, 5, 10, 15 and 30 % w/v (0 – 0.23 M of RAMEB and 0 – 0.20 M of HP β CD).

Constants calculations

In the hypothesis that cyclodextrin-rifampicin complexes (CD-RIF) were of 1:1 stoichiometry, the apparent stability constant K_s was calculated using the following equation:

$$K_s = \frac{[CD - RIF]}{[RIF]_{free} \cdot [CD]_{free}} \quad CD + RIF \xrightleftharpoons{K_s} CD-RIF \quad \text{Equation 1}$$

where $[CD-RIF]$, $[RIF]_{free}$ and $[CD]_{free}$ are the concentrations of the CD-RIF complex, free rifampicin and free cyclodextrin respectively.

In excess of rifampicin, $[RIF]_{free}$ can be considered as equal to rifampicin solubility S_0 and the equation becomes:

$$K_s = \frac{[CD - RIF]}{S_0 \cdot [CD]_{free}} \quad \text{Equation 2}$$

Thus K_s was calculated as defined in equation 3, using the slope of the linear regression obtained from the phase diagram representing the total rifampicin molar concentration ($[RIF]_{free} + [CD-RIF]$) versus the total CD molar concentration.

$$K_s = \frac{\text{slope}}{S_0 (1 - \text{slope})} \quad \text{Equation 3}$$

The complexation efficiency (CE), corresponding to the complex to free cyclodextrin concentration ratio, was calculated from the phase diagram slope according to equation 4 [20].

$$CE = \frac{[CD - Rif]}{[CD]_{free}} = \frac{\text{slope}}{1 - \text{slope}} \quad \text{Equation 4}$$

Complex stability studies.

Solutions of complex formed with rifampicin (59 mM) and RAMEB (0.23 M) in 0.05 M of sodium tetraborate (pH 9) were diluted 6 times in Ringer-HEPES medium (pH 7.4) or in 0.1 M of citric acid/ citrate buffer (pH 4). These dilutions did not modify the initial pH of buffers. After 1H or 2 days of stirring, solutions were filtered on 0.2 µm filter before rifampicin assay.

Calu-3 cell culture and rifampicin transepithelial transport experiments

Calu-3 cells were obtained from the American Type Culture Collection and used between passages 22 and 30. The cells were cultured in DMEM/Ham's F12 (1/1) supplemented with L-glutamine (2 mM; final concentration), fetal calf serum (10 % v/v; final concentration) and gentamycin (50 µg/mL; final concentration). Cell monolayer were obtained by seeding cells (5×10^5 cells/cm²) onto tissue culture inserts (Nunc polycarbonate filters, with a 4.2 cm² growth surface area) placed in six-well plates and maintained at 37°C in a humidified atmosphere of 5 % v/v CO₂ in air. Growth medium, 1 mL in the donor (upper) compartment and 1 mL in the acceptor (lower) compartment, was replaced with fresh medium every other day. The epithelial monolayer confluence was estimated by visual inspection under photonic microscope and was generally achieved 8 days after seeding. Tight junction integrity was verified for each insert by fluorescein permeability assay performed concomitantly with rifampicin transport experiments (see below).

Transport experiments were carried out in Ringer-HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM MgCl₂·6H₂O, 6 mM NaHCO₃, 5 mM HEPES, 2.8mM Glucose, pH = 7.4). Inserts containing cell monolayer at confluence were first equilibrated with Ringer-HEPES solution (1 mL in each compartment) for 15 min. The medium was then discarded and the inserts were transferred into six-well plates containing 1 mL of Ringer-HEPES

solution per well (acceptor compartment). Then 400 μL of the test solutions supplemented with fluorescein (10 $\mu\text{g}/\text{mL}$; final concentration) used as the tight junction integrity marker was added to the donor compartment and cells were incubated at 37°C . After 60 min, 500 μL samples were collected from the acceptor compartments for rifampicin and fluorescein assay by HPLC.

For rifampicin transport experiments rifampicin (rifampicin concentrations range: 0.34 – 2.5 mM) was dissolved in the Ringer-HEPES solution. For transport experiments performed with cyclodextrin-rifampicin complexes, the borate-buffered rifampicin-cyclodextrin solutions (pH 9) were buffered at pH 7.4 by dilution in a modified Ringer HEPES solution pH 7.4 (i.e. 100 mM HEPES, pH 7.4, and NaCl concentration adjusted to obtain a 290 mOsm/kg osmolarity). Total rifampicin concentration ranged from 1.25 to 22 mM. In all cases, samples contained fluorescein (10 $\mu\text{g}/\text{mL}$ final concentrations) to check for the tight junction integrity. Three inserts were used for each condition. In all cases, the amount of rifampicin transferred from the donor to the acceptor compartments never exceeded 5% of the input.

Fluxes J_{a-b} in the apical-to-basal direction were calculated as follows:

$$J_{a-b} = (C_a \cdot V_a) / (S \cdot t) \quad \text{Equation 5}$$

where C_a is the concentration in rifampicin and V_a the volume (1 mL) of solution in the acceptor compartment, t the incubation time (60 min) and S the insert surface area (4.2 cm^2).

The total, i.e. filter-plus-cell, permeability coefficients (P_{Total}) for both rifampicin and fluorescein in the apical-to-basal direction were calculated using the following equation:

$$P_{Total} = J_{a-b} / C_d \quad \text{Equation 6}$$

where C_d is the initial concentration in the donor compartment.

P_{Total} is the resultant of epithelial cell barrier permeability P_e and filter permeability P_f . For diffusion through membranes in series, the mathematical relationship between permeability coefficients is: $1/P_{Total} = 1/P_e + 1/P_f$ [21]. Therefore P_e can be calculated from the formula: $1/P_e = 1/P_{Total} - 1/P_f$. However, due to high values of fluorescein and rifampicin P_f ($131 \pm 12 \times 10^{-6}$ and $139 \pm 14 \times 10^{-6}$ cm/s respectively) compared to P_{Total} , $1/P_f$ was neglected and $P_e = P_{total}$. Mean P_e value for 10 μ g/mL fluorescein (n = 6) was calculated to be $0.57 \pm 0.06 \times 10^{-6}$ cm/s with extremes of 0.40 - 0.70 $\times 10^{-6}$ cm/s. The threshold P_e value of 0.7×10^{-6} cm/s for fluorescein was retained for the tight junction integrity rejection parameter.

Determination of minimum inhibitory concentration (MIC) of rifampicin

MIC of rifampicin, free or after complexation with CD, was determined by a serial two-fold dilution method using Mueller–Hinton medium. Reference strain *A. baumannii* CIP7010 (Institut Pasteur Strain Collection, Paris, France) was used at a concentration of 5.10^7 CFU/mL and incubated in the presence of rifampicin for 18h at 37°C. The total rifampicin concentrations ranged from 1 to 70 mg/L (1.21×10^{-3} – 85×10^{-3} mM). The bacteria growth was evaluated visually (clear or cloudy media).

Rifampicin and fluorescein assay

Stock standard solution of rifampicin (2.8 mg/mL – 3 mM) was prepared by dissolving 28 mg of rifampicin in 10 ml acetonitrile and was stored protected from light at –20°C. Stock standard solution of fluorescein (1 g/L) was prepared by dissolving 10 mg of fluorescein in 10 ml Ringer-HEPES and was stored protected from light at 4°C. Rifampicin calibration standards (1 – 140 μ g/mL; 1.21×10^{-3} – 170×10^{-3} mM) were prepared on the days of assays by diluting the stock solutions with water (for CD complexation assays), or with Ringer solution supplemented with EDTA (0.2 g/L final concentration) and with standard fluorescein

(0.3 to 0.01 mg/L concentrations) (for *in vitro* diffusion studies and simultaneous determination of rifampicin and fluorescein concentrations). Standards and samples were analyzed by HPLC with the following method. Samples containing Ringer were all supplemented with EDTA (0.2 g/L final concentration). Twenty μL were injected in the chromatograph with Waters 717 plus autosampler. The mobile phase consisted of 0.05M of KH_2PO_4 aqueous solution (pH 4.5), and acetonitrile in 67:33 (v/v) ratio, and was run at a flow-rate of 1 mL/min. Separation was achieved with a Kromasil[®] C18 reversed-phase column (250 mm length \times 3 mm i.d., 5 μm particle size) thermostated at 30°C. The eluate was monitored for 15 minutes at 333 nm with a Jasco UV-1570 detector for rifampicin determination, and, when applicable, with a Jasco FP-920 fluorescence detector mounted in series for fluorescein determination (excitation wavelength: 490 nm; detection wavelength: 530 nm). Signals were integrated with EZchrom Elite 3.1 software. Retention times were 9 and 11 minutes for rifampicin and fluorescein respectively. Means of coefficient of determination (r^2) of the linear regression equations performed on five concentration series of fluorescein or rifampicin, built at various days, were greater than 0.999.

Results - Discussions

1. Complexation studies

Rifampicin molecule (Fig.1) contains several ionizable groups leading to the formation of various ionic species as a function of pH. The two main ionizable groups between pH 1 and 11 are the hydroxyl in C4 and the 3'-piperazin nitrogen, with pKa of 1.7 and 7.9 respectively [22]. In order to evaluate the effect of rifampicin ionized groups on the formation of complexes with cyclodextrins, apparent rifampicin solubility values were determined at pH 4, 7.4 and 9 in the presence of 5% w/v of HP β CD or RAMEB and compared to solubility of rifampicin alone. As shown on Fig. 2A, the rifampicin solubility was the lowest at pH 4 and increased with pH, as already described by Agrawal et al. [16]. This increase was of low magnitude between pH 4 and pH 7.4 (2-time increase) and higher from pH 7.4 to 9 (further 3.6-time increase). The increase in rifampicin solubility with pH may be related to the change in rifampicin ionization states. Indeed, three ionic species can be expected in the pH range 1-11; a cationic form predominating at pH < 1.7, a globally neutral zwitterionic form between pH 1.7 and pH 7.9 and an anionic form above 7.9 (Fig. 2B). The lower solubility observed at pH 4 was attributed to predominant intra/intermolecular charge neutralization of zwitterions at the expense of interactions with water molecules. From pH 6 to 9, zwitterions are replaced by anionic rifampicin possessing higher solubility. In the presence of cyclodextrins, rifampicin apparent solubility also varied as a function of the pH (Fig. 2A). At pH 4, 5% w/v HP β CD or RAMEB concentration had no effect on rifampicin apparent solubility, demonstrating an ineffective complexation. At pH 7.4, with 5% w/v of RAMEB, rifampicin apparent solubility was two times higher than rifampicin solubility, whereas HP β CD at similar concentration had no effect. At pH 9, 5% of HP β CD or RAMEB noticeably increased rifampicin apparent solubility. As proposed by Rao et al. [23] from I.R. spectroscopic measurements, rifampicin –

CD interaction is mediated through the piperazin group. Increase in pH from 7.4 to 9 led to the deprotonation of the N-3' of the piperazin group (pKa 7.9) and favoured higher interaction between this group and the apolar interior of CD, as showed by the increase in rifampicin apparent solubility.

Shown to be favorable to the formation of rifampicin CD complexes, pH 9 was chosen to perform phase solubility diagrams (Fig 3). In the 0 to 0.23 M RAMEB concentration range, the rifampicin apparent solubility increased linearly and corresponded to an A_L type curve, suggesting the formation of $1/X$ ($X \geq 1$) stoichiometry RAMEB/rifampicin complex [24]. As suggested above, the rifampicin piperazin group is internalized in CD. Due to the size of this group, only one piperazin group is likely to be included in the β CD hydrophobic cavity and the hypothesis of 1/1 complex between RAMEB and rifampicin was made. For HP β CD, the phase solubility diagram constructed in the 0 to 0.20 M concentration range corresponded to a B_S -type curve: rifampicin apparent solubility increased linearly with HP β CD concentration in the range of 0 – 0.066 M, then plateaued. Consequently, the hypothesis of 1/1 complex can be made only for concentrations up to 0.066 M. Above 0.066 M, the observed deviation from linearity obtained with HP β CD could be attributed to the saturation in free and complexed rifampicin.

Stability constants K_s and complexation efficiencies calculated for 1:1 rifampicin-CD complexes in the 0 – 0.23 M RAMEB concentration range and in 0 – 0.066 M HP β CD concentration range are inserted in Figure 3. K_s and CE values were close, suggesting the same type of interactions between the two CDs and rifampicin, i.e. an interaction limited to the internal cavity without involvement of methyl or hydroxypropyl pending groups.

In order to aerosolize the rifampicin complex solutions into the lungs, their pH (pH 9) need to be lowered to physiological pH (7.4). As this may result in complex destabilization and rifampicin precipitation, the physical stability of complexes was assessed after 1/6th dilution in Ringer-HEPES medium pH 7.4 (Table 1). After one hour in Ringer-HEPES pH 7.4, no loss of rifampicin due to precipitation was observed. After 2 days in pH 7.4, 97.9 % of rifampicin remained solubilized. Hence, rifampicin-CD complexes can be formulated at pH 9 for the best complexation efficiency and then extemporaneously buffered at pH 7.4 for their nebulisation in the lung. Further studies performed after dilution in citrate buffer pH 4 showed a rifampicin loss less than 5% after 1 hour and of 19.5% after 1 day. Rifampicin loss was attributed to rifampicin precipitation from supersaturated solutions. Supersaturation resulted from the dissociation of complexes induced by dilution and from a pH-related decrease in rifampicin solubility. Higher rifampicin loss observed at pH 4 compared to pH 7.4 could be attributed to the inability of CD to complex rifampicin at this pH as observed in Fig. 2A, due to the protonation of piperazin group of 100 % rifampicin molecules.

Formation of complexes between rifampicin and cyclodextrin resulted in an increase in rifampicin apparent solubility. This increase was linear with all tested RAMEB concentrations and, for a RAMEB concentration of 0.23 M, was equal to 22 times the rifampicin solubility determined at pH 7.4 (2.9 mM). With 0.066 M of HP β CD the increase in rifampicin apparent solubility is equal to 7.6 times the rifampicin solubility determined at pH 7.4. The increase of rifampicin apparent solubility by CD inclusion allows for an increase in the maximal dose nebulizable in the lungs. For example, in rats, usual volumes that are nebulised into lungs range from 10 to 400 μ L [25]. Therefore at pH 7.4, based on rifampicin solubility, the maximal amount of rifampicin that can be administered into lungs is 0.96 mg. In contrast, by increasing rifampicin apparent solubility with 0.066 M of HP β CD or 0.23 M of RAMEB, this amount would reach 7.5 mg or 20.8 mg respectively. In the purpose to administer rifampicin

complex as a dry aerosol (e.g. obtained after freeze drying of the solution), the CE values allow to calculate the maximal amount of rifampicin per dosing. The CE values calculated for the two types of complexes formed with rifampicin are about 0.35 (Fig. 3), meaning that on average, about one out of four cyclodextrin molecules in solution forms a complex with one rifampicin molecule. The maximum amount of solid that can be administered in rat lungs is around 10 mg [25]. Therefore, the amounts of rifampicin contained in 10 mg of powder would be 1.36 mg and 1.20 mg, for RAMEB and HP β CD complexes respectively, which is lower than with the complexes in solution. Furthermore, this amount of rifampicin is low in comparison to the aerosolization of 10 mg of pure rifampicin. However, due to higher solubility of the complex than free rifampicin, the solubilisation rate should be faster with the complex. As a consequence, the maximal rifampicin concentration reachable in the ELF could be higher when rifampicin will be administered as CD complex than pure.

2. Transport across an epithelial cell barrier model

The goal of our study was to increase rifampicin concentration in ELF. This concentration is a balanced value depending on the amount of rifampicin administered into the lung and its rate of elimination. The passive diffusion of molecules from the lung lumen to the blood is mainly limited by the lung epithelium, due to the presence of tight junctions between type I pneumocytes. In order to evaluate the effect of rifampicin complexation with CD upon its diffusion, we studied the rifampicin linear flux, as defined by Fick's law, under free and complexed form across a Calu-3 cell layer, an epithelial alveolar model forming high level of tight junction [19].

HP β CD and especially RAMEB are known to potentially reduce cell membrane viscosity due to cholesterol extraction, resulting in an increase in membrane permeability and cell toxicity [26]. Therefore, to assess the effects of CD on epithelial cells, the permeability of fluorescein and rifampicin was determined in the absence or in the presence of 0.066 M of free HP β CD or 0.076 M of free RAMEB (Figure 4). Fluorescein permeability was not increased by the presence of CDs. This demonstrated that CDs at these concentrations did not alter the paracellular transport and that tight junction integrity was maintained, suggesting the absence of toxicity for the cell monolayer in the timescale of 1 hour. A recent toxicity study performed by incubating Calu-3 cells with various CDs for a longer periods of 4 hours showed that at a 0.050 M concentration HP β CD caused no LDH leakage from Calu-3 cell and minor reduction of the number of viable cells, while RAMEB induced cell death and membrane damage [27]. In our studies, rifampicin permeability measured in the presence of HP β CD or RAMEB was slightly lower (1.4 times) than with rifampicin alone, indicating that after 1 hour incubation CDs did not increase rifampicin transcellular transport due to interactions with the epithelial cell membrane. The slightly lower permeability measured in presence of CDs was attributed

to a complexation of the free rifampicin with CDs in the Ringer-HEPES medium, even though the ability of rifampicin to be complexed with CD was weak at pH 7.4.

As the presence of CDs did not modify the integrity of the cell monolayer in the timescale of 1 hour, rifampicin transport across the alveolar epithelial cell model was performed (Fig. 5). For all experiments, rifampicin fluxes were proportional to the total rifampicin concentration. This suggests that at least in the rifampicin concentration ranges studied, in the presence or absence of CD, transport of rifampicin across the cell layer was a diffusion-driven process, excluding the involvement of membrane transporter [28]. As shown by the slopes of the curve in Figure 5, for a given total concentration of rifampicin, flux values were lower with rifampicin complex than with rifampicin alone. Results also showed that rifampicin flux measured in presence of complex was independent of the nature of CDs.

Considering free rifampicin, permeability value ($1.69 \pm 0.40 \times 10^{-6}$ cm/s) calculated from the slope of the linear regression of the rifampicin flux versus concentration curve (Fig.5), was close to values generally reported in literature across rat jejunum or ileum [28, 29], or across Caco-2 cell monolayer model [30] (2×10^{-6} cm/s). This value is lower than values observed on everted rat gut sac model for low permeable drugs listed by the US FDA (furosemide, ranitidine) [29]. This suggests that rifampicin is a drug with low lung permeability and hence qualifies to be used for lung-targeted aerosol therapy.

As shown on Figure 5, the highest flux value found with free rifampicin alone was 5×10^{-12} mol/cm²/s and was observed when rifampicin reached saturation concentration, i.e. 2.6 mM. When rifampicin was complexed with RAMEB, this flux value was reached for a much higher total rifampicin concentration (21.6 mM). Due to their large Mw and their polar character, CDs and CD complexes are indeed generally considered as not being able to diffuse across cell layers. As a matter of fact, CD permeability values recently measured across Calu-3 cell layers were close to 6.7×10^{-8} cm/s [27], which is 25 times lower than the rifampicin

permeability value determined in the present study ($1.69 \pm 0.40 \times 10^{-6}$ cm/s). Hence, the flux of the rifampicin was only related to free molecules fraction, and for a given total rifampicin concentration, rifampicin fluxes in the presence of CD complex were lower than in the absence of CD. The linear increase in flux values as a function of the total rifampicin concentration was attributed to the proportional increase in free rifampicin concentration. As for both complexes K_s values were close, rifampicin fluxes measured with both RAMEB or HP β CD were similar for a given total rifampicin concentration. For the highest total rifampicin concentration tested on the cells, i.e. 21.6 mM with RAMEB complexes, [obtained with a 1/3rd dilution of the highest point observed in the phase diagram (Fig. 3)], the rifampicin flux was similar to the one measured with free rifampicin at saturation concentration (2.6 mM), suggesting that free rifampicin was close to saturation. Therefore, increase in apparent rifampicin concentrations over 21.6 mM would not in theory result in higher rifampicin fluxes through the cell layers. From a formulation viewpoint, this means that solutions of rifampicin complex at or above a 21.6 mM rifampicin total concentration should maintain saturation of rifampicin in the epithelial lining fluid and should act as a sustained release system.

3. Antibacterial activity

In the objective to evaluate the bacteriostatic activity of rifampicin complex, we measured the MIC values against *A. baumannii* CIP7010T. Value observed for rifampicin alone was comprised in the interval]10; 20[$\mu\text{g/mL}$ ($12 \times 10^{-3} - 24 \times 10^{-3}$ mM). When rifampicin was present under HP β CD or RAMEB complexes, MIC values were between]3.75; 7.5[$\mu\text{g/mL}$ ($4.6 \times 10^{-3} - 9.1 \times 10^{-3}$ mM) and]17.5; 35[$\mu\text{g/mL}$ ($21 \times 10^{-3} - 42 \times 10^{-3}$ mM) respectively. Hence, rifampicin-CD solutions had bacteriostatic activities against *A. baumannii* CIP7010T equivalent to (RAMEB) or higher than (HP β CD) a solution of rifampicin alone, suggesting that the complexation process does not alter the rifampicin bacteriostatic activity. Similar results were found on *in vitro* anti-tubercular activity of rifampicin by Rao *et al.* [23]. Complexation of rifampicin with β -CD or hydroxyl-ethyl- β -cyclodextrin reduced MIC values to half. Furthermore, considering that a fraction rifampicin was complexed with CD and that only the free fraction of rifampicin should be active, RAMEB and HP β CD should improve rifampicin activity.

Conclusion

In the aim to improve treatment efficiency against pulmonary infections and avoid the apparition of MDR gram negative bacteria, a new rifampicin – cyclodextrin formulation was developed for rifampicin administration by nebulization. The targeting of lung by direct administration of rifampicin by nebulization should be well adapted since its permeability across cell monolayer is low. Formulation parameters such as pH and cyclodextrin type were optimized to increase the complexation efficiency and rifampicin apparent solubility. Complexes formed with RAMEB or HP β CD maintained similar or higher bacteriostatic activity against *A. Baumannii*. Furthermore, the use of CD allows increase in the administrable dose and did not increase rifampicin lung absorption. This would allow maintaining high rifampicin concentration in the ELF which is a benefit since rifampicin pharmacodynamic is concentration dependant. Further bacteriological and *in vivo* pharmacokinetic studies are envisaged to evaluate this new formulation.

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Figure legends

Figure 1: Rifampicin chemical structure

Figure 2: A) Variation of apparent rifampicin solubility versus pH in absence and presence of 5% (m/v) of HP β CD or RAMEB. Suspensions were stirred during 7 days at 37°C, and then filtered on 0.2 μ m filters. Buffers used: pH 4 (0.05 M citrate-phosphate buffer), pH 7.4 (0.05M PBS) and pH 9 (0.05 M borate buffer). Data are means \pm SD of 3 samples. B) Variation of rifampicin ionic state versus pH, calculated using Henderson-Hasselbach equation and considering only the pKa of 1.7 and 7.9.

Figure 3: Phase solubility diagrams of rifampicin in the presence of HP β CD or of RAMEB. Conditions: pH = 9; temperature: 37°C; stirring time: 7 days; samples were filtered on 0.2 μ m filters. Data are means \pm SD of 3 samples. Dotted line corresponds to linear regression performed with values obtained with RAMEB. Solid line corresponds to linear regression performed with values obtained with HP β CD in the range 0 – 0.066 M.

Figure 4: Rifampicin permeability measured for an initial rifampicin concentration of 0.34 mM in the presence of 0.066 M of free HP β CD or 0.076 M of free RAMEB. Incubation time of one hour in Ringer – HEPES buffered at pH = 7.4, 37°C. Data are mean \pm SD of 3 samples.

Figure 5: Rifampicin transport study across Calu-3 cell monolayer. Grey solid line: rifampicin flux measured without CD. Dotted line: Rifampicin flux measured in the presence of RAMEB. Solid line: Rifampicin flux measured in presence of HP β CD. Cells were incubated at 37°C in Ringer–HEPES pH = 7.4. Fluxes were measured in initial conditions, i.e. $C_a \leq 5\%$ of C_d (for experimental details see materials and methods). Data are means \pm SD of 3 samples

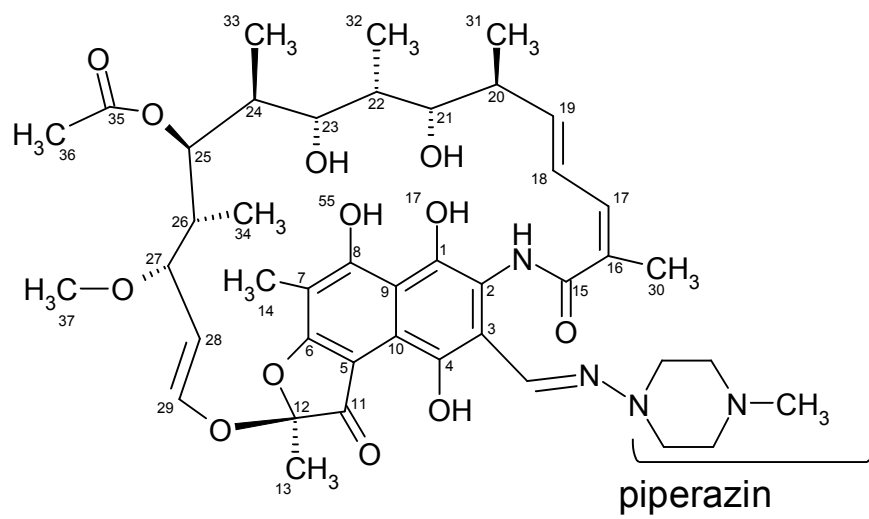


Figure 1

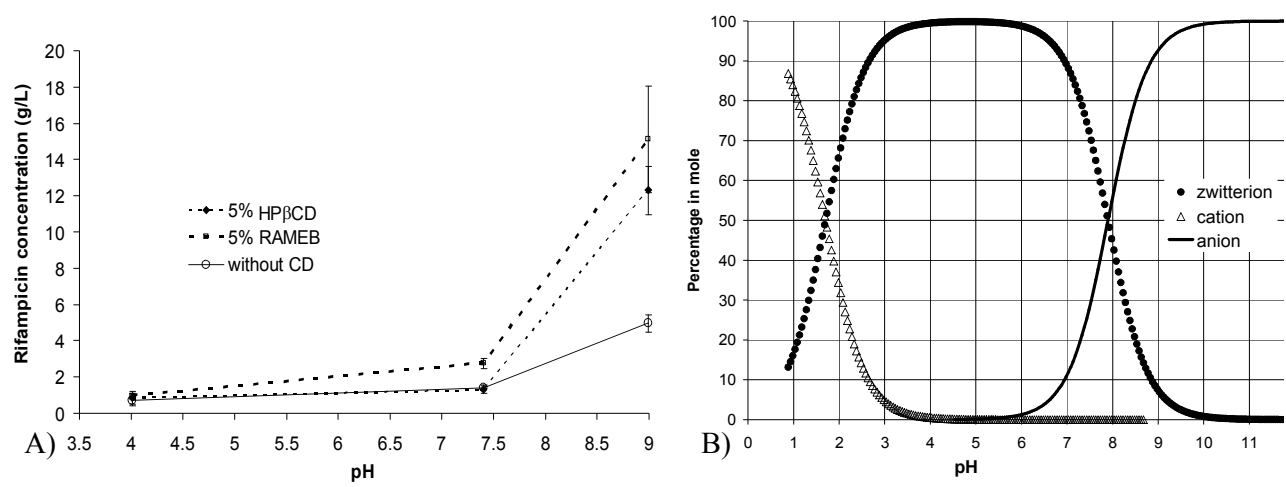


Figure 2

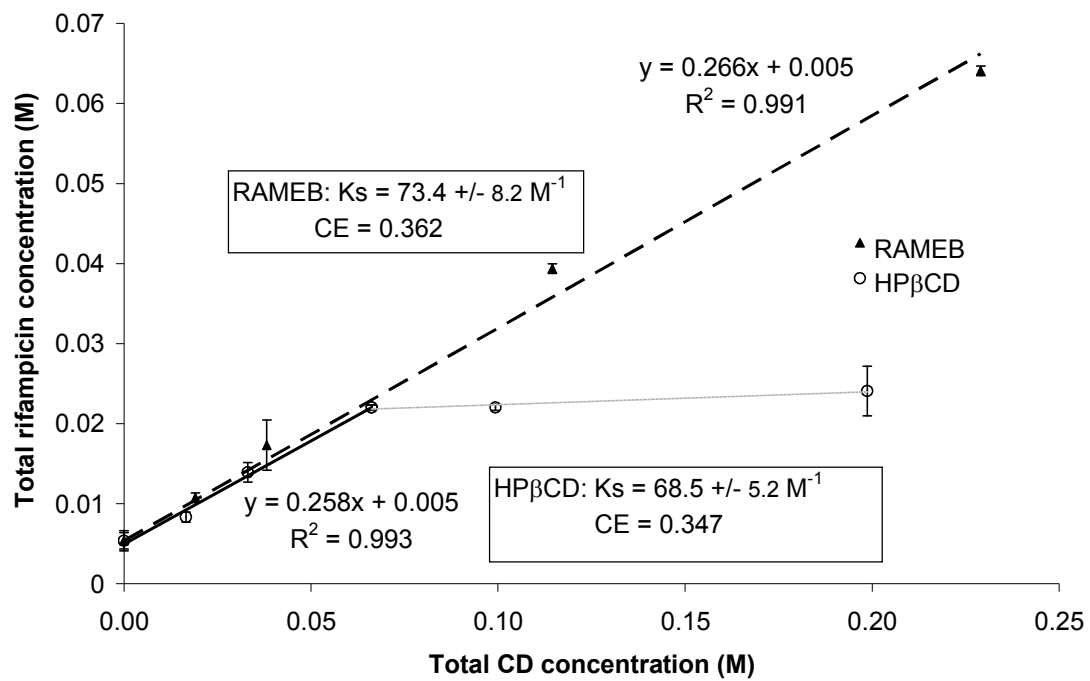


Figure 3

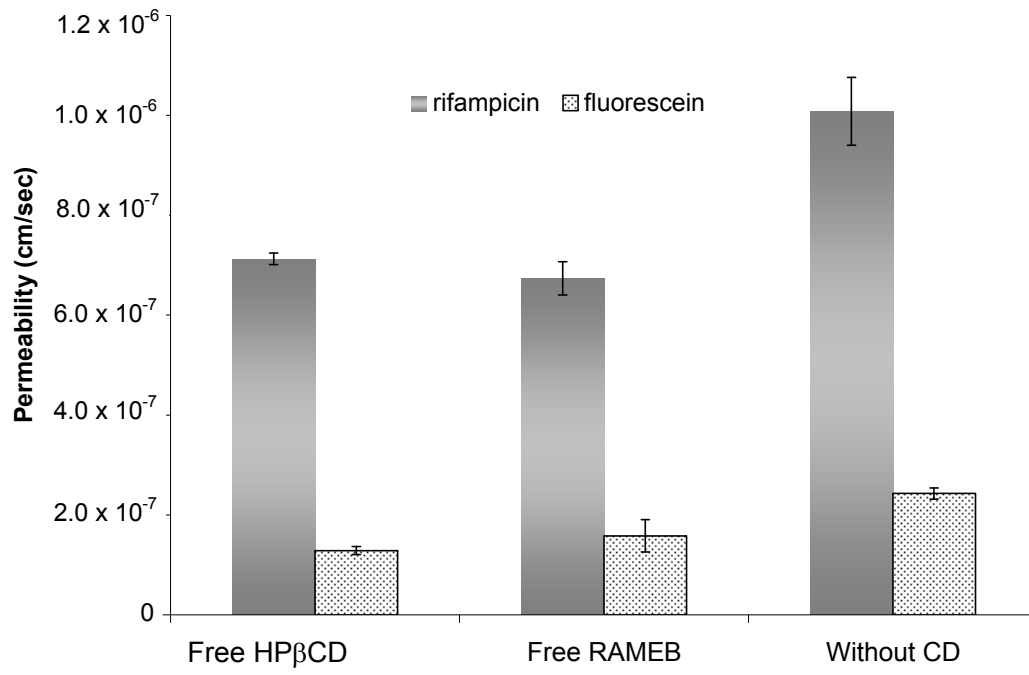


Figure 4

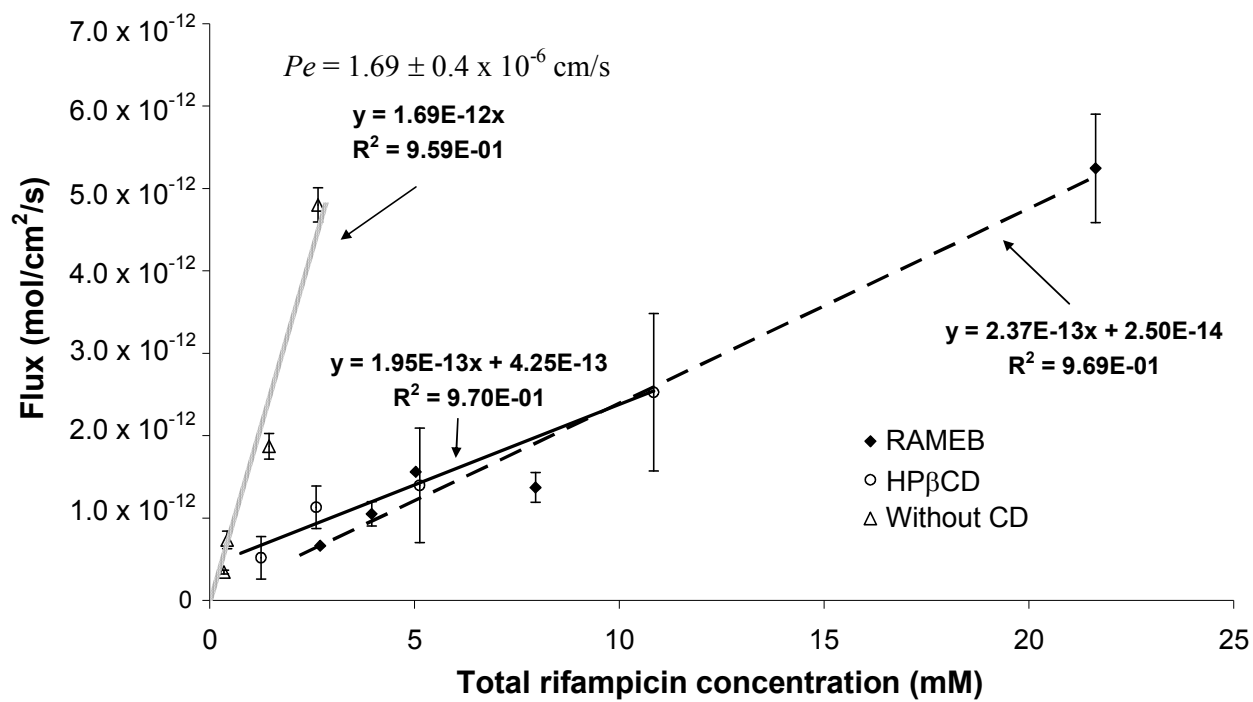


Figure 5

Table 1: Percentage of the rifampicin remained after diluting 6 times the RAMEB (0.23 M) – rifampicin solution (59 mM) in Ringer-HEPES medium (pH 7.4) or in 0.1 M of citric acid /citrate buffer (pH 4). Samples were stirred at room temperature. Solutions were filtered on 0.2 µm filter before rifampicin assay.

	One hour	2 days
pH 9 → pH 4	96.7 %	80.5 %
pH 9 → pH 7.4	100 %	97.9 %