Development of inhaled formulation of modified clofazimine as an alternative to treatment of tuberculosis

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Short Title: **Clofazimine inhaled formulation for tuberculosis**

Abstract

Inhalation drug delivery provides a possible useful alternative to oral drug delivery in the treatment of tuberculosis (TB). This work evaluated inclusion complexes of clofazimine (CFZ), an anti-TB drug with low aqueous solubility and potential gastric degradation, in β -cyclodextrin (β CD), γ -cyclodextrin, (2-hydroxypropyl)- β -cyclodextrin and sulfobutyl-ether- β -cyclodextrin. A phase solubility study indicated that β CD showed the best inclusion capacity for CFZ, so a CFZ: β CD complex (1:7) was selected for further studies. Particle engineering was performed using spray drying to obtain powders with suitable characteristics for pulmonary delivery and L-leucine was added to enhance powder dispersibility. Thermal and spectroscopic analyses indicated the CFZ: β CD integrity after spray drying, and the presence of L-leucine resulted in less hygroscopic and rougher particles, and a less agglomerated powder. All formulations, and especially those containing L-leucine, showed suitable *in vitro* deposition performance in the Next Generation Impactor and presented higher aqueous solubility compared to the free drug. *In vitro* studies showed low toxicity against Calu-3 and CFZ retention in the cell monolayer and apical compartment. These results suggest that the inhalation formulation composed of CFZ: β CD plus L-leucine should improve CFZ pulmonary bioavailability and provide an alternative treatment for TB, acting on the main infection site of the disease.

Keywords: pulmonary delivery; tuberculosis; clofazimine; cyclodextrin; L-leucine

1. Introduction

Tuberculosis (TB) is an infectious disease caused by a unique pathogen, *Mycobacterium tuberculosis* (*M. tuberculosis*). TB is the ninth leading cause of death worldwide, ranking above HIV/AIDS. In 2017, the World Health Organization (WHO) estimated that 10.0 million people fell ill with TB (90% adults) and there were 1.3 million deaths [1–3]. Although controlled in certain developing countries and industrialized areas, the current and continuous migratory movement from Asia and Africa (where there is a high incidence) is a potential risk to the spread of TB to Europe and North America [4]. The disease mainly affects the lungs (within alveolar

macrophages and granulomas structures) but can also affect other sites [2]. Erratic and discontinuous treatments, associated with *M. tuberculosis* adapting mechanisms (efflux pump mechanisms) and population heterogeneity, have triggered multi-drug-resistant (MDR) strains of this mycobacteria and also extremely drug-resistant (XDR) strains [5,6]. Drug-resistant TB is a continuing threat since the first recorded case more than 12 years ago, and the development of new drugs or therapeutic strategies is necessary [4].

Inhalation is a non-invasive administration route used to deliver a drug substance into the lungs and treat local (asthma, cystic fibrosis) or systemic (diabetes, schizophrenia) diseases [7–9]. In recent years pulmonary drug delivery has received attention for TB treatment because it provides high drug concentrations at the main site of the disease, prevents hepatic-first pass metabolism and gastric intolerance and overcomes poor and erratic oral bioavailability of some drugs that are used in clinical practice [10,11]. The United States Pharmacopeia categorizes the dosage forms for pulmonary drug delivery into: (i) inhalation spray, (ii) inhalation solution, (iii) inhalation suspension, (iv) inhalation aerosols or metered-dose inhaler (MDI) and (v) inhalation powder or dry powder inhaler (DPI) [8]. DPIs are commonly employed in therapy for several reasons, including the fact that the formulation is propellant-free, while they also often offer better chemical stability, with a portable device which is easy to use [12].

The efficacy of formulations delivered by means of a DPI depends on the device delivery performance, breathing pattern of the patient, powder deposition pattern in the respiratory tract, the fine particle fraction (FPF) and the disease that is being treated [13]. The deposition site is determined in accordance with the aerosol aerodynamic properties, namely mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD); these parameters are derived from the aerodynamic particle size distribution (APSD) [7]. MMAD is the aerodynamic diameter (d_a) for which 50% of the aerosol mass is above and below that diameter. GSD shows the level of dispersity of the aerosol particles. To avoid particle deposition in the upper respiratory tract (oropharyngeal and tracheo-bronchial region) and exhalation before deposition, it is desirable to obtain an MMAD of between 1 and 5 μm and a $\text{GSD} < 2$. Powder physicochemical properties and morphological characteristics of the particles also determine the deposition profile and drug bioavailability [7]. Additionally, the literature describes the use of different L-leucine concentrations (10-30% w/w) in DPI

formulations, as a dispersion aid, to obtain aerosol particles with the desired *in vitro* deposition profile [14–18].

Once deposition occurs in the respiratory tract, the interaction between the particles and the biological environment also determines the therapeutic efficacy of the formulation. The interaction with mucus (aqueous mixture of glycoproteins), which is secreted in the upper airways, and pulmonary surfactant (90% phospholipids and 10% proteins), which is secreted in the alveolar region of the lungs, is determined by the physicochemical characteristics of the particles, whereby hydrophilicity favors dissolution in the mucus. The mucus is a biological barrier that blocks the entrance of foreign microorganisms and substances, including drugs. The mucociliary clearance removes insoluble particles deposited in the trachea-bronchial region, within 24h, resulting in a short retention time in the lung [9]. Drug solubility in the mucus minimizes the mucociliary clearance effect, favoring the desired drug effect [19]. Lipophilic drugs tends to be insoluble in mucus and thereby exhibit reduced cell permeability [19,20]. On the other hand, insoluble particles (1-2 μm) that reach the alveolar spaces tend to be internalized by macrophages (alveolar clearance), that are surrounded by a thin surfactant layer, and are able to reside for a long time inside them. Thus macrophage uptake represents a strategy to combat microorganisms that infect this cellular type, such as in TB [11,21].

The World Health Organization (WHO) recommends the use of oral clofazimine (CFZ) in combination with other drugs for drug-resistant TB treatment, although its efficacy and long-term safety have not yet been established [2,22]. CFZ's poor clinical efficacy is strongly attributed to low aqueous solubility and gastric degradation, resulting in suboptimal delivery to the target organ. On the other hand, *in vitro* studies demonstrated good inhibitory activity against MDR strains (minimal inhibitory concentration 0.5 – 2 mg/L) [22]. *In vivo* studies showed that administration of CFZ together with first or second-line anti-TB drugs promoted a 100 times reduction of bacteria colony count in the lung, suggesting its potential to shorten the duration of TB treatment [23,24]. These characteristics point towards CFZ as a potential drug for pulmonary delivery.

The use of cyclodextrins (CD) in formulations has allowed many products containing poorly soluble drugs to reach the market, and eventually help to treat many life-threatening disease conditions [25]. The efficacy of CFZ inclusion complexes based on sulfated CD has already been described in the literature, and they showed activity

against *Mycobacterium avium* and *Mycobacterium intracellulare* [26]. The literature has also described the successful inclusion of CFZ into negatively charged sulfobutyl ether β -cyclodextrin (SBE β CD) / epichlorohydrin oligomer to increase its water solubility and bioavailability [27]. Although the improved solubility results of the sulfated CDs are promising, the cost of these carriers are higher than the traditional (non-sulfated) CDs. Furthermore, in the previously reported studies powder products were not adjusted in terms of aerodynamic properties in order to optimize pulmonary drug delivery, since it was not the objective of these studies.

For these reasons, the aim of the present study was to prepare and undertake the physicochemical characterization of novel CFZ-CD and L-leucine-CFZ-CD complexes, developed to enhance CFZ aqueous solubility with a view to improving dissolution in lung mucus. Particle engineering *via spray drying* was performed to develop a dry powder formulation for the local delivery of CFZ to the lung.

2. Materials and Methods

2.1. Materials

Clofazimine (CFZ), (2-hydroxypropyl)- β -cyclodextrin (2-HP β CD), L-leucine and L-isoleucine were purchased from Sigma Aldrich (St Louis, USA). β -cyclodextrin (β CD) and γ -cyclodextrin (γ CD) were purchased from Wacker Chemie AG (Munich, Germany). Sulfobutyl ether β -cyclodextrin (SBE β CD) (Captisol®) was kindly supplied by CyDex Pharmaceuticals (Kansas, USA). The organic solvents (Analytical Degree, A.D.), ethanol, dimethylsulfoxide and acetonitrile, were purchased from TEDIA BRAZIL (Rio de Janeiro, Brazil). Purified water for preparing solutions and performing HPLC studies was obtained by water distillation treatment (Quimis, Diadema, Brazil).

2.2. Methods

2.2.1. Inclusion complex preparation

Complexation efficacy studies were performed by adding an excess amount of CFZ to water or ethanol:water mixtures (from 7:3 to 0:1, ethanol:water) containing increasing concentrations of a CD (β CD, γ CD, 2-HP β CD or SBE β CD), ranging from 1 to 10 mM (Table 1). Each system was stirred at 30 °C for 72 h, 40° C for 72 h, 50° C for 72 h or 30° C for 144 h, and subsequently filtered through a 0.45 μ m pore size filter

membrane (ChromPure syringe filter, PVDF, 25 mm diameter). CFZ concentration in solution was determined by HPLC with UV detection at 495 nm, using the methodology described below. Calculation of complexation efficacy (CE) was according to the following equation [28]:

$$CE = \text{Slope} / (1 - \text{slope}) \quad (1)$$

Where “slope” was the slope of the complexation profile (concentration of CFZ on the y-axis versus concentration of CD on the x-axis).

The molar proportion of CFZ:CD was determined using the following equation [28] :

$$CFZ:CD = 1CFZ : (CE + 1) / CE \quad (2)$$

Where CE was the complexation efficacy according to the complexation profile.

The CD inclusion process was then performed by mixing CFZ and the CDs (β CD, γ CD, 2HP β CD or SBE β CD) by the solution method, in purified water and ethanol:water (7:3) mixture, under agitation using the previously established ideal conditions/proportions. After cooling the mixture, it was filtered through a 0.45 μ m pore size filter membrane (ChromPure syringe filter, PVDF, 25 mm diameter), then ethanol was removed in a rotary evaporator Gehaka RD – 180 (Gehaka, São Paulo, Brazil) at 45 °C for 6 h and the sample was frozen using liquid nitrogen. The frozen sample was immediately submitted to lyophilization (as described below). The powder obtained was stored under a nitrogen atmosphere in a closed glass vial in a desiccator with silica gel.

Physical mixtures were prepared by pulverization and mixing CFZ and the CD (1:7 molar ratio) in a glass mortar.

2.2.2. Determination of CFZ content

The CFZ content in inclusion complex and permeation studies was determined by HPLC (Shimadzu, pump LC- 30AD, autosampler SIL-30AC, detector SPD-M 20A). A Perkin Elmer C18 column (150 x 4.6 mm, particle size 3 μ m) was used at 35 °C. The mobile phase was a mixture of methanol and phosphate buffer (80:20, v/v), and the flow rate was 1.0 ml/min. The elution was carried out isocratically and the injection volume was 20 μ l. The eluant was monitored at 284 nm. Retention time was 4 minutes.

2.2.3. Freeze drying process

Freeze drying was carried out using a laboratory scale freeze dryer Liotop L101 (Liobras, São Carlos, Brazil). Each sample was frozen using liquid nitrogen and then freeze-dried for 72 h to allow the complete removal of water. The resulting powders were characterized by differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD) and ¹³C-CP/MAS NMR spectroscopy. The freeze drying process was used only during preliminary drying evaluation studies; spray drying was used thereafter.

2.2.4. Spray drying process

Production of CFZ-CD inclusion complex or CFZ-CD inclusion complex/L-leucine or L-isoleucine complex spray dried powders was carried out using a laboratory scale spray dryer LabMaq MSD 1.0 (Labmaq do Brasil, Ribeirão Preto, Brazil) with a 0.7 mm nozzle tip. An ethanol-water solution (1:1) was prepared containing the CFZ-CD complex (1:7 molar ratio) and a total solute concentration of 0.70-0.85% (w/v), depending on the L-leucine/isoleucine concentration. The amino acids, L-leucine or L-isoleucine, and the organic solvent dichloromethane were added after the preparation of the CFZ-CD inclusion complex in ethanol-water. Amino acid concentration ranged from 10% to 15% (w/w) in relation to the total solid amount, and the dichloromethane concentration was 2% (v/v). Spray drying parameters are presented in Table 3. The dichloromethane was used as a co-solvent (and potential “blowing/pore-forming agent”) due to its low boiling point (39.8 °C) and fast evaporation rate, allowing particle size reduction and porosity increase [29–31]. Spray drying was used both to obtain the dry complexes and to adjust the properties of these complexes in order to prepare formulations that were suitable for pulmonary delivery. The resulting powders were characterized by DSC, XRPD, Fourier-transform infra-red spectroscopy (FTIR), dynamic vapour sorption (DVS), specific surface area analysis (SSA), particle sizing, morphology, ¹³C-CP/MA NMR spectroscopy and aerodynamic assessment using a Next Generation Impactor (NGI).

2.2.5. Powder characterization

A differential scanning calorimeter DSC-60 (Shimadzu, Kyoto, Japan) was used at a heating rate of 5 °C/min. Accurately weighed samples (1 - 2 mg) were placed into sealed aluminum pans and heated from 30 to 300 °C.

XRPD patterns of raw materials, physical mixtures and inclusion complexes were obtained using a Lab X XRD-6100 X-ray (Shimadzu, Kyoto, Japan) with a Cu K α line as the source of radiation. Measurements were carried out using a voltage of 40 kV, a current of 30 mA and a scanning rate of 2°/min over a range of 2°- 80°, on the two theta scale at a step size of 0.02°.

The infrared spectra of raw materials, physical mixtures and inclusion complexes were obtained from KBr pellets using a Prestige-21 spectrometer (Shimadzu, Kyoto, Japan). Spectra were recorded between 4000 and 600 cm⁻¹ with a resolution of 4 cm⁻¹.

¹³C solid-state NMR spectra were recorded on a Bruker Avance III 400 (9.4 T) spectrometer (Bruker, Massachusetts, USA), operating at 100.64 MHz. A CPMAS 3.2 mm probe head and ZrO₂ rotors spinning at 9.3 KHz were employed. Cross polarization magic angle spinning with ramp in the ¹H channel (CPMAS) combined with high power proton decoupling (HPDEC) were used as the pulse sequence. The contact time varied from 100 μ s to 10,000 μ s. The recycle delay was 4 s, and the $\pi/2$ pulse length was 4 μ s. The spectra were transformed after accumulation of 512 scans. The line broadening used to process the data was 20 Hz. Adamantane (CH at 29.46 ppm) was used as an external chemical shift reference.

Particle size distributions of prepared formulations for inhalation were determined using a Coulter Counter, Multisizer 4 (Beckman Coulter, California, USA). Each formulation was dispersed in an electrolyte solution (Isoton®) and measurement was performed using an aperture tube size of 0.4 – 16 μ m and current of 600 μ A.

Bulk density (bp) was calculated by determining the weight of powder required to occupy a 1 ml volume in a graduated glass syringe, by pouring under gravity. The tapped density (tp) was determined by vertically tapping this sample onto a level bench-top surface, from a height of 5 cm, until there was no observed volume change (100 times) [29]. The tapped density was calculated as the ratio of the mass to the tapped volume of the sample. Each measurement was performed in triplicate. The theoretical aerodynamic diameter (d_a) was calculated based on the following definition $d_a = \sqrt{tp/\rho_l}$; where $\rho_l = 1\text{g/cm}^3$ and “d” is the geometric diameter of the particle [32].

The surface morphology of CFZ:CD complexes was observed using a scanning electron microscope (SEM), TM 3030 Plus (Hitachi, Tokyo, Japan). The SEM was operated under high vacuum, at 15 kV, small spot size, while images were acquired in

the backscattered electrons mode (BSE) with an acceleration voltage of 15 kV and a secondary detector. The powder was deposited on double-sided adhesive tape, vacuum-coated with gold and directly analyzed under SEM with magnifications of x500, x1000, x3000 and x5000.

The water vapour sorption-desorption isotherms of CFZ inclusion complexes were obtained by means of an automated gravimetric vapour sorption analyser, DVS Advantage-1 (Surface Measurements Systems). Samples were equilibrated at 0% relative humidity (RH) until a steady dry reference mass was recorded. The samples were exposed to the following RH profile: 0 - 90% in 10% steps and the reverse for desorption at $25.0 \pm 0.1^\circ\text{C}$. At each stage, the sample mass was allowed to reach equilibrium (i.e. weight change over 10 min $(dm/dt) \leq 0.002$ mg/min).

The specific surface area was determined by the Brunauer, Emmett, Teller (BET) isotherm method using a Micromeritics Gemini VI (Micromeritics, Norcross, USA) surface area analyser. The amount of nitrogen adsorbed at 6 relative pressure points in the relative pressure (P/Po) range of 0.05 to 0.3, with an equilibration time of 10 s, was used for the BET analysis. Each average result was calculated on the basis of three measurements.

2.2.6. In vitro aerosol deposition studies using the Next Generation Impactor (NGI)

The aerodynamic properties of the powders were assessed using an NGI (Copley Scientific Limited, Nottingham, UK), apparatus 5, operated under pharmacopeial conditions [8]. The flow rate was adjusted to achieve a pressure drop of 4 kPa in the powder inhaler, (Handihaler®, Boehringer Ingelheim, Ingelheim, Germany) and the time of aspiration was adjusted to obtain 4 L air flow. The pre-separator was used to remove coarse particles. The dry powder inhaler was loaded with a number 3 hard gelatin capsule loaded with 20 ± 2 mg of powder for each test. NGI stages (stages 1–7 and filter) were coated with 1 mL of HPLC mobile phase to wet the collection surfaces. The deposition profile of each formulation was determined in triplicate and the results presented are the average results of the replicated analyses. After dissolution in an appropriate volume of HPLC mobile phase, particle deposition in the device, the throat and all the stages and the filter was determined by the HPLC method described above. Each test was repeated three times. The emitted recovered dose (ED) was determined as the percent of total powder mass exiting the capsule and device. The total amount of particles with aerodynamic diameters smaller than 5 and $3\ \mu\text{m}$ was calculated by

interpolation from the inverse of the standard normal cumulative mass distribution less than stated size cut-off against the natural logarithm of the cut-off diameter of the respective stages. This amount was considered as the fine particle fraction (FPF%) below 5 μ m and 3 μ m, and expressed as a percentage of the ED. The mass median aerodynamic diameter (MMAD) of the particles was determined from the same plot as the particle size corresponding to the 50% point of the cumulative distribution, and the geometric standard deviation (GSD) as $GSD = \sqrt{(Size\ X/Size\ Y)}$, where Size X is the particle size corresponding to the 84% point and size Y is the particle size corresponding to the 16% point of the cumulative distribution.

2.2.7. Solubility studies

The solubility studies were determined using a shaker Innova 44 (New Brunswick Scientific, New Jersey, USA). It was performed by the 24 h shake flask method at 100 rpm and 37 °C (Wermuth, 2008; The Practice of Medicinal Chemistry, 3rd ed. Elsevier Ltd, p. 750). Therefore, an excess of pure CFZ, and formulations were individually added to 10 ml of pure water in glass tubes. After the appropriate time, the tubes were opened, and the supernatant withdrawn and filtered through 0.45 μ m membrane filters (ChromPure syringe filter, PVDF, 25 mm diameter). CFZ concentrations in the supernatant were determined by HPLC as previously described.

2.2.8. Cytotoxicity studies

Exponentially growing human lung adenocarcinoma cells (Calu-3) were seeded at an initial density of 5×10^4 cells/mL in 96-well plates in DMEM medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin/streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% CO₂ atmosphere, for 24 h. After this time, the medium was removed and replaced with fresh medium containing either pure β CD (25 - 200 μ M), pure CFZ, the physical mixture or the inclusion complex. All samples that contained CFZ ranged from 3.125 to 25 μ M, in relation to CFZ concentration. The plate was incubated at 37 °C in a humidified, 5% CO₂ atmosphere. The highest concentration tested when CFZ was present was limited to the equivalent of pure CFZ solubility (25 μ M). Cell viability was determined after 24 h by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide method (MTT) [33].

The absorbance was measured at 570 nm in a microplate reader, Perkin Elmer Victor X5 (Perkin Elmer, Massachusetts, USA).

A second approach to cytotoxicity testing was used to evaluate the effect of higher CFZ equivalent concentrations (50 and 100 μ M) for a shorter incubation time (4h). Results from this study were needed to support the permeability study conditions (detailed below), where initial low drug concentration (25 μ M) were found to result in levels falling outside the detection limit of the analytical method employed (HPLC) after permeation. This issue has previously been reported for poorly water soluble molecules in permeability testing [34].

2.2.9. Permeability studies

Calu-3 cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin/streptomycin. Cell monolayers were obtained by seeding 5.5×10^5 cells/cm² onto tissue culture inserts (Costar polyester filters, with a 1.12 cm² growth surface area and 0.4 μ m pore size) placed in twelve-well plates and maintained at 37 °C in a humidified, 5% CO₂ atmosphere. During the first seven days, cells were cultured under liquid-liquid interface and growth medium (0.5 ml on the apical side and 1.5 ml on the basal side) was replaced with fresh medium every 2-3 days. From the eighth to twelfth day, cells were cultured in an air-liquid interface (0.6 ml on the basal side)

The epithelial monolayer confluence was estimated by visual inspection under an optical microscope. The monolayer integrity was evaluated by transepithelial electrical resistance (TEER) measurements, using a Millicell® ERS-2 (Merck Millipore, Massachusetts, USA). TEER was calculated by subtracting the resistance of a cell-free culture insert and correcting for the surface area of the Transwell cell culture support. The increase in the values of TEER accompanied the growth of cells in each filter to reach a constant value of $> 700\Omega \text{ cm}^2$, indicating suitable cellular maturity for the test [35]. TEER was measured at the beginning, during and after the experiment, to check cell layer integrity. Transport experiments were evaluated in the apical to basolateral direction and were carried out in Hank's Balanced Salt Solution (HBSS). The apical side was filled with 0.5 ml of sample suspension (100 μ M, in relation to CFZ) and the basolateral side with 1.0 ml of HBSS. After 0.5 h, 1 h, 2 h, 3 h and 4 h all the basolateral side volume was collected and immediately replaced with fresh HBSS. At the last time point, the apical content was also collected. The cell monolayer was

washed with HBSS and disrupted using DMSO (200 µl), after which this volume was collected. CFZ concentration in all collected samples was determined by HPLC as previously described.

~~*In vitro aerosol deposition studies using the Next Generation Impactor (NGI)*~~
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2.2.10. Statistical analysis

Results are presented as mean ± standard deviation. Significant differences were evaluated using analysis of variance (ANOVA) with post-hoc test. All statistical

analyses were performed with the software GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and Discussion

3.1. Inclusion complex preparation

The efficacy of CFZ inclusion complexes based on sulfated CD has previously been described, showing these complexes' activity against two different TB strains (*M. avium*/*M. intracellulare*) [26]. Thus, the use of CD to formulate a CFZ pulmonary delivery system was considered the first-choice option. This approach was reinforced by the reported successful inclusion of CFZ in negatively charged SBE β CD / epichlorhydrin oligomer to increase its water solubility and bioavailability [27].

The inclusion complexes between CFZ and the CDs (β CD, γ CD, 2-HP β CD and SBE β CD) were obtained using different conditions, described in Table 1.

The association efficiency between CFZ and each CD was verified after the inclusion process (Figure 1). It can be observed that β CD (ethanol:water, β CD_b condition) provided the highest percentage of drug inclusion (included drug mass*100/total drug mass). The highest affinity of CFZ for β CD compared to other CDs was an unexpected result, since other water insoluble drugs, such as curcumin, ~~a drug structurally related to CFZ~~, have shown higher affinities for more hydrophobic CDs, like RM β CD (randomly methylated β -cyclodextrin) and HP γ CD (hydroxypropyl- γ -cyclodextrin). Another interesting result was the low affinity observed for the negatively charged SBE β CD, when the least affinity observed for curcumin was for the cationic HTA β CD (hydroxytrimethylammoniumpropyl- β -cyclodextrin) [36].

3.2. Cyclodextrin Complexation studies

The complexation efficacy (CE) determined for the condition ethanol: water (named β CD_b, described in Table 1), which provided a higher percentage of recovered CFZ, was 0.1738, with the ideal CFZ: β CD molar ratio being 1:7. For comparative purposes, the CE and the molar ratio were also determined for the β CD_a (water, in Table 1) condition. Compared to the β CD_a, the β CD_b condition, as expected, showed greater CE and a molar ratio comprising 12-fold lower β CD, compared to condition β CD_a. Figure 2 shows the complexing profiles between CFZ and β CD under conditions a and b. Phase solubility curves are represented as dissolved drug

concentration against the concentration of β CD used. The binding constant (K) for complexes formed under conditions β CD_a and β CD_b were calculated from the slope of the straight-line portion of the curve, according to the following equation:

$$K = \text{slope} / S_0(1 - \text{slope}) \quad (3)$$

Where S_0 was the solubility of the drug in the absence of β CD. The obtained K value for the complex formed under condition β CD_b (5.03 M^{-1}) was higher than that of the complex obtained under condition β CD_a (2.61 M^{-1}), therefore, indicating a more stable complex for condition β CD_b, using ethanol as co-solvent.

Despite the increased powder volume associated with a system comprised of drug and CD in a 1:7 ratio, (i.e. 1CFZ:7 β CD), the bulk of the formulation to be delivered is not of concern, since a reduction in the therapeutic concentration of the drug to be delivered (and the powder volume used, less than 100mg) is expected, due to an improvement in its bioavailability by means of pulmonary administration [13,22]

3.3. Characterization studies

The characterization studies of the complexes were performed on the dry powder obtained by lyophilization and spray drying, resulting from the complexation obtained under condition β CD_b (1CFZ:7 β CD). The inclusion of a molecule in the CD cavity generally promotes a change in the melting point, with a reduction in signal intensity or the disappearance of the fusion event signal in the DSC [37]. The thermograms of the raw materials (CFZ and β CD), physical mixture and the 1CFZ:7 β CD complex powders (dried by lyophilization and spray drying) are shown in Figure 3. The CFZ has a typical DSC curve of a crystalline substance, i.e. with a sharp and narrow melting event with a peak at approximately 223°C . β CD exhibits a large endothermic signal related to loss of water, peaking at 81.5°C . The physical mixture presents the signal corresponding to the loss of water from β CD, followed by the melting peak of CFZ with a reduced signal intensity. The thermograms of the products obtained by lyophilization and spray drying maintained the thermal event related to the loss of water from the CD, and show the disappearance of the drug melting peak, suggesting the formation of the inclusion complex in both cases (Figure 3). Thus, the use of the spray drying technique can be proposed for the formation of the complex, while optimizing the powder properties to obtain the desired pulmonary formulation.

At the same time, complexation of CFZ tends to promote changes in its diffractogram, seen as a signal shift or change in signal intensity when compared to isolated molecules or the physical mixture [37–39]. XRPD analysis of CFZ and β CD demonstrates typical crystalline substance diffraction patterns, corresponding to those deposited in the Cambridge Structural Database (CSD). The diffraction pattern of the CFZ raw material was typical of polymorphic form I of the drug (9.2, 13.4, 19.8 and 21.7° 2 θ), according to CSD reference (DAKUI01), Figure 4.

The diffractogram of spray-dried β CD (Figure 5C) showed powder amorphization after processing, with broad peaks (at 6.6°, 11.6° and 18.3° 2 θ) evident. In a previous study, [Amaro et. al \(2015\)-\[40\]](#), observed a similar diffraction pattern for spray-dried 2-HP β CD, with diffuse peaks in a similar position [\[40\]](#). The diffraction pattern was reported to be associated with the molecular arrangement of the CD as a cup that can form small domains with some degree of short-range order. Despite this, the resulting powder was classified as amorphous [40]. The diffractogram of the physical mixture was similar to that of combined diffraction patterns of the individual raw materials (Figure 5). The physical mixture peak pattern (Table 2) indicated a simple mixture (lack of chemical interaction) as it was the sum of the diffraction peaks of the raw materials. On the other hand, for the inclusion complex a new peak (7.7° 2 θ), peak displacements (12-13.4° and 18.3-19.8° 2 θ) and peak absences (4.5°, 8.9-9.2°, 10.8°, 15.4° and 22.0° 2 θ) were observed (Table 2), indicating complex formation by means of CFZ- β CD interaction. The DSC result presented in the previous section, corroborates with the XRPD result pointing to inclusion complex formation. The diffractogram of the 1CFZ:7 β CD complex obtained by freeze drying exhibited broad peaks while spray drying appeared to promote a higher degree of amorphization, based on the XRPD patterns (Figure 5). The formation of completely or partially amorphous material can represent complex formation, but it can also be a characteristic resulting from lyophilization and spray drying processes [15,41]. Therefore, XRPD results should be considered in association with results from thermal (DSC – described above) and spectroscopy analyses (discussed below) for the appropriate characterization of the inclusion complex [37].

The FTIR spectra of the dried complexes compared to the physical mixture and the raw materials, did not show any relevant alterations of the characteristic absorption peaks of CFZ, indicating only the presence of the drug, as shown in Figure 6.

Nuclear magnetic resonance (NMR) spectra can reveal more structural information of the analyzed material than the techniques used above, where the occurrence of a chemical shift (δ) in the carbon (^{13}C) signals reveals the formation of an inclusion complex [37]. From the ^{13}C -CP/MAS solid phase NMR spectra (Figure 7), the main signals (60.77, 72.89 and 102.89 ppm) of the βCD present in the physical mixture and the CFZ: βCD complex (1:7) were analyzed. The CFZ: βCD (1:7) complex shows, in relation to βCD , chemical shift variations of 0.37 ppm, 0.61 ppm and 1.22 ppm, whereas in the physical mixture the same displacements were lower and correspond to 0.35 ppm, 0.06 ppm and 0.21 ppm. The change in multiplicity plus the greater variation in chemical displacement were indicative of inclusion complex formation. The CFZ signals were not exploited due to its low concentration in the complex.

3.4. Spray drying for pulmonary delivery formulations

Spray dryer settings and formulation constituents are known to influence the final particle properties, such as size, density, morphology and moisture content, which in turn determine powder aerosolisation characteristics and site of lung deposition [1,42–44]. Different formulations and spray drying conditions were evaluated, as presented in Table 3. The geometric mean diameter (GD) and the polydispersity index obtained for the different formulations are shown in Figure 8. The polydispersity index (span value) was calculated from the equation: $(d_{90}-d_{10})/d_{50}$, where d_{90} , d_{50} and d_{10} correspond respectively to the geometric diameter of 90%, 50% and 10% of the distribution fraction (data not shown) [15,40,42].

Initial spray dryer settings were shown to be sub-optimal due to a high GD (11.59 μm , Figure 8). The high inlet temperature (95 $^{\circ}\text{C}$) and pump flow (0.55 L / h) used to generate the SP1 formulation (Table 3) probably caused this behavior, as previously observed by Wang and Wang [45]. These authors indicated that the high temperature and flow caused, respectively, fast solvent evaporation and larger droplet size formation, and consequently a larger particle GD [45]. The inlet temperature was reduced to 65 $^{\circ}\text{C}$, as previously described in a study to determine the influence of spray dryer conditions on the preparation of a beclomethasone: γCD inclusion complex using the same co-solvent system as used in this work [44]. The lower inlet temperature and flow resulted in small but statistically insignificant decrease in GD for SP2 compared to SP1 (Figure 8).

Formulations SP3-9 tested the inclusion of L-leucine or L-isoleucine to evaluate the influence of these excipients on particle size. These formulations were also fine-tuned with respect to amino acid concentration, pump flow and co-solvent used (Table 3). A pump feed increase to 0.35 L/h for SP6, SP7 and SP8 formulations led to the lowest values of GD, corresponding to $5.73 \pm 1.20 \mu\text{m}$, $5.45 \pm 1.62 \mu\text{m}$ and $2.18 \pm 0.110 \mu\text{m}$, respectively, and narrowest size distribution, observed for SP8 (span=0.03). These GD values were promising with respect to the production of powder with appropriate aerodynamic characteristics for pulmonary delivery [7]. L-leucine addition led to a particle size reduction, while L-isoleucine (in SP5) did not. Likewise, the addition of 2% dichloromethane (SP9) did not result in a significant reduction in GD.

3.5. Particle morphology

The morphological evaluation of formulations SP6 to SP9 (Figure 9) revealed a near-spherical shape. Among the formulations spray dried from ethanol:water (1:1) and with the same spray drying conditions (SP6 – SP8), L-leucine incorporation led to the production of powders comprised of fewer agglomerates, with a more homogeneous size distribution and particle surfaces that were slightly corrugated, consistent with previous reports [14,15]. The lesser agglomeration and increased particle corrugation may favor powder dispersibility and the effective fraction of breathable powder ($<5 \mu\text{m}$) [46]. The SP9 formulation (CFZ: βCD + L-leucine 15%, dispersed in dichloromethane 2%, in addition to ethanol:water), differs considerably in its appearance from the other powders, since the particles appear to be cohesive, forming rod structures with smooth surfaces, i.e., without pores (unlike what may have been expected when using a blowing agent). A more heterogeneous particle size distribution (larger span) was measured for this system (Table 3).

3.6. Bulk density (bp), Tapped density (tp), Specific Surface Area (SSA) and Aerodynamic Characteristics

The bp, tp, SSA and the aerodynamic characteristics were measured for formulations with greatest potential for pulmonary delivery (SP6, SP7 and SP8), given their GD and morphology (Table 4). The bp and tp ranged between 0.113 and 0.265 g/cm³ and were considered suitable for pulmonary delivery, as previously reported by some authors ($< 0.3\text{g/cm}^3$) [15,43,47]. The theoretical d_a calculated from tp and GD for SP6, SP7 and SP8 were $2.36 \mu\text{m}$, $2.28 \mu\text{m}$ and $1.12 \mu\text{m}$ respectively, further indicating

suitability for pulmonary delivery (1 – 5 μm) [9]. The L-leucine promoted an increase in bp and tp, related to the higher solid content and formation of dense particles, in agreement with previously reported data [15]. As expected, when compared to the other formulations, the presence of 15% L-leucine significantly increased the SSA ($P < 0.001$) due to the formation of rougher/corrugated/more textured particles (Figure 9-D) [1]. The roughness tends to reduce the interparticle contact, thus favoring powder dispersibility. The aerodynamic behavior of the formulation is influenced by the interrelationship of factors related to the particle (geometric diameter, morphology, surface area) and to the powder (bp, tp, hygroscopicity). The emitted recovered dose (ED) (i.e. total dose less the amount remaining in the capsule and device) for all evaluated formulations was similar, and high (94.13 to 95.19%). This result indicated adequate release from the capsule and the inhalation device and at the same time demonstrated good powder flow properties. The addition of L-leucine did not change the %ED (probably due to the fact that the %ED for the equivalent formulation without leucine was already high). Previous studies have commonly indicated an increased ED corresponding to the presence and increasing concentration of this amino acid [17,48]. However, here the fine particle fraction (FPF) of the selected formulations ranged from 46.20 to 51.56%, with no significant difference between different formulations S6, S7, S8. A comparison between MMADs (Table 4) and theoretical aerodynamic diameter values for SP6 – SP8 (given above) showed that $\text{MMAD} > d_a$ in all cases, suggesting that during aerosolisation, the powder remains aggregated rather than behaving as individual particles. Other researchers had already noted the same $\text{MMAD} > d_a$ relation, with an even larger difference between values [32,49]. However, despite the aggregates, approximately fifty percent of ED corresponded to FPF in all tested formulations. The MMAD of all formulations was less than 5 μm and the geometric standard deviation (GSD) was less than 2. These were considered adequate values for pulmonary formulations and delivery, since the combination of these parameters indicates a narrow particle size distribution in the FPF region [11,43].

3.7. Dynamic vapour sorption (DVS)

DVS analysis was performed to evaluate the behavior of SP6, SP7 and SP8 formulations when exposed to different moisture conditions (Figure 10). The adsorption isotherms of the formulations evaluated were similar and showed considerable incorporation of vapor (water) even at low relative humidities (RH). As expected, due to

the amorphous characteristics of these powders, the three formulations exhibited considerable and continuous mass gain with increasing relative humidity (RH). Li *et. al.* (2016) demonstrated that water uptake by spray dried L-leucine alone was very low (<1%) at 90% RH, being classified as non-hygroscopic. Thus, the L-leucine contribution to water uptake when incorporated in a powder should be insignificant [50]. However, the presence of L-leucine made the water sorption both slower and overall lower and provided some protection to the powder against increasing relative humidity. When comparing the formulations containing L-leucine (SP7 and SP8) to that without (SP6), it was observed that, up to 80% RH, the weight gain due to water sorption was much lower for the L-leucine-containing formulations, but from 90% RH the difference in the sorption profiles was negligible. At the end of the sorption process, the mass increase was 23.22%, 22.03% and 19.78% for the formulations SP6, SP7 and SP8 respectively, demonstrating the anti-hygroscopic effect of L-leucine [48,50]. The absence of inflection in the adsorption isotherms indicated the absence of recrystallization for all formulations. The diffractograms obtained after the DVS runs (Figure 11) still demonstrated high degrees of amorphization. The broad peaks in the XRPDs can be attributed to the spray-dried β CD which was present in a large proportion in all formulations. The peak position displacements for the complexes 1CFZ: 7 β CD (SP6) (12.7° and 19.2° 2 θ), 1CFZ: 7 β CD + L-leucine 10% (SP7) (13.4° and 19.3° 2 θ) and 1CFZ: 7 β CD + L-leucine 15% (SP8) (6.2°, 13.6° and 19.1° 2 θ) when compared to spray-dried β CD (6.6°, 11.6° and 18.3° 2 θ - Figure 5C) can be explained by differences in powder composition, leading to slight variations in molecular interactions. In addition, the diffractogram of spray-dried L-leucine after the DVS analysis exhibited peaks at 2 θ of 6.2° and 19.1°, as previously reported [15]. The intensity of the peak ranging in position from 19.1° to 19.3° 2 θ reflected the L-leucine content variation in formulations SP6-8 (Figure 11). For the desorption in the range of 80 - 10%, the presence of L-leucine slowed down the water loss. However, after the desorption process, all formulations returned to the initial mass levels, indicating a reversible water sorption process.

3.8. In vitro aerosol deposition studies using the Next Generation Impactor (NGI)

The deposition profiles of SP6, SP7 and SP8 formulations show that 58-62% of emitted dose (ED) was able to cross the mouthpiece adapter/induction port (MA/IC),

which *in vivo* would correspond to the oro-pharyngeal region and indicates suitability for deposition in the respiratory tract (Figure 12). Particle deposition on MA/IC can be explained by agglomerate formation as a consequence of powder hygroscopicity, due to its amorphous state. Regarding the NGI stages, the particle deposition of the evaluated formulations at a 100 L/min flow rate was concentrated in stages 2, 3 and 4, which have cut-off diameters of 3.42 μ m, 2.18 μ m and 1.31 μ m respectively [8]. Stage 2 was the only one that exhibited significant deposition differences ($P<0.001$) between formulations SP6 (without L-leucine) and SP8 (L-leucine 15%). The higher SP6 deposition was in line with its larger MMAD ($3.38 \pm 0.19 \mu\text{m}$ for SP6 compared to $2.98 \pm 0.08 \mu\text{m}$ for SP8) and the stage cut-off diameter (3.42 μ m). The SP8 formulation presented lower deposition at this stage, which is an advantage and can enable better pulmonary retention. The percentage of particle deposition in the stages corresponding to the lower respiratory tract (stages 3 – 7) was 30-40%, with, perhaps surprisingly, no significant difference between formulations with or without L-leucine, in spite of significant difference in SSA ($P<0.001$) and MMAD ($P<0.05$) for SP8 (with 15% L-leucine) compared to other formulations.

When considering exclusively the aerodynamic powder characteristics, the formulations SP6 – SP8 all showed appropriate parameters for use in a DPI. Efficacy tests should be performed in the future to confirm this hypothesis.

3.9. Solubility studies

The aqueous solubility of SP6, SP7 and SP8 formulations were evaluated (Figure 13). When compared to the free drug, formation of the CFZ: β CD complex (1:7) increased the aqueous solubility of CFZ by sixteen-fold ($P<0.001$) and incorporation of 10% L-leucine, promoted an even greater increase (almost twenty seven-fold). A further increase in L-leucine content from 10% to 15% did not modify the water solubility of the complex ($P>0.05$). The CFZ solubility improvement when complexed with β CD may be explained not only by their association, but also by the formation of amorphous particles during the spray drying process [1,25]. On the other hand the L-leucine effect on solubility is likely to be due to its surfactant-like properties [17], which has already been observed for azithromycin co-spray-dried particles [51]. The higher solubility for L-leucine containing formulations improves their therapeutic potential, since they may be better able to overcome the mucus barrier, present prolonged lung retention time and higher CFZ bioavailability.

3.10. Cytotoxicity studies

The *in vitro* cytotoxicity of free CFZ and β CD, and the CFZ: β CD complex, Figure 14, was evaluated after 24 h exposure in a Calu-3 cell line, due to its representativeness of the constituents of the respiratory tract [52]. The cytotoxicity assay was performed not only to identify the cytotoxicity of the raw material itself, but also to identify if the complexation could reduce this. The β CD complex revealed >90% cell viability even when exposed to the highest equivalent CFZ concentration (25 μ M). This value was not significantly different from that observed for CFZ or a CFZ-CD physical mixture. The β CD toxicity at Calu-3 cells (Figure 14B) revealed that it was dependent on β CD concentration. Cell viability for a β CD concentration $\leq 50 \mu$ M was higher than 90%, while it was higher than 70% for a concentration of 200 μ M. These results indicated a high tolerability of Calu-3 cells for the CFZ: β CD complex, enabling its lung administration. The international guide ISO 10993-5, states that cell viability > 70% is not considered a cytotoxic effect [53]. The cytotoxicity test based on the cell viability assay condition to inform the permeability study used CFZ equivalent concentrations of 50 – 100 μ M incubated for 4h. Cell viability results were: (i) for the free CFZ: $78.2 \pm 9.9\%$ (50 μ M) and $77.0 \pm 6.1\%$ (100 μ M); and (ii) for SP8 formulation (selected for permeability study, as justified in the next section): $75.1 \pm 11.34\%$ (50 μ M) and $72.5 \pm 3.9\%$ (100 μ M). Then, in terms of cytotoxicity, these results demonstrated the suitability of both concentrations for permeability studies and 100 μ M was the concentration selected for this study.

3.11. Permeability studies

As described in the literature, the *in vitro* permeability study is a good indicator for drug delivery in the respiratory tract [54]. Because of its lower MMAD (2.98 μ m), higher FPF (51.6%), promising deposition profile, improved water solubility and specially low hygroscopicity compared to other tested formulations, SP8 was selected for this evaluation. The formulation was added as a suspension to the surface of the Calu-3 monolayer, as reported in other studies [55]. The maintenance of the integrity of the monolayer was confirmed by the stability of TEER values, which was verified during and after the experiment (Figure S145). After 4 h, there was no CFZ detected in the basolateral compartment for wells containing the free drug or for SP8. ~~This result~~

~~points to the possibility of obtaining a high drug concentration directly at the site of pulmonary infection, while minimizing systemic CFZ biodistribution and toxicity.~~ The quantification of intracellular CFZ was similar for the free drug ($44.4 \pm 14.0\%$) and for SP8 ($43.7 \pm 4.6\%$), indicating that both exhibit the same penetrating capacity in the monolayer and availability for local action in the lungs. This result points to the possibility of obtaining a high drug concentration directly at the site of pulmonary infection and thus a more effective therapy, while minimizing systemic CFZ biodistribution and toxicity. Hence, reducing systemic side effects like reddish-brown discoloration of the skin and conjunctiva [22], a relevant issue at TB prolonged treatment regime (minimum 6 months) [2,10]. At the same time, pulmonary delivery of CFZ microparticles can be an interesting strategy to improve mycobacteria death by favoring two inherent mechanisms: (i) CFZ distribution to the mononuclear phagocyte system, and (ii) the macrophages uptake of microparticles [10,56]. In addition, the CFZ inside macrophages also can act as a drug reservoir, prolonging the residence time at the lungs, reaching granulomas and supporting the TB latent cases treatment [10]. ~~Cellular internalization can act as a drug reservoir and thus prolong the residence time of the drug in the lungs and favor treatment for latent cases of TB.~~

3.12. Pulmonary dose calculation

The CFZ WHO treatment regimen for TB has established a daily oral dose of 100 mg [57], this drug also has an oral bioavailability of 45-62% [22]. The calculation of the inhaled estimated dose (4.5 mg/day) considered that the pulmonary delivery drug amount usually is about ten-times lower compared to oral one [43,58,59]. Considering that the best formulation (SP8) exhibited a drug:powder ratio of about 1:20 (w/w), the total powder mass amount was of 89.3 mg/day. This mass is smaller than those of the marketed tobramycin and colistimethate dry powder inhaled formulation, and like the examples can be used in multiple doses or inhalations [60–62].

4. Conclusions

In the present study, CFZ:CD complexes were studied with a view to developing pulmonary delivery systems for TB treatment. We produced inhalable microparticles of CFZ:CD by spray drying to facilitate the particle formation process, with water soluble CD complex formation, able to overcome the mucus barrier and prolong the lung

retention time. The 1CFZ:7CD complex improved the CFZ water solubility and L-leucine incorporation promoted an even greater solubility. Inhalable microparticles were produced under optimized spray drying conditions of: 65 °C inlet temperature, 0.35 L/h pump rate, and 35 L/min gas flow rate. DPI formulations with or without L-leucine were developed, comprising suitable aerodynamic properties and improved CFZ water solubility. The addition of 15% L-leucine had the advantage of reducing powder hygroscopicity while also enhancing *in vitro* powder deposition in the lower airways. *In vitro* studies showed a viability of at least 70% for Calu-3 cells after 24 hours of exposure to CFZ and β CD (high concentrations) and permeability testing indicated that the drug was retained at the apical compartment and achieved cellular internalization.

The inhaled estimated dose was 4.5 mg in a powder mass of 89.3 mg/day, allowing dry powder device use.

In conclusion, the current work presented dry powder formulations for inhalation of cyclodextrin complex representing a potential new local treatment for lung tuberculosis infection and should be tested *in vivo* in the near future.

Data availability: The dataset analyzed during the current study is available from the corresponding author on reasonable request. Data generated during this study are included in this published article.

Declaration of interest: None.

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Table 1. Complexation conditions of CFZ and CD.

Cyclodextrin condition	Solvent	Molar proportion CFZ:CD (mM)	Temperature (°C)	Time (h)
β CD_a	Water	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
β CD_b	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
γ CD_a	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
2-HP β CD_a	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
2-HP β CD_b	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
2-HP β CD_c	Ethanol:Water (7:3)	1:1 / 1:5 / 1:10	50	72
2-HP β CD_d	Ethanol:Water (4:6)	1:1 / 1:5 / 1:10	40	72
2-HP β CD_e	Ethanol:Water (2:8)	1:1 / 1:5 / 1:10	40	72
2-HP β CD_f	Ethanol:Water (1:9)	1:1 / 1:5 / 1:10	40	72
SBE β CD_a	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	30	144
SBE β CD_b	Water	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	30	72

The solution method was used for all complexation conditions.

Table 2. The characteristic peaks of CFZ, CDs, physical mixture and their complexes in the XRPD diffractograms.

	Main peaks 2θ (degrees)							
1CFZ:7βCD Spray dried	---	---	---	---	13.1	---	18.8	---
1CFZ:7βCD Freeze dried	---	7.7	---	---	12.3	---	18.3	---
Physical mixture	4.4	---	8.9	10.5	12.4	15.3	19.5	22.7
βCD	4.5	---	8.9	10.8	12.5	15.4	19.6	---
Clofazimine	---	---	9.2	---	13.4	---	19.8	22.0

Table 3. Clofazimine powder formulations and spray dryer settings.

Formulation	Solid constituents	Liquid constituents	Spray dryer settings			
			Inlet temperature (°C)	Outlet temperature (°C)	Pump flow (L/h)	Gas flow (L/min)
SP1	CFZ:βCD (1:7)	Ethanol:Water (1:1)	95	45 – 48	0.55	35
SP2	CFZ:βCD (1:7)	Ethanol:Water (1:1)	65	41 – 43	0.30	35
SP3	CFZ:βCD (1:7) L-leucine 10%	Ethanol:Water (1:1)	65	41 – 43	0.30	35
SP4	CFZ:βCD (1:7) L-leucine 15%	Ethanol:Water (1:1)	65	37 – 42	0.30	35
SP5	CFZ:βCD (1:7) Isoleucine 15%	Ethanol:Water (1:1)	65	38 – 41	0.30	35
SP6	CFZ:βCD (1:7)	Ethanol:Water (1:1)	65	44 – 47	0.35	35
SP7	CFZ:βCD (1:7) L-leucine 10%	Ethanol:Water (1:1)	65	44 – 47	0.35	35
SP8	CFZ:βCD (1:7) L-leucine 15%	Ethanol:Water (1:1)	65	44 – 47	0.35	35
SP9	CFZ:βCD (1:7) L-leucine 15%	Ethanol:Water (1:1) Dichloromethane 2%	65	34 – 46	0.35	35

Table 4. Bulk (bp) and tapped density (tp), specific surface area (SSA) and aerodynamic characteristics of L-leucine free (SP6) and L-leucine spray dried formulations (SP7 – SP8).

	Aerodynamics characteristics						
	Bulked density (g/cm ³)	Tapped density (g/cm ³)	Specific surface area (m ² /g)	Emitted dose (%)	FPF < 5µm (%)	MMAD (µm)	GSD
SP6	0.113 ± 0.005 ^a	0.170 ± 0.003 ^c	3.504 ± 0.075 ^{e,f}	95.19 ± 1.83	48.41 ± 2.79	3.38 ± 0.19 ^h	1.65 ± 0.12
SP7	0.128 ± 0.002 ^b	0.176 ± 0.010 ^d	2.766 ± 0.054 ^{e,g}	95.02 ± 1.39	46.20 ± 6.81	3.06 ± 0.09	1.82 ± 0.13
SP8	0.185 ± 0.004 ^{a,b}	0.265 ± 0.006 ^{c,d}	6.104 ± 0.285 ^{f,g}	94.13 ± 1.52	51.56 ± 5.16	2.98 ± 0.08 ^h	1.80 ± 0.11

FPF: fine particle fraction. MMAD: mass median aerodynamic diameter. GSD: geometric standard deviation. Results were presented as mean ± SD (n = 3). Difference significance: ^{a,b,c,d,f,g} denote P<0.001; ^e denotes P<0.01 and ^h denotes P<0.05 (one-way ANOVA, with a post-hoc test).

Figure captions:

Figure 1. Percentage of total mass of clofazimine recovered after complexation process. Each condition is described in Table 1. Results are presented as mean $\pm$ SD (n = 3).

Figure 2. Clofazimine's phase solubility profile determined under different conditions (A) Condition β CD_a in Table 1: excess amount of CFZ was added to aqueous solutions containing increasing concentrations of β CD (1-10 mM) and were stirred at 40 °C for 72 h, and subsequently filtered through a 0.45 μ m membrane. (B) Condition β CD_b in Table 1: excess amount of CFZ was added to ethanol:water (1:1) solutions containing increasing concentrations of β CD (1-10mM) and were stirred at 40 °C for 72 h, and subsequently filtered through a 0.45 μ m membrane. "CE" was the complexation efficacy. Results are presented as mean $\pm$ SD (n=3).

Figure 3. DSC curves of CFZ systems and β CD: (A) CFZ raw material; (B) β CD raw material; (C) physical mixture of CFZ and β CD raw materials; (D) inclusion complex, freeze-dried; (E) inclusion complex, spray-dried.

Figure 4. XRPD diffractograms of CFZ from CSD (polymorph form I, DAKUI01) and CFZ raw material.

Figure 5. XRPD diffractograms of CFZ and β CD systems: (A) CFZ raw material; (B) β CD raw material; (C) spray-dried β CD; (D) physical mixture of CFZ and β CD raw materials; (E) inclusion complex, freeze-dried; (F) inclusion complex, spray-dried.

Figure 6. FTIR spectra of CFZ and β CD systems: (A) CFZ raw material; (B) β CD raw material; (C) physical mixture of CFZ and β CD raw materials; (D) inclusion complex, spray-dried. The dotted lines (---) and the circle indicates the main vibrational bands of CFZ (aromatic C-H stretching and C=N stretching).

Figure 7. ^{13}C -CP/MAS NMR spectra of CFZ systems and β CD: (A) CFZ raw material; (B) β CD raw material; (C) physical mixture of CFZ and β CD raw materials; (D) inclusion complex, freeze-dried.

Figure 8. Geometric mean diameter and polydispersity index (span) of raw material (CFZ and β CD) and spray dried formulations (SP). The SP formulation constituents and spray dryer settings are provided in Table 3. Results are presented as mean $\pm$ SD (n = 3). Difference significance: * P<0.05 and *** P<0.001 (one-way ANOVA, with a post-hoc test).

Figure 9. Scanning electron micrographs of physical mixture of CFZ and β CD and spray dried formulations. (A) physical mixture of CFZ and β CD raw materials; (B) SP6; (C) SP7; (D) SP8 and (E) SP9.

Figure 10. Water vapour isotherms (sorption and desorption). (a) SP6 – CFZ: β CD (1:7); (b) SP7 - CFZ: β CD (1:7) + L-leucine 10% and (c) SP8 - CFZ: β CD (1:7) + L-leucine 15%

Figure 11. XRPD profile after DVS analyses. (A) SP6 – CFZ: β CD (1:7); (B) SP7 - CFZ: β CD (1:7) + L-leucine 10% and (C) SP8 - CFZ: β CD (1:7) + L-leucine 15%.

Figure 12. *In vitro* aerosol deposition profile by use of NGI for spray dried formulations as calculated % of recovered emitted dose vs. NGI stages. (A) SP6 – CFZ: β CD (1:7); (B) SP7 - CFZ: β CD (1:7) + L-leucine 10% and (C) SP8 - CFZ: β CD (1:7) + L-leucine 15%. MA: mouthpiece adapter / IP: induction port. MOC: micro-orifice collector. Results are presented as mean $\pm$ SD (n = 3). Difference significance: *** P<0.001 (two-way ANOVA, with a post-hoc test).

Figure 13. Water solubility of clofazimine as free drug and formulations SP6 – CFZ: β CD (1:7); SP7 - CFZ: β CD (1:7) + L-leucine 10% and SP8 - CFZ: β CD (1:7) + L-leucine 15%. The water pH = 6.5. n.d: not detected. Results are presented as mean $\pm$ SD (n = 3). Difference significance: ** P<0.01 and *** P<0.001 (two-way ANOVA, with a post-hoc test).

Figure 14. (A) Viability of Calu-3 grown as cell layer after 24h exposure to 3.125 - 25 μ M of equivalent clofazimine. CFZ: Clofazimine raw material. CFZ: β CD (1:7) complex. PM: physical mixture of CFZ and β CD raw materials. (B) Viability of Calu-3 grown as cell layer after 24h exposure to 25 – 200 μ M β CD. Both experiments were

measured by MTT test (mean $\pm$ SD, n = 3). The dotted lines mark 70% and 90% viability. Difference significance: ** P<0.01 and *** P<0.001 (two-way ANOVA, with a post-hoc test).

Supplementary material:

Figure ~~S14~~^{S145}. TEER values of Calu-3 culture during the permeability study. Control: no treatment; CFZ: Clofazimine raw material; SP8: CFZ: β CD (1:7) + L-leucine 15%. The measurements were at the sampling time points.

1. Clofazimine:β-cyclodextrin (1:7) with L-leucine were submitted to spray drying.
2. Dry powder formulations showed suitable performance in Next Generation Impactor.
3. This inhalable formulation should improve clofazimine pulmonary bioavailability.
4. This study presented a potential local treatment for lung tuberculosis infection.

Development of inhaled formulation of modified clofazimine as an alternative to treatment of tuberculosis

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Short Title: **Clofazimine inhaled formulation for tuberculosis**

Abstract

Inhalation drug delivery provides a possible useful alternative to oral drug delivery in the treatment of tuberculosis (TB). This work evaluated inclusion complexes of clofazimine (CFZ), an anti-TB drug with low aqueous solubility and potential gastric degradation, in β -cyclodextrin (β CD), γ -cyclodextrin, (2-hydroxypropyl)- β -cyclodextrin and sulfobutyl-ether- β -cyclodextrin. A phase solubility study indicated that β CD showed the best inclusion capacity for CFZ, so a CFZ: β CD complex (1:7) was selected for further studies. Particle engineering was performed using spray drying to obtain powders with suitable characteristics for pulmonary delivery and L-leucine was added to enhance powder dispersibility. Thermal and spectroscopic analyses indicated the CFZ: β CD integrity after spray drying, and the presence of L-leucine resulted in less hygroscopic and rougher particles, and a less agglomerated powder. All formulations, and especially those containing L-leucine, showed suitable *in vitro* deposition performance in the Next Generation Impactor and presented higher aqueous solubility compared to the free drug. *In vitro* studies showed low toxicity against Calu-3 and CFZ retention in the cell monolayer and apical compartment. These results suggest that the inhalation formulation composed of CFZ: β CD plus L-leucine should improve CFZ pulmonary bioavailability and provide an alternative treatment for TB, acting on the main infection site of the disease.

Keywords: pulmonary delivery; tuberculosis; clofazimine; cyclodextrin; L-leucine

1. Introduction

Tuberculosis (TB) is an infectious disease caused by a unique pathogen, *Mycobacterium tuberculosis* (*M. tuberculosis*). TB is the ninth leading cause of death worldwide, ranking above HIV/AIDS. In 2017, the World Health Organization (WHO) estimated that 10.0 million people fell ill with TB (90% adults) and there were 1.3 million deaths [1–3]. Although controlled in certain developing countries and industrialized areas, the current and continuous migratory movement from Asia and Africa (where there is a high incidence) is a potential risk to the spread of TB to Europe and North America [4]. The disease mainly affects the lungs (within alveolar

macrophages and granulomas structures) but can also affect other sites [2]. Erratic and discontinuous treatments, associated with *M. tuberculosis* adapting mechanisms (efflux pump mechanisms) and population heterogeneity, have triggered multi-drug-resistant (MDR) strains of this mycobacteria and also extremely drug-resistant (XDR) strains [5,6]. Drug-resistant TB is a continuing threat since the first recorded case more than 12 years ago, and the development of new drugs or therapeutic strategies is necessary [4].

Inhalation is a non-invasive administration route used to deliver a drug substance into the lungs and treat local (asthma, cystic fibrosis) or systemic (diabetes, schizophrenia) diseases [7–9]. In recent years pulmonary drug delivery has received attention for TB treatment because it provides high drug concentrations at the main site of the disease, prevents hepatic-first pass metabolism and gastric intolerance and overcomes poor and erratic oral bioavailability of some drugs that are used in clinical practice [10,11]. The United States Pharmacopeia categorizes the dosage forms for pulmonary drug delivery into: (i) inhalation spray, (ii) inhalation solution, (iii) inhalation suspension, (iv) inhalation aerosols or metered-dose inhaler (MDI) and (v) inhalation powder or dry powder inhaler (DPI) [8]. DPIs are commonly employed in therapy for several reasons, including the fact that the formulation is propellant-free, while they also often offer better chemical stability, with a portable device which is easy to use [12].

The efficacy of formulations delivered by means of a DPI depends on the device delivery performance, breathing pattern of the patient, powder deposition pattern in the respiratory tract, the fine particle fraction (FPF) and the disease that is being treated [13]. The deposition site is determined in accordance with the aerosol aerodynamic properties, namely mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD); these parameters are derived from the aerodynamic particle size distribution (APSD) [7]. MMAD is the aerodynamic diameter (d_a) for which 50% of the aerosol mass is above and below that diameter. GSD shows the level of dispersity of the aerosol particles. To avoid particle deposition in the upper respiratory tract (oropharyngeal and tracheo-bronchial region) and exhalation before deposition, it is desirable to obtain an MMAD of between 1 and 5 μm and a $\text{GSD} < 2$. Powder physicochemical properties and morphological characteristics of the particles also determine the deposition profile and drug bioavailability [7]. Additionally, the literature describes the use of different L-leucine concentrations (10-30% w/w) in DPI

formulations, as a dispersion aid, to obtain aerosol particles with the desired *in vitro* deposition profile [14–18].

Once deposition occurs in the respiratory tract, the interaction between the particles and the biological environment also determines the therapeutic efficacy of the formulation. The interaction with mucus (aqueous mixture of glycoproteins), which is secreted in the upper airways, and pulmonary surfactant (90% phospholipids and 10% proteins), which is secreted in the alveolar region of the lungs, is determined by the physicochemical characteristics of the particles, whereby hydrophilicity favors dissolution in the mucus. The mucus is a biological barrier that blocks the entrance of foreign microorganisms and substances, including drugs. The mucociliary clearance removes insoluble particles deposited in the trachea-bronchial region, within 24h, resulting in a short retention time in the lung [9]. Drug solubility in the mucus minimizes the mucociliary clearance effect, favoring the desired drug effect [19]. Lipophilic drugs tends to be insoluble in mucus and thereby exhibit reduced cell permeability [19,20]. On the other hand, insoluble particles (1-2 μm) that reach the alveolar spaces tend to be internalized by macrophages (alveolar clearance), that are surrounded by a thin surfactant layer, and are able to reside for a long time inside them. Thus macrophage uptake represents a strategy to combat microorganisms that infect this cellular type, such as in TB [11,21].

The World Health Organization (WHO) recommends the use of oral clofazimine (CFZ) in combination with other drugs for drug-resistant TB treatment, although its efficacy and long-term safety have not yet been established [2,22]. CFZ's poor clinical efficacy is strongly attributed to low aqueous solubility and gastric degradation, resulting in suboptimal delivery to the target organ. On the other hand, *in vitro* studies demonstrated good inhibitory activity against MDR strains (minimal inhibitory concentration 0.5 – 2 mg/L) [22]. *In vivo* studies showed that administration of CFZ together with first or second-line anti-TB drugs promoted a 100 times reduction of bacteria colony count in the lung, suggesting its potential to shorten the duration of TB treatment [23,24]. These characteristics point towards CFZ as a potential drug for pulmonary delivery.

The use of cyclodextrins (CD) in formulations has allowed many products containing poorly soluble drugs to reach the market, and eventually help to treat many life-threatening disease conditions [25]. The efficacy of CFZ inclusion complexes based on sulfated CD has already been described in the literature, and they showed activity

against *Mycobacterium avium* and *Mycobacterium intracellulare* [26]. The literature has also described the successful inclusion of CFZ into negatively charged sulfobutyl ether β -cyclodextrin (SBE β CD) / epichlorohydrin oligomer to increase its water solubility and bioavailability [27]. Although the improved solubility results of the sulfated CDs are promising, the cost of these carriers are higher than the traditional (non-sulfated) CDs. Furthermore, in the previously reported studies powder products were not adjusted in terms of aerodynamic properties in order to optimize pulmonary drug delivery, since it was not the objective of these studies.

For these reasons, the aim of the present study was to prepare and undertake the physicochemical characterization of novel CFZ-CD and L-leucine-CFZ-CD complexes, developed to enhance CFZ aqueous solubility with a view to improving dissolution in lung mucus. Particle engineering *via spray drying* was performed to develop a dry powder formulation for the local delivery of CFZ to the lung.

2. Materials and Methods

2.1. Materials

Clofazimine (CFZ), (2-hydroxypropyl)- β -cyclodextrin (2-HP β CD), L-leucine and L-isoleucine were purchased from Sigma Aldrich (St Louis, USA). β -cyclodextrin (β CD) and γ -cyclodextrin (γ CD) were purchased from Wacker Chemie AG (Munich, Germany). Sulfobutyl ether β -cyclodextrin (SBE β CD) (Captisol®) was kindly supplied by CyDex Pharmaceuticals (Kansas, USA). The organic solvents (Analytical Degree, A.D.), ethanol, dimethylsulfoxide and acetonitrile, were purchased from TEDIA BRAZIL (Rio de Janeiro, Brazil). Purified water for preparing solutions and performing HPLC studies was obtained by water distillation treatment (Quimis, Diadema, Brazil).

2.2. Methods

2.2.1. Inclusion complex preparation

Complexation efficacy studies were performed by adding an excess amount of CFZ to water or ethanol:water mixtures (from 7:3 to 0:1, ethanol:water) containing increasing concentrations of a CD (β CD, γ CD, 2-HP β CD or SBE β CD), ranging from 1 to 10 mM (Table 1). Each system was stirred at 30 °C for 72 h, 40° C for 72 h, 50° C for 72 h or 30° C for 144 h, and subsequently filtered through a 0.45 μ m pore size filter

membrane (ChromPure syringe filter, PVDF, 25 mm diameter). CFZ concentration in solution was determined by HPLC with UV detection at 495 nm, using the methodology described below. Calculation of complexation efficacy (CE) was according to the following equation [28]:

$$CE = \text{Slope} / (1 - \text{slope}) \quad (1)$$

Where “slope” was the slope of the complexation profile (concentration of CFZ on the y-axis versus concentration of CD on the x-axis).

The molar proportion of CFZ:CD was determined using the following equation [28] :

$$CFZ:CD = 1CFZ : (CE + 1) / CE \quad (2)$$

Where CE was the complexation efficacy according to the complexation profile.

The CD inclusion process was then performed by mixing CFZ and the CDs (β CD, γ CD, 2HP β CD or SBE β CD) by the solution method, in purified water and ethanol:water (7:3) mixture, under agitation using the previously established ideal conditions/proportions. After cooling the mixture, it was filtered through a 0.45 μ m pore size filter membrane (ChromPure syringe filter, PVDF, 25 mm diameter), then ethanol was removed in a rotary evaporator Gehaka RD – 180 (Gehaka, São Paulo, Brazil) at 45 °C for 6 h and the sample was frozen using liquid nitrogen. The frozen sample was immediately submitted to lyophilization (as described below). The powder obtained was stored under a nitrogen atmosphere in a closed glass vial in a desiccator with silica gel.

Physical mixtures were prepared by pulverization and mixing CFZ and the CD (1:7 molar ratio) in a glass mortar.

2.2.2. Determination of CFZ content

The CFZ content in inclusion complex and permeation studies was determined by HPLC (Shimadzu, pump LC- 30AD, autosampler SIL-30AC, detector SPD-M 20A). A Perkin Elmer C18 column (150 x 4.6 mm, particle size 3 μ m) was used at 35 °C. The mobile phase was a mixture of methanol and phosphate buffer (80:20, v/v), and the flow rate was 1.0 ml/min. The elution was carried out isocratically and the injection volume was 20 μ l. The eluant was monitored at 284 nm. Retention time was 4 minutes.

2.2.3. Freeze drying process

Freeze drying was carried out using a laboratory scale freeze dryer Liotop L101 (Liobras, São Carlos, Brazil). Each sample was frozen using liquid nitrogen and then freeze-dried for 72 h to allow the complete removal of water. The resulting powders were characterized by differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD) and ¹³C-CP/MAS NMR spectroscopy. The freeze drying process was used only during preliminary drying evaluation studies; spray drying was used thereafter.

2.2.4. Spray drying process

Production of CFZ-CD inclusion complex or CFZ-CD inclusion complex/L-leucine or L-isoleucine complex spray dried powders was carried out using a laboratory scale spray dryer LabMaq MSD 1.0 (Labmaq do Brasil, Ribeirão Preto, Brazil) with a 0.7 mm nozzle tip. An ethanol-water solution (1:1) was prepared containing the CFZ-CD complex (1:7 molar ratio) and a total solute concentration of 0.70-0.85% (w/v), depending on the L-leucine/isoleucine concentration. The amino acids, L-leucine or L-isoleucine, and the organic solvent dichloromethane were added after the preparation of the CFZ-CD inclusion complex in ethanol-water. Amino acid concentration ranged from 10% to 15% (w/w) in relation to the total solid amount, and the dichloromethane concentration was 2% (v/v). Spray drying parameters are presented in Table 3. The dichloromethane was used as a co-solvent (and potential “blowing/pore-forming agent”) due to its low boiling point (39.8 °C) and fast evaporation rate, allowing particle size reduction and porosity increase [29–31]. Spray drying was used both to obtain the dry complexes and to adjust the properties of these complexes in order to prepare formulations that were suitable for pulmonary delivery. The resulting powders were characterized by DSC, XRPD, Fourier-transform infra-red spectroscopy (FTIR), dynamic vapour sorption (DVS), specific surface area analysis (SSA), particle sizing, morphology, ¹³C-CP/MA NMR spectroscopy and aerodynamic assessment using a Next Generation Impactor (NGI).

2.2.5. Powder characterization

A differential scanning calorimeter DSC-60 (Shimadzu, Kyoto, Japan) was used at a heating rate of 5 °C/min. Accurately weighed samples (1 - 2 mg) were placed into sealed aluminum pans and heated from 30 to 300 °C.

XRPD patterns of raw materials, physical mixtures and inclusion complexes were obtained using a Lab X XRD-6100 X-ray (Shimadzu, Kyoto, Japan) with a Cu K α line as the source of radiation. Measurements were carried out using a voltage of 40 kV, a current of 30 mA and a scanning rate of 2°/min over a range of 2°- 80°, on the two theta scale at a step size of 0.02°.

The infrared spectra of raw materials, physical mixtures and inclusion complexes were obtained from KBr pellets using a Prestige-21 spectrometer (Shimadzu, Kyoto, Japan). Spectra were recorded between 4000 and 600 cm⁻¹ with a resolution of 4 cm⁻¹.

¹³C solid-state NMR spectra were recorded on a Bruker Avance III 400 (9.4 T) spectrometer (Bruker, Massachusetts, USA), operating at 100.64 MHz. A CPMAS 3.2 mm probe head and ZrO₂ rotors spinning at 9.3 KHz were employed. Cross polarization magic angle spinning with ramp in the ¹H channel (CPMAS) combined with high power proton decoupling (HPDEC) were used as the pulse sequence. The contact time varied from 100 μ s to 10,000 μ s. The recycle delay was 4 s, and the $\pi/2$ pulse length was 4 μ s. The spectra were transformed after accumulation of 512 scans. The line broadening used to process the data was 20 Hz. Adamantane (CH at 29.46 ppm) was used as an external chemical shift reference.

Particle size distributions of prepared formulations for inhalation were determined using a Coulter Counter, Multisizer 4 (Beckman Coulter, California, USA). Each formulation was dispersed in an electrolyte solution (Isoton®) and measurement was performed using an aperture tube size of 0.4 – 16 μ m and current of 600 μ A.

Bulk density (bp) was calculated by determining the weight of powder required to occupy a 1 ml volume in a graduated glass syringe, by pouring under gravity. The tapped density (tp) was determined by vertically tapping this sample onto a level bench-top surface, from a height of 5 cm, until there was no observed volume change (100 times) [29]. The tapped density was calculated as the ratio of the mass to the tapped volume of the sample. Each measurement was performed in triplicate. The theoretical aerodynamic diameter (d_a) was calculated based on the following definition $d_a = \sqrt{tp/\rho_l \cdot d}$; where $\rho_l = 1\text{g/cm}^3$ and “d” is the geometric diameter of the particle [32].

The surface morphology of CFZ:CD complexes was observed using a scanning electron microscope (SEM), TM 3030 Plus (Hitachi, Tokyo, Japan). The SEM was operated under high vacuum, at 15 kV, small spot size, while images were acquired in

the backscattered electrons mode (BSE) with an acceleration voltage of 15 kV and a secondary detector. The powder was deposited on double-sided adhesive tape, vacuum-coated with gold and directly analyzed under SEM with magnifications of x500, x1000, x3000 and x5000.

The water vapour sorption-desorption isotherms of CFZ inclusion complexes were obtained by means of an automated gravimetric vapour sorption analyser, DVS Advantage-1 (Surface Measurements Systems). Samples were equilibrated at 0% relative humidity (RH) until a steady dry reference mass was recorded. The samples were exposed to the following RH profile: 0 - 90% in 10% steps and the reverse for desorption at $25.0 \pm 0.1^\circ\text{C}$. At each stage, the sample mass was allowed to reach equilibrium (i.e. weight change over 10 min $(\text{dm}/\text{dt}) \leq 0.002 \text{ mg}/\text{min}$).

The specific surface area was determined by the Brunauer, Emmett, Teller (BET) isotherm method using a Micromeritics Gemini VI (Micromeritics, Norcross, USA) surface area analyser. The amount of nitrogen adsorbed at 6 relative pressure points in the relative pressure (P/P_0) range of 0.05 to 0.3, with an equilibration time of 10 s, was used for the BET analysis. Each average result was calculated on the basis of three measurements.

2.2.6. *In vitro* aerosol deposition studies using the Next Generation Impactor (NGI)

The aerodynamic properties of the powders were assessed using an NGI (Copley Scientific Limited, Nottingham, UK), apparatus 5, operated under pharmacopeial conditions [8]. The flow rate was adjusted to achieve a pressure drop of 4 kPa in the powder inhaler, (Handihaler®, Boehringer Ingelheim, Ingelheim, Germany) and the time of aspiration was adjusted to obtain 4 L air flow. The pre-separator was used to remove coarse particles. The dry powder inhaler was loaded with a number 3 hard gelatin capsule loaded with $20 \pm 2 \text{ mg}$ of powder for each test. NGI stages (stages 1–7 and filter) were coated with 1 mL of HPLC mobile phase to wet the collection surfaces. The deposition profile of each formulation was determined in triplicate and the results presented are the average results of the replicated analyses. After dissolution in an appropriate volume of HPLC mobile phase, particle deposition in the device, the throat and all the stages and the filter was determined by the HPLC method described above. Each test was repeated three times. The emitted recovered dose (ED) was determined as the percent of total powder mass exiting the capsule and device. The total amount of particles with aerodynamic diameters smaller than 5 and $3 \mu\text{m}$ was calculated by

interpolation from the inverse of the standard normal cumulative mass distribution less than stated size cut-off against the natural logarithm of the cut-off diameter of the respective stages. This amount was considered as the fine particle fraction (FPF%) below 5 μm and 3 μm , and expressed as a percentage of the ED. The mass median aerodynamic diameter (MMAD) of the particles was determined from the same plot as the particle size corresponding to the 50% point of the cumulative distribution, and the geometric standard deviation (GSD) as $\text{GSD} = \sqrt{(\text{Size X}/\text{Size Y})}$, where Size X is the particle size corresponding to the 84% point and size Y is the particle size corresponding to the 16% point of the cumulative distribution.

2.2.7. Solubility studies

The solubility studies were determined using a shaker Innova 44 (New Brunswick Scientific, New Jersey, USA). It was performed by the 24 h shake flask method at 100 rpm and 37 °C (Wermuth, 2008; The Practice of Medicinal Chemistry, 3rd ed. Elsevier Ltd, p. 750). Therefore, an excess of pure CFZ, and formulations were individually added to 10 ml of pure water in glass tubes. After the appropriate time, the tubes were opened, and the supernatant withdrawn and filtered through 0.45 μm membrane filters (ChromPure syringe filter, PVDF, 25 mm diameter). CFZ concentrations in the supernatant were determined by HPLC as previously described.

2.2.8. Cytotoxicity studies

Exponentially growing human lung adenocarcinoma cells (Calu-3) were seeded at an initial density of 5×10^4 cells/mL in 96-well plates in DMEM medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin/streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere, for 24 h. After this time, the medium was removed and replaced with fresh medium containing either pure βCD (25 - 200 μM), pure CFZ, the physical mixture or the inclusion complex. All samples that contained CFZ ranged from 3.125 to 25 μM , in relation to CFZ concentration. The plate was incubated at 37 °C in a humidified, 5% CO_2 atmosphere. The highest concentration tested when CFZ was present was limited to the equivalent of pure CFZ solubility (25 μM). Cell viability was determined after 24 h by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide method (MTT) [33].

The absorbance was measured at 570 nm in a microplate reader, Perkin Elmer Victor X5 (Perkin Elmer, Massachusetts, USA).

A second approach to cytotoxicity testing was used to evaluate the effect of higher CFZ equivalent concentrations (50 and 100 μ M) for a shorter incubation time (4h). Results from this study were needed to support the permeability study conditions (detailed below), where initial low drug concentration (25 μ M) were found to result in levels falling outside the detection limit of the analytical method employed (HPLC) after permeation. This issue has previously been reported for poorly water soluble molecules in permeability testing [34].

2.2.9. Permeability studies

Calu-3 cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin/streptomycin. Cell monolayers were obtained by seeding 5.5×10^5 cells/cm² onto tissue culture inserts (Costar polyester filters, with a 1.12 cm² growth surface area and 0.4 μ m pore size) placed in twelve-well plates and maintained at 37 °C in a humidified, 5% CO₂ atmosphere. During the first seven days, cells were cultured under liquid-liquid interface and growth medium (0.5 ml on the apical side and 1.5 ml on the basal side) was replaced with fresh medium every 2-3 days. From the eighth to twelfth day, cells were cultured in an air-liquid interface (0.6 ml on the basal side)

The epithelial monolayer confluence was estimated by visual inspection under an optical microscope. The monolayer integrity was evaluated by transepithelial electrical resistance (TEER) measurements, using a Millicell® ERS-2 (Merck Millipore, Massachusetts, USA). TEER was calculated by subtracting the resistance of a cell-free culture insert and correcting for the surface area of the Transwell cell culture support. The increase in the values of TEER accompanied the growth of cells in each filter to reach a constant value of $> 700\Omega \text{ cm}^2$, indicating suitable cellular maturity for the test [35]. TEER was measured at the beginning, during and after the experiment, to check cell layer integrity. Transport experiments were evaluated in the apical to basolateral direction and were carried out in Hank's Balanced Salt Solution (HBSS). The apical side was filled with 0.5 ml of sample suspension (100 μ M, in relation to CFZ) and the basolateral side with 1.0 ml of HBSS. After 0.5 h, 1 h, 2 h, 3 h and 4 h all the basolateral side volume was collected and immediately replaced with fresh HBSS. At the last time point, the apical content was also collected. The cell monolayer was

washed with HBSS and disrupted using DMSO (200 μ l), after which this volume was collected. CFZ concentration in all collected samples was determined by HPLC as previously described.

2.2.10. Statistical analysis

Results are presented as mean $\pm$ standard deviation. Significant differences were evaluated using analysis of variance (ANOVA) with post-hoc test. All statistical analyses were performed with the software GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and Discussion

3.1. Inclusion complex preparation

The efficacy of CFZ inclusion complexes based on sulfated CD has previously been described, showing these complexes' activity against two different TB strains (*M. avium*/*M. intracellulare*) [26]. Thus, the use of CD to formulate a CFZ pulmonary delivery system was considered the first-choice option. This approach was reinforced by the reported successful inclusion of CFZ in negatively charged SBE β CD / epichlorohydrin oligomer to increase its water solubility and bioavailability [27].

The inclusion complexes between CFZ and the CDs (β CD, γ CD, 2-HP β CD and SBE β CD) were obtained using different conditions, described in Table 1.

The association efficiency between CFZ and each CD was verified after the inclusion process (Figure 1). It can be observed that β CD (ethanol:water, β CD_b condition) provided the highest percentage of drug inclusion (included drug mass*100/total drug mass). The highest affinity of CFZ for β CD compared to other CDs was an unexpected result, since other water insoluble drugs, such as curcumin, have shown higher affinities for more hydrophobic CDs, like RM β CD (randomly methylated β -cyclodextrin) and HP γ CD (hydroxypropyl- γ -cyclodextrin). Another interesting result was the low affinity observed for the negatively charged SBE β CD, when the least affinity observed for curcumin was for the cationic HTA β CD (hydroxytrimethylammoniumpropyl- β -cyclodextrin) [36].

3.2. Cyclodextrin Complexation studies

The complexation efficacy (CE) determined for the condition ethanol: water (named β CD_b, described in Table 1), which provided a higher percentage of recovered CFZ, was 0.1738, with the ideal CFZ: β CD molar ratio being 1:7. For comparative purposes, the CE and the molar ratio were also determined for the β CD_a (water, in Table 1) condition. Compared to the β CD_a, the β CD_b condition, as expected, showed greater CE and a molar ratio comprising 12-fold lower β CD, compared to condition β CD_a. Figure 2 shows the complexing profiles between CFZ and β CD under conditions a and b. Phase solubility curves are represented as dissolved drug concentration against the concentration of β CD used. The binding constant (K) for complexes formed under conditions β CD_a and β CD_b were calculated from the slope of the straight-line portion of the curve, according to the following equation:

$$K = \text{slope} / S_0(1 - \text{slope}) \quad (3)$$

Where S_0 was the solubility of the drug in the absence of β CD. The obtained K value for the complex formed under condition β CD_b (5.03 M^{-1}) was higher than that of the complex obtained under condition β CD_a (2.61 M^{-1}), therefore, indicating a more stable complex for condition β CD_b, using ethanol as co-solvent.

Despite the increased powder volume associated with a system comprised of drug and CD in a 1:7 ratio, (i.e. 1CFZ:7 β CD), the bulk of the formulation to be delivered is not of concern, since a reduction in the therapeutic concentration of the drug to be delivered (and the powder volume used, less than 100mg) is expected, due to an improvement in its bioavailability by means of pulmonary administration [13,22]

3.3. Characterization studies

The characterization studies of the complexes were performed on the dry powder obtained by lyophilization and spray drying, resulting from the complexation obtained under condition β CD_b (1CFZ:7 β CD). The inclusion of a molecule in the CD cavity generally promotes a change in the melting point, with a reduction in signal intensity or the disappearance of the fusion event signal in the DSC [37]. The thermograms of the raw materials (CFZ and β CD), physical mixture and the 1CFZ:7 β CD complex powders (dried by lyophilization and spray drying) are shown in Figure 3. The CFZ has a typical DSC curve of a crystalline substance, i.e. with a sharp and narrow melting event with a peak at approximately 223°C . β CD exhibits a large endothermic signal related to loss of

water, peaking at 81.5 °C. The physical mixture presents the signal corresponding to the loss of water from β CD, followed by the melting peak of CFZ with a reduced signal intensity. The thermograms of the products obtained by lyophilization and spray drying maintained the thermal event related to the loss of water from the CD, and show the disappearance of the drug melting peak, suggesting the formation of the inclusion complex in both cases (Figure 3). Thus, the use of the spray drying technique can be proposed for the formation of the complex, while optimizing the powder properties to obtain the desired pulmonary formulation.

At the same time, complexation of CFZ tends to promote changes in its diffractogram, seen as a signal shift or change in signal intensity when compared to isolated molecules or the physical mixture [37–39]. XRPD analysis of CFZ and β CD demonstrates typical crystalline substance diffraction patterns, corresponding to those deposited in the Cambridge Structural Database (CSD). The diffraction pattern of the CFZ raw material was typical of polymorphic form I of the drug (9.2, 13.4, 19.8 and 21.7° 2 θ), according to CSD reference (DAKUI01), Figure 4.

The diffractogram of spray-dried β CD (Figure 5C) showed powder amorphization after processing, with broad peaks (at 6.6°, 11.6° and 18.3° 2 θ) evident. In a previous study, Amaro et. al (2015), observed a similar diffraction pattern for spray-dried 2-HP β CD, with diffuse peaks in a similar position [40]. The diffraction pattern was reported to be associated with the molecular arrangement of the CD as a cup that can form small domains with some degree of short-range order. Despite this, the resulting powder was classified as amorphous [40]. The diffractogram of the physical mixture was similar to that of combined diffraction patterns of the individual raw materials (Figure 5). The physical mixture peak pattern (Table 2) indicated a simple mixture (lack of chemical interaction) as it was the sum of the diffraction peaks of the raw materials. On the other hand, for the inclusion complex a new peak (7.7° 2 θ), peak displacements (12-13.4° and 18.3-19.8° 2 θ) and peak absences (4.5°, 8.9-9.2°, 10.8°, 15.4° and 22.0° 2 θ) were observed (Table 2), indicating complex formation by means of CFZ- β CD interaction. The DSC result presented in the previous section, corroborates with the XRPD result pointing to inclusion complex formation. The diffractogram of the 1CFZ:7 β CD complex obtained by freeze drying exhibited broad peaks while spray drying appeared to promote a higher degree of amorphization, based on the XRPD patterns (Figure 5). The formation of completely or partially amorphous material can

represent complex formation, but it can also be a characteristic resulting from lyophilization and spray drying processes [15,41]. Therefore, XRPD results should be considered in association with results from thermal (DSC – described above) and spectroscopy analyses (discussed below) for the appropriate characterization of the inclusion complex [37].

The FTIR spectra of the dried complexes compared to the physical mixture and the raw materials, did not show any relevant alterations of the characteristic absorption peaks of CFZ, indicating only the presence of the drug, as shown in Figure 6.

Nuclear magnetic resonance (NMR) spectra can reveal more structural information of the analyzed material than the techniques used above, where the occurrence of a chemical shift (δ) in the carbon (^{13}C) signals reveals the formation of an inclusion complex [37]. From the ^{13}C -CP/MAS solid phase NMR spectra (Figure 7), the main signals (60.77, 72.89 and 102.89 ppm) of the βCD present in the physical mixture and the CFZ: βCD complex (1:7) were analyzed. The CFZ: βCD (1:7) complex shows, in relation to βCD , chemical shift variations of 0.37 ppm, 0.61 ppm and 1.22 ppm, whereas in the physical mixture the same displacements were lower and correspond to 0.35 ppm, 0.06 ppm and 0.21 ppm. The change in multiplicity plus the greater variation in chemical displacement were indicative of inclusion complex formation. The CFZ signals were not exploited due to its low concentration in the complex.

3.4. Spray drying for pulmonary delivery formulations

Spray dryer settings and formulation constituents are known to influence the final particle properties, such as size, density, morphology and moisture content, which in turn determine powder aerosolisation characteristics and site of lung deposition [1,42–44]. Different formulations and spray drying conditions were evaluated, as presented in Table 3. The geometric mean diameter (GD) and the polydispersity index obtained for the different formulations are shown in Figure 8. The polydispersity index (span value) was calculated from the equation: $(d_{90}-d_{10})/d_{50}$, where d_{90} , d_{50} and d_{10} correspond respectively to the geometric diameter of 90%, 50% and 10% of the distribution fraction (data not shown) [15,40,42].

Initial spray dryer settings were shown to be sub-optimal due to a high GD (11.59 μm , Figure 8). The high inlet temperature (95 $^{\circ}\text{C}$) and pump flow (0.55 L / h) used to generate the SP1 formulation (Table 3) probably caused this behavior, as

previously observed by Wang and Wang [45]. These authors indicated that the high temperature and flow caused, respectively, fast solvent evaporation and larger droplet size formation, and consequently a larger particle GD [45]. The inlet temperature was reduced to 65 °C, as previously described in a study to determine the influence of spray dryer conditions on the preparation of a beclomethasone: γ CD inclusion complex using the same co-solvent system as used in this work [44]. The lower inlet temperature and flow resulted in small but statistically insignificant decrease in GD for SP2 compared to SP1 (Figure 8).

Formulations SP3-9 tested the inclusion of L-leucine or L-isoleucine to evaluate the influence of these excipients on particle size. These formulations were also fine-tuned with respect to amino acid concentration, pump flow and co-solvent used (Table 3). A pump feed increase to 0.35 L/h for SP6, SP7 and SP8 formulations led to the lowest values of GD, corresponding to $5.73 \pm 1.20 \mu\text{m}$, $5.45 \pm 1.62 \mu\text{m}$ and $2.18 \pm 0.110 \mu\text{m}$, respectively, and narrowest size distribution, observed for SP8 (span=0.03). These GD values were promising with respect to the production of powder with appropriate aerodynamic characteristics for pulmonary delivery [7]. L-leucine addition led to a particle size reduction, while L-isoleucine (in SP5) did not. Likewise, the addition of 2% dichloromethane (SP9) did not result in a significant reduction in GD.

3.5. Particle morphology

The morphological evaluation of formulations SP6 to SP9 (Figure 9) revealed a near-spherical shape. Among the formulations spray dried from ethanol:water (1:1) and with the same spray drying conditions (SP6 – SP8), L-leucine incorporation led to the production of powders comprised of fewer agglomerates, with a more homogeneous size distribution and particle surfaces that were slightly corrugated, consistent with previous reports [14,15]. The lesser agglomeration and increased particle corrugation may favor powder dispersibility and the effective fraction of breathable powder ($<5 \mu\text{m}$) [46]. The SP9 formulation (CFZ: β CD + L-leucine 15%, dispersed in dichloromethane 2%, in addition to ethanol:water), differs considerably in its appearance from the other powders, since the particles appear to be cohesive, forming rod structures with smooth surfaces, i.e., without pores (unlike what may have been expected when using a blowing agent). A more heterogeneous particle size distribution (larger span) was measured for this system (Table 3).

3.6. Bulk density (bp), Tapped density (tp), Specific Surface Area (SSA) and Aerodynamic Characteristics

The bp, tp, SSA and the aerodynamic characteristics were measured for formulations with greatest potential for pulmonary delivery (SP6, SP7 and SP8), given their GD and morphology (Table 4). The bp and tp ranged between 0.113 and 0.265 g/cm³ and were considered suitable for pulmonary delivery, as previously reported by some authors (< 0.3g/cm³) [15,43,47]. The theoretical d_a calculated from tp and GD for SP6, SP7 and SP8 were 2.36 μ m, 2.28 μ m and 1.12 μ m respectively, further indicating suitability for pulmonary delivery (1 – 5 μ m) [9]. The L-leucine promoted an increase in bp and tp, related to the higher solid content and formation of dense particles, in agreement with previously reported data [15]. As expected, when compared to the other formulations, the presence of 15% L-leucine significantly increased the SSA ($P < 0.001$) due to the formation of rougher/corrugated/more textured particles (Figure 9-D) [1]. The roughness tends to reduce the interparticle contact, thus favoring powder dispersibility. The aerodynamic behavior of the formulation is influenced by the interrelationship of factors related to the particle (geometric diameter, morphology, surface area) and to the powder (bp, tp, hygroscopicity). The emitted recovered dose (ED) (i.e. total dose less the amount remaining in the capsule and device) for all evaluated formulations was similar, and high (94.13 to 95.19%). This result indicated adequate release from the capsule and the inhalation device and at the same time demonstrated good powder flow properties. The addition of L-leucine did not change the %ED (probably due to the fact that the %ED for the equivalent formulation without leucine was already high). Previous studies have commonly indicated an increased ED corresponding to the presence and increasing concentration of this amino acid [17,48]. However, here the fine particle fraction (FPF) of the selected formulations ranged from 46.20 to 51.56%, with no significant difference between different formulations S6, S7, S8. A comparison between MMADs (Table 4) and theoretical aerodynamic diameter values for SP6 – SP8 (given above) showed that $MMAD > d_a$ in all cases, suggesting that during aerosolisation, the powder remains aggregated rather than behaving as individual particles. Other researchers had already noted the same $MMAD > d_a$ relation, with an even larger difference between values [32,49]. However, despite the aggregates, approximately fifty percent of ED corresponded to FPF in all tested formulations. The MMAD of all formulations was less than 5 μ m and the geometric standard deviation (GSD) was less than 2. These were considered adequate values for pulmonary

formulations and delivery, since the combination of these parameters indicates a narrow particle size distribution in the FPF region [11,43].

3.7. *Dynamic vapour sorption (DVS)*

DVS analysis was performed to evaluate the behavior of SP6, SP7 and SP8 formulations when exposed to different moisture conditions (Figure 10). The adsorption isotherms of the formulations evaluated were similar and showed considerable incorporation of vapor (water) even at low relative humidities (RH). As expected, due to the amorphous characteristics of these powders, the three formulations exhibited considerable and continuous mass gain with increasing relative humidity (RH). Li *et. al.* (2016) demonstrated that water uptake by spray dried L-leucine alone was very low (<1%) at 90% RH, being classified as non-hygroscopic. Thus, the L-leucine contribution to water uptake when incorporated in a powder should be insignificant [50]. However, the presence of L-leucine made the water sorption both slower and overall lower and provided some protection to the powder against increasing relative humidity. When comparing the formulations containing L-leucine (SP7 and SP8) to that without (SP6), it was observed that, up to 80% RH, the weight gain due to water sorption was much lower for the L-leucine-containing formulations, but from 90% RH the difference in the sorption profiles was negligible. At the end of the sorption process, the mass increase was 23.22%, 22.03% and 19.78% for the formulations SP6, SP7 and SP8 respectively, demonstrating the anti-hygroscopic effect of L-leucine [48,50]. The absence of inflection in the adsorption isotherms indicated the absence of recrystallization for all formulations. The diffractograms obtained after the DVS runs (Figure 11) still demonstrated high degrees of amorphization. The broad peaks in the XRPDs can be attributed to the spray-dried β CD which was present in a large proportion in all formulations. The peak position displacements for the complexes 1CFZ: 7 β CD (SP6) (12.7° and 19.2° 2 θ), 1CFZ: 7 β CD + L-leucine 10% (SP7) (13.4° and 19.3° 2 θ) and 1CFZ: 7 β CD + L-leucine 15% (SP8) (6.2°, 13.6° and 19.1° 2 θ) when compared to spray-dried β CD (6.6°, 11.6° and 18.3° 2 θ - Figure 5C) can be explained by differences in powder composition, leading to slight variations in molecular interactions. In addition, the diffractogram of spray-dried L-leucine after the DVS analysis exhibited peaks at 2 θ of 6.2° and 19.1°, as previously reported [15]. The intensity of the peak ranging in position from 19.1° to 19.3° 2 θ reflected the L-leucine

content variation in formulations SP6-8 (Figure 11). For the desorption in the range of 80 - 10%, the presence of L-leucine slowed down the water loss. However, after the desorption process, all formulations returned to the initial mass levels, indicating a reversible water sorption process.

3.8. *In vitro* aerosol deposition studies using the Next Generation Impactor (NGI)

The deposition profiles of SP6, SP7 and SP8 formulations show that 58-62% of emitted dose (ED) was able to cross the mouthpiece adapter/induction port (MA/IC), which *in vivo* would correspond to the oro-pharyngeal region and indicates suitability for deposition in the respiratory tract (Figure 12). Particle deposition on MA/IC can be explained by agglomerate formation as a consequence of powder hygroscopicity, due to its amorphous state. Regarding the NGI stages, the particle deposition of the evaluated formulations at a 100 L/min flow rate was concentrated in stages 2, 3 and 4, which have cut-off diameters of 3.42 μ m, 2.18 μ m and 1.31 μ m respectively [8]. Stage 2 was the only one that exhibited significant deposition differences ($P < 0.001$) between formulations SP6 (without L-leucine) and SP8 (L-leucine 15%). The higher SP6 deposition was in line with its larger MMAD (3.38 $\pm$ 0.19 μ m for SP6 compared to 2.98 $\pm$ 0.08 μ m for SP8) and the stage cut-off diameter (3.42 μ m). The SP8 formulation presented lower deposition at this stage, which is an advantage and can enable better pulmonary retention. The percentage of particle deposition in the stages corresponding to the lower respiratory tract (stages 3 – 7) was 30-40%, with, perhaps surprisingly, no significant difference between formulations with or without L-leucine, in spite of significant difference in SSA ($P < 0.001$) and MMAD ($P < 0.05$) for SP8 (with 15% L-leucine) compared to other formulations.

When considering exclusively the aerodynamic powder characteristics, the formulations SP6 – SP8 all showed appropriate parameters for use in a DPI. Efficacy tests should be performed in the future to confirm this hypothesis.

3.9. *Solubility studies*

The aqueous solubility of SP6, SP7 and SP8 formulations were evaluated (Figure 13). When compared to the free drug, formation of the CFZ: β CD complex (1:7) increased the aqueous solubility of CFZ by sixteen-fold ($P < 0.001$) and incorporation of 10% L-leucine, promoted an even greater increase (almost twenty seven-fold). A further increase in L-leucine content from 10% to 15% did not modify the water solubility of

the complex ($P>0.05$). The CFZ solubility improvement when complexed with β CD may be explained not only by their association, but also by the formation of amorphous particles during the spray drying process [1,25]. On the other hand the L-leucine effect on solubility is likely to be due to its surfactant-like properties [17], which has already been observed for azithromycin co-spray-dried particles [51]. The higher solubility for L-leucine containing formulations improves their therapeutic potential, since they may be better able to overcome the mucus barrier, present prolonged lung retention time and higher CFZ bioavailability.

3.10. Cytotoxicity studies

The *in vitro* cytotoxicity of free CFZ and β CD, and the CFZ: β CD complex, Figure 14, was evaluated after 24 h exposure in a Calu-3 cell line, due to its representativeness of the constituents of the respiratory tract [52]. The cytotoxicity assay was performed not only to identify the cytotoxicity of the raw material itself, but also to identify if the complexation could reduce this. The β CD complex revealed $>90\%$ cell viability even when exposed to the highest equivalent CFZ concentration (25 μ M). This value was not significantly different from that observed for CFZ or a CFZ-CD physical mixture. The β CD toxicity at Calu-3 cells (Figure 14B) revealed that it was dependent on β CD concentration. Cell viability for a β CD concentration $\leq 50\mu$ M was higher than 90%, while it was higher than 70% for a concentration of 200 μ M. These results indicated a high tolerability of Calu-3 cells for the CFZ: β CD complex, enabling its lung administration. The international guide ISO 10993-5, states that cell viability $>70\%$ is not considered a cytotoxic effect [53]. The cytotoxicity test based on the cell viability assay condition to inform the permeability study used CFZ equivalent concentrations of 50 – 100 μ M incubated for 4h. Cell viability results were: (i) for the free CFZ: $78.2 \pm 9.9\%$ (50 μ M) and $77.0 \pm 6.1\%$ (100 μ M); and (ii) for SP8 formulation (selected for permeability study, as justified in the next section): $75.1 \pm 11.34\%$ (50 μ M) and $72.5 \pm 3.9\%$ (100 μ M). Then, in terms of cytotoxicity, these results demonstrated the suitability of both concentrations for permeability studies and 100 μ M was the concentration selected for this study.

3.11. Permeability studies

As described in the literature, the *in vitro* permeability study is a good indicator

for drug delivery in the respiratory tract [54]. Because of its lower MMAD (2.98 μm), higher FPF (51.6%), promising deposition profile, improved water solubility and specially low hygroscopicity compared to other tested formulations, SP8 was selected for this evaluation. The formulation was added as a suspension to the surface of the Calu-3 monolayer, as reported in other studies [55]. The maintenance of the integrity of the monolayer was confirmed by the stability of TEER values, which was verified during and after the experiment (Figure S1). After 4 h, there was no CFZ detected in the basolateral compartment for wells containing the free drug or for SP8. The quantification of intracellular CFZ was similar for the free drug ($44.4 \pm 14.0\%$) and for SP8 ($43.7 \pm 4.6\%$), indicating that both exhibit the same penetrating capacity in the monolayer and availability for local action in the lungs. This result points to the possibility of obtaining a high drug concentration directly at the site of pulmonary infection and thus a more effective therapy, while minimizing systemic CFZ biodistribution and toxicity. Hence, reducing systemic side effects like reddish-brown discoloration of the skin and conjunctiva [22], a relevant issue at TB prolonged treatment regime (minimum 6 months) [2,10]. At the same time, pulmonary delivery of CFZ microparticles can be an interesting strategy to improve mycobacteria death by favoring two inherent mechanisms: (i) CFZ distribution to the mononuclear phagocyte system, and (ii) the macrophages uptake of microparticles [10,56]. In addition, the CFZ inside macrophages also can act as a drug reservoir, prolonging the residence time at the lungs, reaching granulomas and supporting the TB latent cases treatment [10].

3.12. Pulmonary dose calculation

The CFZ WHO treatment regimen for TB has established a daily oral dose of 100 mg [57], this drug also has an oral bioavailability of 45-62% [22]. The calculation of the inhaled estimated dose (4.5 mg/day) considered that the pulmonary delivery drug amount usually is about ten-times lower compared to oral one [43,58,59]. Considering that the best formulation (SP8) exhibited a drug:powder ratio of about 1:20 (w/w), the total powder mass amount was of 89.3 mg/day. This mass is smaller than those of the marketed tobramycin and colistimethate dry powder inhaled formulation, and like the examples can be used in multiple doses or inhalations [60–62].

4. Conclusions

In the present study, CFZ:CD complexes were studied with a view to developing pulmonary delivery systems for TB treatment. We produced inhalable microparticles of CFZ:CD by spray drying to facilitate the particle formation process, with water soluble CD complex formation, able to overcome the mucus barrier and prolong the lung retention time. The 1CFZ:7CD complex improved the CFZ water solubility and L-leucine incorporation promoted an even greater solubility. Inhalable microparticles were produced under optimized spray drying conditions of: 65 °C inlet temperature, 0.35 L/h pump rate, and 35 L/min gas flow rate. DPI formulations with or without L-leucine were developed, comprising suitable aerodynamic properties and improved CFZ water solubility. The addition of 15% L-leucine had the advantage of reducing powder hygroscopicity while also enhancing *in vitro* powder deposition in the lower airways. *In vitro* studies showed a viability of at least 70% for Calu-3 cells after 24 hours of exposure to CFZ and β CD (high concentrations) and permeability testing indicated that the drug was retained at the apical compartment and achieved cellular internalization. The inhaled estimated dose was 4.5 mg in a powder mass of 89.3 mg/day, allowing dry powder device use.

In conclusion, the current work presented dry powder formulations for inhalation of cyclodextrin complex representing a potential new local treatment for lung tuberculosis infection and should be tested *in vivo* in the near future.

Data availability: The dataset analyzed during the current study is available from the corresponding author on reasonable request. Data generated during this study are included in this published article.

Declaration of interest: None.

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Table 1. Complexation conditions of CFZ and CD.

Cyclodextrin condition	Solvent	Molar proportion CFZ:CD (mM)	Temperature (°C)	Time (h)
β CD_a	Water	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
β CD_b	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
γ CD_a	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
2-HP β CD_a	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
2-HP β CD_b	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	40	72
2-HP β CD_c	Ethanol:Water (7:3)	1:1 / 1:5 / 1:10	50	72
2-HP β CD_d	Ethanol:Water (4:6)	1:1 / 1:5 / 1:10	40	72
2-HP β CD_e	Ethanol:Water (2:8)	1:1 / 1:5 / 1:10	40	72
2-HP β CD_f	Ethanol:Water (1:9)	1:1 / 1:5 / 1:10	40	72
SBE β CD_a	Ethanol:Water (1:1)	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	30	144
SBE β CD_b	Water	1:1 / 1:2 / 1:4 / 1:6 / 1:8 / 1:10	30	72

The solution method was used for all complexation conditions.

Table 2. The characteristic peaks of CFZ, CDs, physical mixture and their complexes in the XRPD diffractograms.

	Main peaks 2θ (degrees)							
1CFZ:7βCD Spray dried	---	---	---	---	13.1	---	18.8	---
1CFZ:7βCD Freeze dried	---	7.7	---	---	12.3	---	18.3	---
Physical mixture	4.4	---	8.9	10.5	12.4	15.3	19.5	22.7
βCD	4.5	---	8.9	10.8	12.5	15.4	19.6	---
Clofazimine	---	---	9.2	---	13.4	---	19.8	22.0

Table 3. Clofazimine powder formulations and spray dryer settings.

Formulation	Solid constituents	Liquid constituents	Spray dryer settings			
			Inlet temperature (°C)	Outlet temperature (°C)	Pump flow (L/h)	Gas flow (L/min)
SP1	CFZ:βCD (1:7)	Ethanol:Water (1:1)	95	45 – 48	0.55	35
SP2	CFZ:βCD (1:7)	Ethanol:Water (1:1)	65	41 – 43	0.30	35
SP3	CFZ:βCD (1:7) L-leucine 10%	Ethanol:Water (1:1)	65	41 – 43	0.30	35
SP4	CFZ:βCD (1:7) L-leucine 15%	Ethanol:Water (1:1)	65	37 – 42	0.30	35
SP5	CFZ:βCD (1:7) Isoleucine 15%	Ethanol:Water (1:1)	65	38 – 41	0.30	35
SP6	CFZ:βCD (1:7)	Ethanol:Water (1:1)	65	44 – 47	0.35	35
SP7	CFZ:βCD (1:7) L-leucine 10%	Ethanol:Water (1:1)	65	44 – 47	0.35	35
SP8	CFZ:βCD (1:7) L-leucine 15%	Ethanol:Water (1:1)	65	44 – 47	0.35	35
SP9	CFZ:βCD (1:7) L-leucine 15%	Ethanol:Water (1:1) Dichloromethane 2%	65	34 – 46	0.35	35

Table 4. Bulk (bp) and tapped density (tp), specific surface area (SSA) and aerodynamic characteristics of L-leucine free (SP6) and L-leucine spray dried formulations (SP7 – SP8).

	Aerodynamics characteristics						
	Bulked density (g/cm ³)	Tapped density (g/cm ³)	Specific surface area (m ² /g)	Emitted dose (%)	FPF < 5µm (%)	MMAD (µm)	GSD
SP6	0.113 ± 0.005 ^a	0.170 ± 0.003 ^c	3.504 ± 0.075 ^{e,f}	95.19 ± 1.83	48.41 ± 2.79	3.38 ± 0.19 ^h	1.65 ± 0.12
SP7	0.128 ± 0.002 ^b	0.176 ± 0.010 ^d	2.766 ± 0.054 ^{e,g}	95.02 ± 1.39	46.20 ± 6.81	3.06 ± 0.09	1.82 ± 0.13
SP8	0.185 ± 0.004 ^{a,b}	0.265 ± 0.006 ^{c,d}	6.104 ± 0.285 ^{f,g}	94.13 ± 1.52	51.56 ± 5.16	2.98 ± 0.08 ^h	1.80 ± 0.11

FPF: fine particle fraction. MMAD: mass median aerodynamic diameter. GSD: geometric standard deviation. Results were presented as mean ± SD (n = 3). Difference significance: ^{a,b,c,d,f,g} denote P<0.001; ^e denotes P<0.01 and ^h denotes P<0.05 (one-way ANOVA, with a post-hoc test).

Figure captions:

Figure 1. Percentage of total mass of clofazimine recovered after complexation process. Each condition is described in Table 1. Results are presented as mean $\pm$ SD (n = 3).

Figure 2. Clofazimine's phase solubility profile determined under different conditions (A) Condition β CD_a in Table 1: excess amount of CFZ was added to aqueous solutions containing increasing concentrations of β CD (1-10 mM) and were stirred at 40 °C for 72 h, and subsequently filtered through a 0.45 μ m membrane. (B) Condition β CD_b in Table 1: excess amount of CFZ was added to ethanol:water (1:1) solutions containing increasing concentrations of β CD (1-10mM) and were stirred at 40 °C for 72 h, and subsequently filtered through a 0.45 μ m membrane. "CE" was the complexation efficacy. Results are presented as mean $\pm$ SD (n=3).

Figure 3. DSC curves of CFZ systems and β CD: (A) CFZ raw material; (B) β CD raw material; (C) physical mixture of CFZ and β CD raw materials; (D) inclusion complex, freeze-dried; (E) inclusion complex, spray-dried.

Figure 4. XRPD diffractograms of CFZ from CSD (polymorph form I, DAKUI01) and CFZ raw material.

Figure 5. XRPD diffractograms of CFZ and β CD systems: (A) CFZ raw material; (B) β CD raw material; (C) spray-dried β CD; (D) physical mixture of CFZ and β CD raw materials; (E) inclusion complex, freeze-dried; (F) inclusion complex, spray-dried.

Figure 6. FTIR spectra of CFZ and β CD systems: (A) CFZ raw material; (B) β CD raw material; (C) physical mixture of CFZ and β CD raw materials; (D) inclusion complex, spray-dried. The dotted lines (---) and the circle indicates the main vibrational bands of CFZ (aromatic C-H stretching and C=N stretching).

Figure 7. ^{13}C -CP/MAS NMR spectra of CFZ systems and β CD: (A) CFZ raw material; (B) β CD raw material; (C) physical mixture of CFZ and β CD raw materials; (D) inclusion complex, freeze-dried.

Figure 8. Geometric mean diameter and polydispersity index (span) of raw material (CFZ and β CD) and spray dried formulations (SP). The SP formulation constituents and spray dryer settings are provided in Table 3. Results are presented as mean $\pm$ SD (n = 3). Difference significance: * P<0.05 and *** P<0.001 (one-way ANOVA, with a post-hoc test).

Figure 9. Scanning electron micrographs of physical mixture of CFZ and β CD and spray dried formulations. (A) physical mixture of CFZ and β CD raw materials; (B) SP6; (C) SP7; (D) SP8 and (E) SP9.

Figure 10. Water vapour isotherms (sorption and desorption). (a) SP6 – CFZ: β CD (1:7); (b) SP7 - CFZ: β CD (1:7) + L-leucine 10% and (c) SP8 - CFZ: β CD (1:7) + L-leucine 15%

Figure 11. XRPD profile after DVS analyses. (A) SP6 – CFZ: β CD (1:7); (B) SP7 - CFZ: β CD (1:7) + L-leucine 10% and (C) SP8 - CFZ: β CD (1:7) + L-leucine 15%.

Figure 12. *In vitro* aerosol deposition profile by use of NGI for spray dried formulations as calculated % of recovered emitted dose vs. NGI stages. (A) SP6 – CFZ: β CD (1:7); (B) SP7 - CFZ: β CD (1:7) + L-leucine 10% and (C) SP8 - CFZ: β CD (1:7) + L-leucine 15%. MA: mouthpiece adapter / IP: induction port. MOC: micro-orifice collector. Results are presented as mean $\pm$ SD (n = 3). Difference significance: *** P<0.001 (two-way ANOVA, with a post-hoc test).

Figure 13. Water solubility of clofazimine as free drug and formulations SP6 – CFZ: β CD (1:7); SP7 - CFZ: β CD (1:7) + L-leucine 10% and SP8 - CFZ: β CD (1:7) + L-leucine 15%. The water pH = 6.5. n.d: not detected. Results are presented as mean $\pm$ SD (n = 3). Difference significance: ** P<0.01 and *** P<0.001 (two-way ANOVA, with a post-hoc test).

Figure 14. (A) Viability of Calu-3 grown as cell layer after 24h exposure to 3.125 - 25 μ M of equivalent clofazimine. CFZ: Clofazimine raw material. CFZ: β CD (1:7) complex. PM: physical mixture of CFZ and β CD raw materials. (B) Viability of Calu-3 grown as cell layer after 24h exposure to 25 – 200 μ M β CD. Both experiments were

measured by MTT test (mean $\pm$ SD, n = 3). The dotted lines mark 70% and 90% viability. Difference significance: ** P<0.01 and *** P<0.001 (two-way ANOVA, with a post-hoc test).

Supplementary material:

Figure S1. TEER values of Calu-3 culture during the permeability study. Control: no treatment; CFZ: Clofazimine raw material; SP8: CFZ: β CD (1:7) + L-leucine 15%. The measurements were at the sampling time points.

Conflict of Interest

Authors have no conflicts of interest to disclose.

Credit Author Statement:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Renata Ribeiro de Castro, Valério Todaro and Alice Simon. Bruno Sarmento, Anne Marie Healy and Lucio Mendes Cabral supervised the preparation and characterization of formulations. Luiz Claudio Rodrigues Pereira da Silva supervised the in vitro studies using Calu-3. The first draft of the manuscript was written by Renata Ribeiro de Castro and Flávia Almada do Carmo. The manuscript was revised and edited by Renata Ribeiro de Castro, Flávia Almada do Carmo, Valeria Pereira de Sousa, Carlos Rangel Rodrigues, Anne Marie Healy and Lucio Mendes Cabral. Valeria Pereira de Sousa, Carlos Rangel Rodrigues, Anne Marie Healy and Lucio Mendes Cabral were responsible for financial support. All authors approved the submitted and final version.