Inhaled Liposomal Ciprofloxacin Nanoparticles Control the Release of Antibiotic at the Bronchial Epithelia

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INTRODUCTION

The cycle of respiratory tract infection (RTI) and inflammation in patients with chronic obstructive lung diseases, such as cystic fibrosis (CF), periodically develops into exacerbations, where chronic colonization of the airway by bacteria causes severe decline in lung function, leading to increased hospitalization and high mortality rates (1, 2). Current antibiotic inhalation treatments approved for the management of chronic airway infections in cystic fibrosis are limited to tobramycin (TOBI®) and more recently, aztreonam (Cayston®). A major drawback to these localized treatments of RTIs is the rapid absorption and clearance of antibiotics from the lungs requiring multiple daily inhalations of high concentration antibiotic solutions. Hence, liposomal ciprofloxacin nanoparticles were developed to prolong lung residence time of the antibiotics, with the view to enhance antimicrobial activity and reduce the burden of therapy for the patients and their relatives who often have to assist them. Although *in vivo* studies with aerosolized delivery of liposomal ciprofloxacin have previously been performed on human and animal subjects, *in vitro* cell models may be better suited to study the transport, interactions of drugs and carrier systems, and drug localization within and on the airway cell epithelium at a molecular level. Therefore, the aim of this study was to investigate the newly developed system allowing nebulized liposomal ciprofloxacin to be delivered directly to the bronchial epithelial surface in an established air interface Calu-3 cell model.

MATERIALS AND METHODS

Two nebulized ciprofloxacin formulations: Liposomal ciprofloxacin (CFI) and free ciprofloxacin (FCI) aqueous systems were analysed in terms of their release profiles and antimicrobial efficacy, using *in vitro* methodologies. The formulations were prepared as previously described (3). Briefly, the CFI was prepared by actively loading ciprofloxacin into ~90 nm unilamellar vesicles via a transmembrane pH gradient generated in response to an ammonium sulphate gradient.

Calu-3, a bronchial epithelial cell line, was seeded at a density of 5 X 10^5 cells/cm^2 on Transwell polyester inserts (Corning Costar, USA). The cells were grown at the air-interface to allow for monolayer differentiation and cell polarization. Experiments were performed between day 11 and 14 in culture (4). The formulations were aerosolized, using a Pari LC Sprint® nebulizer, directly onto the Calu-3 bronchial epithelia cells placed in Stage 2 of an *in vitro* twin-stage impinger (TSI; Copley Scientific, Nottingham, UK) to assess the release kinetics. Two milliliters of diluted CFI and FCI was introduced into the reservoir of the nebulizer and was aerosolized for 5 seconds at a flow rate of 15 L/min, allowing <20 μg of ciprofloxacin to be deposited onto the Calu-3 cells. Samples were taken at pre-determined time points for up to 4 hours post treatment and cells were washed gently with Hank's Balanced Salt Solution (HBSS) (Invitrogen, Australia) at the final time point to collect the remaining drug on the cell monolayer. Cells were subsequently lysed to release and quantify intracellular drug. Samples were analyzed using high-performance liquid chromatography (HPLC). Confocal live cell imaging (LSM 510 Microscope Meta, Carl Zeiss, Australia) was also performed after deposition of the formulation on the Calu-3 cells with 10X objective and UV laser (405nm) at various time points.

The antibacterial activities of the formulations were compared by determining the minimum bactericidal concentrations (MBC) on *Pseudomonas aeruginosa* (NCTC7244). Prior to MBC determination, the minimum inhibitory concentration (MIC) was established using a standard twofold serial dilution method with cation-adjusted Mueller-Hinton broth (CAMHB), according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (5). The drug concentration used ranged from 32 to 0.153 μg/mL. The MBC was subsequently determined by sampling 100 μL from each MIC broth dilution that lacked visible growth, and inoculating on blood agar which was finally incubated at 37°C for 24 hours. The bactericidal endpoint for MBC was defined as the drug concentration that demonstrated a reduction of ≥ 99.9% of the initial inoculum.

RESULTS AND DISCUSSION

The release kinetics of CFI and FCI formulations are shown in Figure 1. CFI effectively controlled the release of ciprofloxacin, with approximately 1.5% of the drug being transported across the epithelial over 4 hours. In comparison, the FCI formulation showed >33% of the drug transported over the same time period. No effects were observed on the epithelial cell integrity following exposure to either formulations or control, as there was no statistical differences in transepithelial electrical resistance observed.

The quantitative analysis was further confirmed by confocal imaging, where CFI showed slow diffusional spreading of the deposited aerosols into the surrounding epithelial lining fluid after 4 hours. In comparison, the FCI diffuses and spreads rapidly into the epithelial lining fluid giving a relatively even antibiotic distribution of antibiotics within 30 minutes. The FCI was hardly detected on the cell surface after 1.5 hours (images not shown). These results are in qualitative agreement with animal and human pharmacokinetic data; in healthy subjects and in patients, the terminal plasma half-life of ciprofloxacin inhaled in the form of CFI was ~10 hours which is much

longer than the terminal plasma half-life following IV or oral administration of ciprofloxacin or inhalation of unencapsulated ciprofloxacin (3, 6, 7). Thus, the different transport kinetics, distribution and interaction with the lung epithelium of the ciprofloxacin formulations were observed in the air-interfaced Calu-3 cell and impactor system providing a qualitatively representative assessment of release and transport kinetics with respect to *in vivo* conditions.

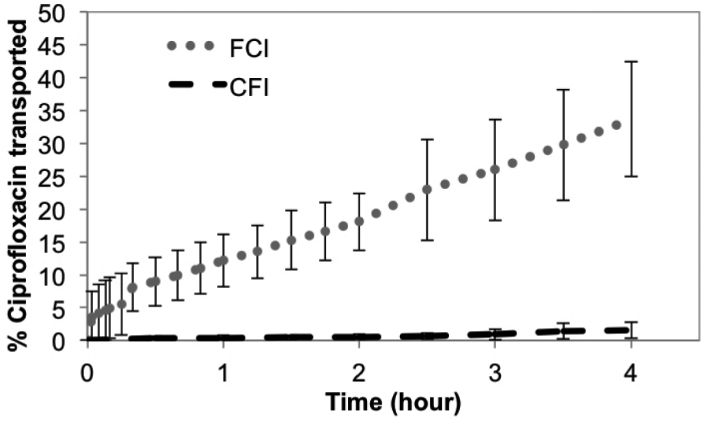

Figure 1. Release profile of nebulized liposomal ciprofloxacin (CFI) (n=6, mean ±S.D, SD<1.5%) and free ciprofloxacin (FCI) (n=6, mean ±SD) formulations on Calu-3 air interface model.

The *in vitro* antimicrobial activities of the ciprofloxacin formulations on laboratory strain *P. aeruginosa* are shown in Table 1. The MBC value for CFI was found to be significantly lower compared to the FCI. It is hypothesized that the enhanced antimicrobial activity against *P. aeruginosa* is due to the fusion of the phospholipids of liposomes with the bacterial outer membrane (8). In addition, the increased retention time of ciprofloxacin in the lungs would further enhance the antimicrobial efficacy of the formulation *in vivo*.

Table 1.

***In vitro* antimicrobial activity of nebulized liposomal ciprofloxacin (CFI) and free ciprofloxacin (FCI) against *Pseudomonas aeruginosa*.**

Formulation	Free ciprofloxacin (FCI)	Liposomal ciprofloxacin (CFI)
Minimum bactericidal concentration (μg/mL)	4	2

CONCLUSIONS

Inhaled formulations of liposomal ciprofloxacin are a promising treatment for pulmonary infections. The use of the combined TSI and Calu-3 air-interface model for the assessment of transport rate after drug deposition from nebulizer could be an attractive model to potentially reduce much animal and human experimentation. The combined TSI and Calu-3 air-interface model requires further refinements to more closely approximate *in vivo* transport rates.

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