

## **Abstract**

The purpose of this study was to present a novel and simple drug deposition method to evaluate drug transport of aerosol microparticles across airway epithelial cells. Microparticles containing ciprofloxacin HCl (Cip) and doxycycline (Dox), alone or in a 50:50% w/w ratio, were spray dried and suspended using 2H, 3H-perfluoropentane, model propellant. The suspension was then used to assess deposition and transport of these drug microparticles across sub-bronchial epithelial Calu-3 cells were also studied. In comparison with other methods of depositing microparticles, this proposed method, using drug suspended in HPFP, provides control over the amount of drugs applied on the surface of the cells. Therefore, cell permeability studies could be conducted with considerably smaller and more reproducible doses, without the physico-chemical characteristics of the drugs being compromised or the use of modified pharmacopeia impactors. The suspension of microparticles in HPFP as presented in this study, has provided a non-toxic, simple and reproducible novel method to deliver and study the permeability of specific quantity of drugs across respiratory epithelial cells *in vitro*.

## Introduction

Over the last decade, the pulmonary route of drug administration has received significant attention by scientists and the pharmaceutical industry. Subsequently, there has been a large number of medicinal aerosol formulations commercialised, with many new formulations and molecules still in the development phase. This interest has resulted in a trend towards the development of improved *in vitro* methods and models that allow the investigation of the respiratory epithelial permeability to inhalable drug formulations (1-6).

Understanding the epithelial permeability of an aerosol formulation is important for predicting therapeutic effectiveness and pharmacokinetics of the active drug. For a short acting  $\beta_2$ -agonist, such as salbutamol sulphate, fast transport across the epithelial barrier resulting in the delivery of the drug to the site of action ( $\beta_2$ -agonist receptors being present on the underlying smooth muscle) is desirable, while, for instance, for an antibiotic, used to treat localized respiratory infection, such as tobramycin, increased residence time in the airways may be more favorable (to ensure sustained minimum inhibitory concentrations).

Several approaches have been developed to study the permeability of aerosol particles *in vitro*. Until the last decade, most approaches utilized liquid covered epithelia models and studied the permeability of either solutions or suspensions of drug in media, transported from the apical to basolateral compartments of Transwell based cell cultures (7-10). However, the major drawback of this method is that the solid-state properties of the formulation are not taken into consideration as parameters affecting cell permeability (i.e. dissolution of the drug in the epithelia surface fluid

and interaction of this drug with the epithelia membrane). Furthermore, when these experiments are conducted in a liquid covered culture, epithelial permeability and surface structure may differ from an air interface culture, more representative of the physiological conditions.

For these reasons there has been a change from liquid covered cultures to air interface cell culture (7, 11) methods, and the application of cascade impactors to study the deposition and transport of 'real' particles across these epithelia (1, 3, 5, 6). Several studies have modified current Pharmacopeia apparatuses, such as the twin-stage impinger (12), Andersen cascade impactor (1), multi-stage liquid impinger (3) and the next generation impactor (6) to achieve these goals.

While integrated cell-based cascade impactor methodology is useful for determining the transport of deposited aerosol particles, all these methods are limited with respect to controlling the initial amount of drug deposited on the cells. One must be careful not to overload the cells (i.e. deposit multilayers of drug). Furthermore, controlling the initial deposited amount of drug require extensive preliminary formulation-specific studies and in some cases may require altering the formulation to reduce the amount deposited, making dose ranging studies particularly difficult.

To overcome this limitation, a complementary approach is required that allows the delivery of very specific amounts of aerosol particles (i.e. 0.01-10  $\mu\text{g}$  drug) to air interface cell cultures, without compromising the physico-chemical properties of the formulation. One approach to achieve this would be to deposit the formulation as a suspension in a non-aqueous (hydrophobic and volatile) media that has a low surface

tension and low boiling point. After addition, the media would rapidly evaporate leaving the drug particles to interact with the surface lining fluid and epithelia cells; transport studies would then be performed. 2H,3H-decafluoropentane (HPFP) is liquid at ambient pressure, with a boiling point of 53.6°C and surface tension of 13.59 mNm<sup>-1</sup>. This liquid has been proposed as a model propellant substitute for current hydrofluoro alkane (HFA) propellants used in metered dose inhalers (13). Thus, it is proposed that HPFP may be used as suitable media for the deposition and study of drug transport across air interface epithelia models.

In a previous study by Adi *et al.* (14), a combination of spray-dried microparticles of doxycycline (Dox) and ciprofloxacin (Cip) for inhalation, have shown potential in treatment of lung diseases which require antibiotic therapy, such as cystic fibrosis. The aim of this study was to deliver a suspension of aerosol microparticles (Dox and Cip) using HPFP as delivery vehicle to study the epithelial permeability of specific amounts of microparticles (between 1 and 3 µg of each antibiotic) across Calu-3 bronchial cells.

## **Materials and Methods**

### **Materials**

Microparticles containing ciprofloxacin HCl (Cip) (MP; Biomedical Australasia Pty Limited, Sydney, Australia) and doxycycline (Dox) (MP; Biomedical Australasia Pty Limited, Sydney, Australia) alone or in a 50:50% w/w ratio, were spray dried using a Büchi B-290 spray dryer (Büchi Mini Spray Dryer B-191, Flawil, Switzerland) using MilliQ water as solvent, equipped with a twin-fluid, coaxial atomizer under the following conditions: inlet temperature 110°C, measured outlet temperature 58°C, solution feed rate 4 mL.min<sup>-1</sup>, aspirator rate 100% and atomizing airflow 700 L.h<sup>-1</sup> (15). Dry powder yields were between 60 and 70%.

### **Particle size measurements**

Particle size measurements of the microparticles were conducted using a Malvern Mastersizer 3000 with Scirocco dry dispersion unit (Malvern Instruments Ltd., Malvern, UK). A feed pressure of 4 bar and feed rate of 50% was used and samples were measured when obscuration values fell between 0.5% and 5%. All samples were analysed in triplicate over 10 s intervals.

### **Solvent purification**

2H, 3H-perfluoropentane (HPFP; Apollo Scientific, Cheshire, UK) was purified with chromatographic grade acidic and basic aluminum oxide (Fluka, Gillingham, UK) to remove impurities according to the method described by Rogueda (13).

### **Cell biology assays**

Calu-3 cells (ATCC, Rockville, USA) were cultured and propagated according ATCC recommendations as described previously (7). Cells were seeded onto polyester Transwell inserts (0.33 cm<sup>2</sup>, 0.4 µm pore size) (Corning Costar, MA, USA) at a density of 5×10<sup>5</sup> cells.ml<sup>-1</sup> to study the uptake of the Cip and Dox antibiotics. The cell monolayers were allowed to differentiate and polarise for 12-14 days under air-interface conditions as previously determined (7).

The viability of Calu-3 cells following evaporation of HPFP was demonstrated by harvesting the cells and confirming the number of viable cells using the trypan blue exclusion method, as described in literature (16).

To ensure the integrity of the cell layer following treatment, 50 µl of HPFP was added to Transwells seeded with Calu-3 cells. Following evaporation of HPFP, the barrier integrity was studied by measuring the transepithelial electrical resistance (TEER) of the monolayers using a Voltohmmeter (EVOM with STX-2 chopstick electrodes, World Precision Instruments, FL, USA). Transport of the paracellular marker fluorescein-Na (Flu-Na) (MW =376 g/mol and log P = -1.52) was also assessed to complement TEER results (7).

To determine the effect of HPFP on the mucus glycoproteins following evaporation of HPFP, the mucus layer was stained using alcian blue according to a previously published method (7).

Uptake of Cip and Dox, as single and combination microparticles, was studied following suspension of 200 and 400 µg of single and combination formulation in 1

ml of HPFP, respectively. The suspensions were then vortexed and sonicated for 30 seconds, and 10  $\mu$ l of each suspension (Dox, Cip and Dox-Cip) was added to Calu-3 cells grown on Transwells in a 24-well plate. The plate remained without a lid for 5-10 minutes in the laminar flow hood until HPFP evaporated from the surface of the cells. This resulted in approximately 2  $\mu$ g of single drug formulations and 4  $\mu$ g of combo formulation on the cell surface. The Transwells were then transferred to a 24-well plate containing 350  $\mu$ l of Hank's balanced salt solution (HBSS) (Gibco, Invitrogen, Sydney, Australia) in the basal chamber. At set time points (30, 60, 90, 120, 150, 180, 210 and 240 mins), samples of the buffer were taken to measure the drug transport. At the end of the experiment the cell surfaces were rinsed with buffer and samples collected for analysis. Furthermore, the amount of Cip and Dox present inside the cells was analysed as described previously (14). The amount of the transported drug, the amount of drug left on the cell surface and the drug retained within the cells were then analysed by high performance liquid chromatography (HPLC) according to the method described by Adi *et al.* (14).

### **Statistical analysis**

Data were analysed using SPSS.17 software. One-way ANOVA with Tukey's post-hoc test (alpha level of  $p < 0.05$ ) was used to test for significance. Data was represented as the mean  $\pm$  standard deviation of at least three independent experiments.

## Results and discussion

Particle size distributions for the spray-dried microparticles are shown in Figure 1. Analysis of the data shows that powders have similar volumetric particle size distributions, suitable for inhalation drug delivery. Specifically, Cip had  $D_{10} = 1.07 \mu\text{m} \pm 0.04$ ;  $D_{50} = 1.90 \pm 0.07$  and  $D_{90} = 3.36 \mu\text{m} \pm 0.11$ ; Dox had  $D_{10} = 0.64 \mu\text{m} \pm 0.84$ ;  $D_{50} = 2.19 \pm 1.61$  and  $D_{90} = 2.56 \mu\text{m} \pm 0.45$ ; and the Dox-Cip combination had Cip had  $D_{10} = 0.87 \mu\text{m} \pm 0.02$ ;  $D_{50} = 1.66 \pm 0.05$  and  $D_{90} = 3.03 \mu\text{m} \pm 0.15$ ,  $n=3$ .

Cell viability results using trypan blue exclusion method showed that  $91.3 \pm 2.7\%$  cells following evaporation of the HPFP were still viable. This value was achieved relative to the number of viable cells in the control Transwell inserts not exposed to HPFP. This finding shows the non-toxic nature of HPFP to Calu-3 cells in culture.

Comparison of the TEER values before addition and after the evaporation of HPFP, showed no significant difference in the measurements. The TEER value was  $451 \pm 28 \Omega \cdot \text{cm}^2$  before, and  $437 \pm 17 \Omega \cdot \text{cm}^2$  after HPFP evaporation, respectively. These results were further confirmed by Flu-Na permeability; the paracellular permeability was  $2.52 \pm 0.45 \times 10^{-7} \text{cm} \cdot \text{s}^{-1}$  before HPFP addition, and  $3.01 \pm 0.58 \times 10^{-7} \text{cm} \cdot \text{s}^{-1}$  after HPFP evaporation, respectively. There was no statistically significant difference observed in TEER and permeability values after HPFP evaporation. Furthermore, results were similar to the barrier integrity values obtained in previous study by Haghi *et al.* that indicated a TEER value of  $474 \pm 44 \Omega \cdot \text{cm}^2$  and permeability value of  $2.33 \pm 0.36 \times 10^{-7} \text{cm} \cdot \text{s}^{-1}$ , where barrier integrity for Calu-3 epithelial cells was established (7).

Mucus staining with alcian blue and the corresponding analysis of the intensity of the blue color using ImageJ software (NIH, USA), showed no statistically significant difference in the blue intensity measured, indicating that exposure of the Calu-3 cells to HPFP did not alter the amount of mucus present at the epithelia. Figure 2 depicts the mucus staining for the control vs. staining of the cells following evaporation of HPFP. According to cell viability, barrier integrity and mucus staining results, exposure to HPFP did not alter any of the characteristics deemed essential for drug transport study investigated in a previous study (7).

Analysis of Dox and Cip following the collection of drug samples, revealed that the total amount of Cip and Dox on Calu-3 cells were as follows:  $2.09 \pm 0.32 \mu\text{g}$  Cip and  $2.15 \pm 0.54 \mu\text{g}$  Cip from the single and combination formulation, respectively; and  $2.15 \pm 0.43 \mu\text{g}$  and  $2.33 \pm 0.35 \mu\text{g}$  Dox from the single and combination formulation, respectively. There was no statistically significant difference observed between the amount of Cip and Dox on Calu-3 cells. The percentage of drug transport illustrates the amount of drug transported relative to the drug recovered after adding together the transported amount, the amount still on the cell surface (apical) and the amount retained within the cells (Figure 3).

When comparing single Dox permeability from single vs. combination formulations, a significant difference is observed in the amount and percentage of Dox transported and retained in the epithelial cells (Figure 3 A). Interestingly, this phenomenon is not observed with Cip formulations and the amount and percentage of Cip transported

from the single formulation was no significantly different from the amount and percentage of Cip transported from the combination formulation (Table 1).

Comparison of the single Dox and Cip formulation revealed that the amount of Cip transported (Figure 3B) was significantly more than Dox, but no significant difference was observed in the amount of either Cip or Dox retained in the cells. Interestingly, in the combination formulation, no Dox was detected in the basal chamber while the amount of Cip transported was not significantly different from transport of the single Cip formulation. Furthermore, analysis of the amount of Dox in the cells from the combination formulation revealed a significant increase in comparison with Dox from the single formulation. Interestingly, this phenomenon was not observed for Cip and the amount of Cip retained in the cells from single and combination formulations were not significantly different.

These findings can be attributed to the fact that, following particles deposition on the epithelial cells, these particles have to dissolve before being able to cross the epithelial barrier. Since water solubility of Cip at pH 7.4 is considerably higher than Dox (135 mg.ml<sup>-1</sup> for Cip vs. 2.15 mg.ml<sup>-1</sup> for Dox), Cip can dissolve in the limited amount of airways surface liquid faster than Dox, and consequently has a faster transport across the epithelial barrier. However, in more complex systems, in the presence of a carrier or a second drug, in this case (Dox-Cip), dissolution of Dox could become a rate-limiting step in transport, resulting in a significantly lower transport rate for Dox from combination formulation. This agrees with a previous study where the presence of glycerol in aerosol particles containing beclomethasone dipropionate (BDP) may influence the disposition of BDP in the lungs (17).

In physiological conditions, the number of deposited particles per unit airway surface area is at submicron levels, therefore in order to gain clear understanding of the interaction between epithelial cells and transport characteristics of these microparticles, development of methods that would allow the deposition of specific quantity of drug on the epithelial cells is essential.

In comparison with other methods of depositing microparticles, this proposed method, using drug suspended in HPFP, provides control over the amount of drugs applied on the surface of the cells. Therefore, cell permeability studies could be conducted with considerably smaller and more reproducible doses, without the physico-chemical characteristics of the drugs being compromised, or the use of modified pharmacopeia impactors.

## **Conclusion**

In conclusion, one important step in the assessment of an aerosol formulation is the establishment of the permeability profile of drug/s across the epithelial barrier. Specifically, when investigating more complex formulations where excipients or multiple drugs are present in the formulation, or when there is very little material to work with, these factors might impact on the permeability of the drug. In these cases, using methods that allow deposition of small amount (<10 µg) of drugs without compromising the physico-chemical properties of the particles becomes essential. The suspension of microparticles in HPFP as presented in this study, has provided a novel, non-toxic and reproducible method to deliver and study the permeability of specific quantity of drugs across respiratory epithelial cells.

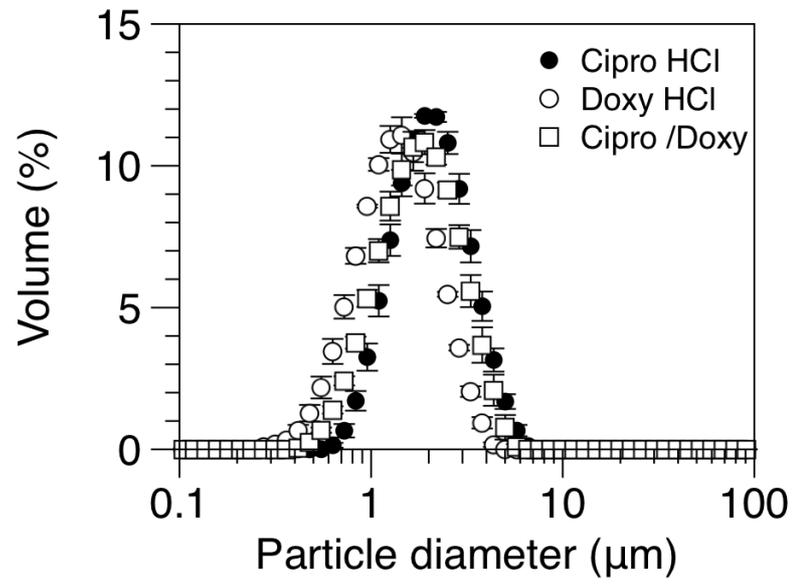
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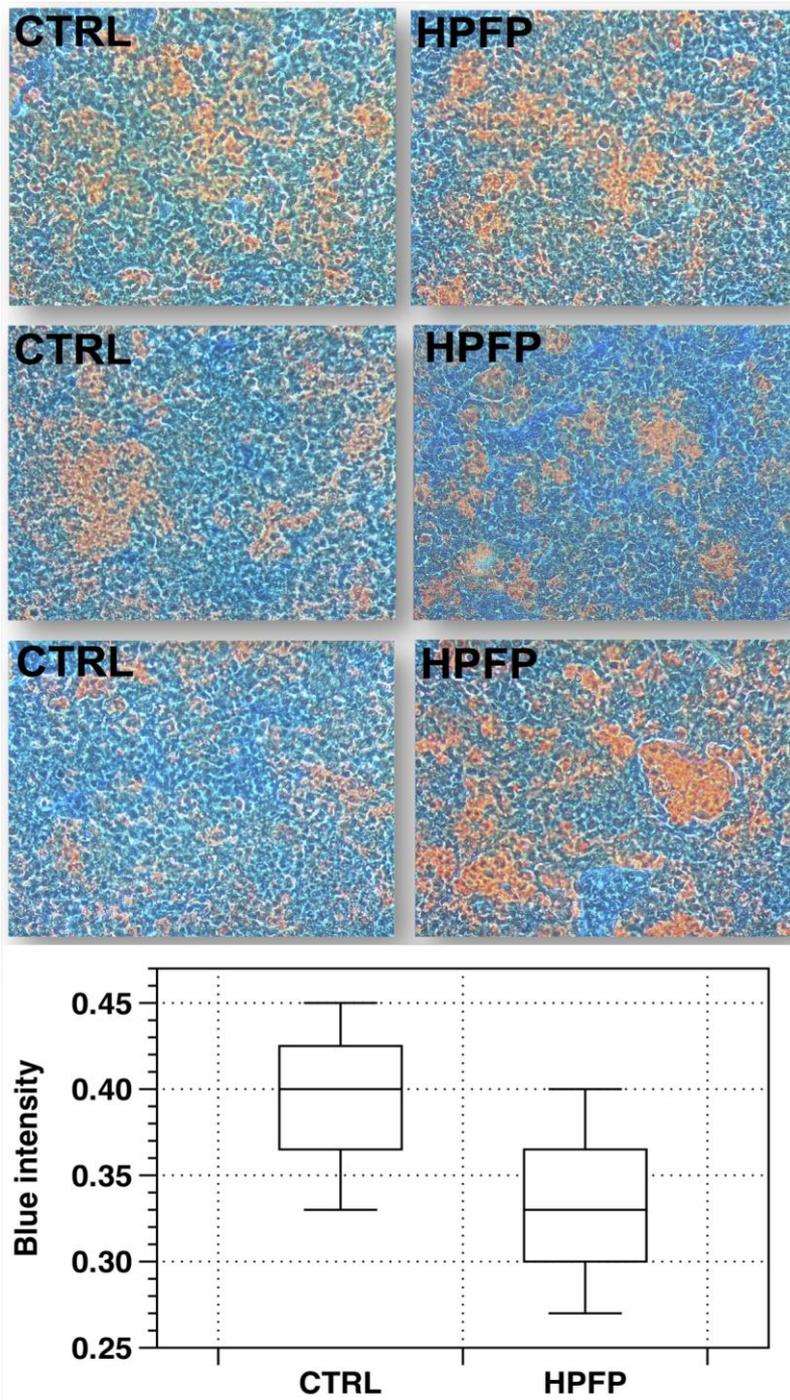
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**Table 1:** Percentage of Dox and Cip transported through and retained in the Calu 3 cells from single and combination formulations (n=3,  $\pm$  StDev).

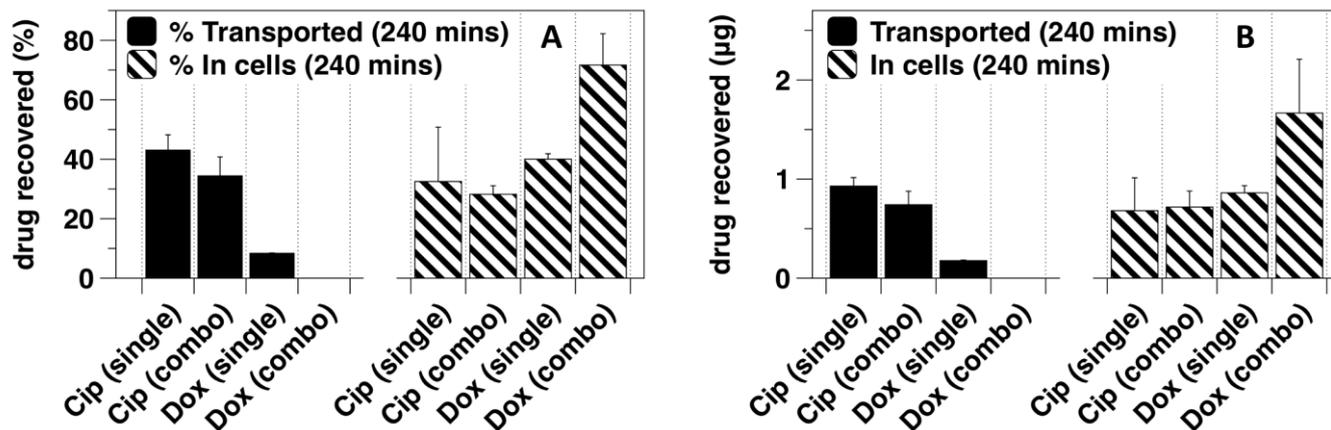
	<b>Transported (<math>\mu\text{g}</math>)</b>	<b>On cells (<math>\mu\text{g}</math>)</b>	<b>In cells (<math>\mu\text{g}</math>)</b>
<b>Dox single</b>	$0.18 \pm 0.01$	$1.12 \pm 0.12$	$0.86 \pm 0.07$
<b>Dox combo</b>	$0.00 \pm 0.00$	$0.66 \pm 0.15$	$1.67 \pm 0.54$
<b>Cip single</b>	$0.9 \pm 0.09$	$0.52 \pm 0.16$	$0.68 \pm 0.33$
<b>Cip combo</b>	$0.74 \pm 0.14$	$0.69 \pm 0.32$	$0.72 \pm 0.16$



**Figure 1:** Particle size distributions for the spray-dried microparticles.



**Figure 2:** Top-Mucus staining for the control (Left panel) vs. staining of the cells following evaporation of HPFP (Right panel). Bottom- corresponding analysis of the intensity of the blue color using ImageJ software (NIH, USA).



**Figure 3:** Percentage of drug recovered (amount of drug transported relative to the drug recovered after adding together the transported amount, the amount still on the cell surface (apical) (A), and the amount retained within the cells (B) for both Dox and Cip (n=3,  $\pm$  StDev).