The use of fatty acids as absorption enhancer for pulmonary drug delivery

Maliheh Ghadiri [a*], Finbarr Canney [b], Cristina Pacciana [c], Gaia Colombo [c], Paul M. Young [a], Daniela Traini [a**]
[a] Respiratory Technology, Woolcock Institute of Medical Research and Discipline of Pharmacology, Sydney Medical School, Sydney, Australia
[b] Imperial College Healthcare NHS Trust, London, UK
[c] Department of Life Sciences and Biotechnology, University of Ferrara, Via Fossato di Mortara 17/19, 44121 Ferrara, Italy

*Corresponding author. Tel.: +61 2912400366; fax: +39 0405583691.
Addresses: Maliheh.ghadiri@sydney.edu.au, Daniela.traini@sydney.edu.au

Abstract

A limitation in the systemic uptake of many inhalable drugs is the restricted permeation through the pulmonary epithelial layer barrier. One strategy to bypass the epithelial layer when delivering non-permeable drugs is to alter the paracellular transport, allowing the uptake of drugs into the systemic circulation. In this study, the potential of sodium decanoate (Na dec), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) as absorption enhancers has been investigated to increase pulmonary paracellular permeability by modulating epithelial cells' tight junctions. By incorporating Na dec, DHA and EPA, separately, into a nebulising formulation, the aim was to enhance the absorption of a fluorescent marker (flu-Na, used as model drug) across pulmonary epithelial cells (Calu-3).

Results indicate that the aerosol performance of all the nebulizing formulations containing absorption enhancers was significantly better than control. Furthermore, the *in vitro* cell assays demonstrated a significant increase in paracellular transport of the fluorescent marker with Na dec and DHA

formulations. This finding supports the potential use of DHA and Na dec to enhance epithelial transport of poorly permeable drugs delivered via inhalation.

Keywords: Fatty acids, Docosahexaenoic Acid, Eicosapentaenoic Acid, Sodium Decanoate, Tight junction, pulmonary drug delivery

Introduction

The use of inhaled medication for systemic drug delivery is a very interesting route of administration due to the highly vascularized respiratory mucosa, large absorptive surface area, thin air-blood barrier, and relatively low enzymatic activity (Patil and Sarasija, 2012). Furthermore, it can also be used as a non-invasive systemic delivery route (Patil and Sarasija, 2012). Over the last 2 decades, the systemic absorption of a broad range of therapeutics delivered via this route has been demonstrated in animals as well as in humans (Gandhimathi et al., 2015). Nevertheless, there are limitations in the uptake of many drugs through lung epithelial barriers before reaching the systemic circulation. Drug candidates do not show biological activity without their absorption into systemic flow, especially if they are hydrophilic, polar or with high molecular weight (macromolecules) (Goldberg and Gomez-Orellana, 2003; McMartin et al.; Ramesan and Sharma, 2009).

There are two main routes of drug absorption via the respiratory system including transcellular and paracellular transport route (Fig. 1). In the transcellular route, drugs are transferred to the sub-epithelia primarily by diffusion across the cell membrane or active transport via a receptor or transporter on the cell membrane or via vesicle mediator (Majumdar et al., 2004). With the paracellular route, molecules diffuse through paracellular canals. It should be noted that the paracellular route in the respiratory tract is tightly sealed for the drug absorption, due to the inherent barriers that prevent systemic absorption of particles (Mizuno et al., 2003).

The mucociliary escalator, intercellular apical junctional complexes which regulate paracellular permeability and respiratory surfactants secreted by the airway epithelial cells are the three primary components of the barrier function of the airway tract. All these factors contribute significantly to the first defence line against pathogens, allergens and particulate materials in the lung (Hussain et al., 2004). Therefore, if systemic delivery of drugs via the lung is to be achieved, these barriers must be overcome, at least transiently, to ensure efficient drug absorption from the respiratory tract. One option to overcome this barrier is the use of absorption enhancers (Aungst, 2012). Absorption enhancers are functional excipients included in inhalable formulations to improve the absorption of a pharmacologically active drug (Asai et al., 2016). The term absorption enhancer usually refers to an agent whose function is to increase absorption by enhancing membrane permeation. The mechanism of action of these absorption enhancers are proposed to be: 1) transient opening of tight junctions, by molecules such as protein kinase C activators (Clarke et al., 2000), cytochalasins B (Feighery et al., 2008), Na dec (Brayden et al., 2015); 2) disruption of lipid bilayer packing by fatty acids (Beguin et al., 2013), and 3) complexation/carrier/ion pairing with molecules such as 1-hydroxy-2-naphthoic acid (Miller et al., 2010). Absorption enhancers have been investigated for a while, particularly for oral and skin drug delivery systems for peptides, proteins (Taverner et al., 2015), and other pharmacologically active compounds with poor membrane permeability (Siew et al., 2012). An ideal absorption enhancer for poorly permeable drugs should reversibly increase epithelial flux without local or systemic toxicity (McCartney et al., 2016). Also, their action should be rapid and should coincide with the presence of the drug at the absorption site (Salama et al., 2006). Although, the use of absorption enhancers has been investigated greatly for transdermal applications, for example, their use in pulmonary drug delivery systems has been limited. Furthermore, although some studies have raised safety concerns regarding possible long-term effects (Bur and Lehr, 2008) of these absorption enhancers, others have shown that the short-term use of enhancers in the lung was safe and their effects reversible (Zhang et al., 2014; Zhang et al., 2015; Zheng et al., 2012).

Modulating tight junctions is one of primary mechanism of action of absorption enhancers. Tight junctions are the most apically located intercellular junctions and appear as membrane fusions spanning the intercellular space to restrict the paracellular zone (Kirschner and Brandner, 2012). There are four primary groups of transmembrane proteins at conventional epithelial tight junctions: occludin, members of the claudin family; the junction-adhesion molecules (JAMs); the Coxsackie virus and Adenovirus Receptor (CAR) proteins (Evans and Martin, 2002).
One interesting tight junction modulators is Sodium decanoate (Na dec), which is a medium chain fatty acid (Brandsch et al., 2008), and it is the only absorption enhancer approved in the pharmaceutical industry (Joint-FAO-WHO-Expert-Comm-Food, 2011) for oral use. Clinical data from Merrion Pharmaceutical's (Ireland) GIPET™ matrix tablet containing Na dec have demonstrated its use for significantly enhancing the oral bioavailability of poorly permeable molecules with very low numbers of adverse events in several hundred volunteers in Phase 1 studies (Karsdal et al., 2015).
The way Na dec acts as an absorption enhancer is highly complex and few mechanisms have been suggested including: 1) phospholipase C-mediated elevation

of intracellular Ca^{2+}, which subsequently regulates tight junction proteins, and 2) detergent-induced membrane fluidization.

Additionally, fatty acids, specifically poly-unsaturated fatty acids, have also been investigated as absorption enhancers for intestinal cells (Beguin et al., 2013; Knoch et al., 2010). Indeed, diets supplemented with poly-unsaturated fatty acids have been shown to increase absorption of poorly absorbed medications (Dunbar et al., 2014). Specifically, two poly-unsaturated fatty acids, namely Docosahexaenoic acid (DHA, (C22:6, n-3)), and eicosapentaenoic acid (EPA, (C20:5, n-3)), have shown an important role in epithelial permeability, dysfunction of the epithelial barrier and redistribution of tight junction proteins in lipid raft fractions by modifying the local lipid environment (Li et al., 2008b). In addition, their effect as tight junction modulators have been shown in a model of human intestinal epithelium (Caco-2 cell) (Beguin et al., 2013), resulting in an increase in the paracellular absorption of hydrophilic substances such as mannitol and aluminum (Aspenstrom-Fagerlund et al., 2009; Vine et al., 2002). Furthermore, it has been demonstrated that EPA, in concentrations between 50 - 200 µM (Antal et al., 2014), and DHA, in a range of 10 - 100 µM (Antal et al., 2014), can induce functional changes to the epithelial tight junctions, without any cytotoxicity effects in intestinal epithelial cells.

Therefore, in this study, the effects of Na dec, and two poly-unsaturated fatty acids, DHA and EPA, have been investigated as possible absorption enhancers to increase airway paracellular permeability of sodium fluorescein as model compound (flu-Na). Flu-Na is a well-known marker for the assessment of the paracellular permeability of epithelia. It is a monocarboxylic acid with two net negative charges at physiological pH and is thus believed to cross the epithelial cells only through tight junctions (Kristl, 2009).

Materials and Methods

Nebulizer formulation and characterization

A nebulizer solution was formulated with flu-Na (model drug and fluorescent marker) in phosphate buffered saline (PBS) at a concentration of 50 µg/ml. Specific amounts of Na dec, EPA and DHA were then added to aliquots of this solution up to 13 mM, 50 µM and 10 µM of each formulation, respectively. Choice of sample concentration was based on literature values that showed these concentrations to be effective on intestinal cell cultures and also based on their cytotoxicity on Calu-3 cells (Anderberg et al., 1993b; Li et al., 2008a). A formulation without fatty acids (50 µg/ml of flu-Na in PBS) was also prepared as a control. Formulations were aerosolized using a Pari LC Sprint® jet nebulizer, powered by the Pari Turbo Boy S Compressor (Starnberg, Germany).

Cascade impaction study

Aerosol performance of each formulation was assessed using the Next Generation Impactor (NGI; COPLEY Scientific, UK) at a flow rate of 15 L/min (to mimic the midpoint of adult tidal breathing) (Marple et al., 2004) using a vacuum pump (Westech W7, Bedfordshire, UK) calibrated using a flow meter (model 3063, TSI Inc., MN, USA). The nebulizer was connected to the induction port of the NGI with a mouthpiece adapter, filled with 2 mL of each formulation, and nebulized for 2 minutes. Following aerosol deposition, the nebulizer and NGI plates were oven dried to evaporate any aqueous phase. NGI plates were then washed in PBS and flu-Na amounts determined on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA). The absorption maximum was set at 485 nm and the emission maximum at 520 nm. Each experiment was run in triplicate and results presented as mean ± StDev.

The following parameters were calculated using CITDAS software (COPLEY Scientific, UK): mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), and fine particle fraction (FPF) for an aerodynamic particle size distribution.

Biological experiments

Calu-3 cells (purchased from American Type Cell Culture Collection (ATCC, Rockville, USA) were cultured in Dulbecco's Modified Eagle's medium: F-12 (Gibco- Australia) containing 10% (v/v) foetal calf serum (Gibco- Australia), 1% (v/v) non-essential amino acid solution and 1% (v/v) L-glutamine solution (Sigma Aldrich-Australia). Cells were maintained in humidified 95% air, 5% CO_2 atmosphere at 37°C and were sub-cultured according to ATCC recommendations. Cells were cultured on Transwell cell culture inserts (Corning -Australia) using air-liquid interface (ALI) method (Haghi et al., 2010) and seeded at a density of 1.65×10^5 cells/insert and the experiment was performed after day 11, to ensure adequate differentiation. The medium was replaced three times a week and any apical surface liquid or mucus removed to make air liquid interface model.

Cytotoxicity of Na dec, DHA and EPA on Calu-3 cells

MTS assay to evaluate cytotoxicity

The cytotoxicity of Na dec, DHA and EPA on Calu-3 cell was determined using CellTiter 96® AQueous One Solution Cell proliferation Assay (Promega, USA). The MTS assay is a colorimetric quantification method of measuring cytotoxicity. It is based on the reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product. In this assay Calu-3 cells were seeded in a sterile 96-well plate at a density of 5×10^4 cell/well and incubated overnight to allow for cell attachment. Cells were treated with Na de, DHA and EPA nebulizing formulations and subsequently incubated at 37°C in 5% CO_2 and 95% humidity for 24 h. The viability of Calu-3 cells were assessed following the addition of 20 µl of MTS reagent to each well and incubated for further 4 hours. Absorbance of each plate was measured at 490 nm using a fluorescence plate reader (SpectraMax M2; Molecular devices, USA). Results were expressed as the percentage of cell viability to the untreated control for each treatment.

Lactate dehydrogenase assay (LDH)

LDH assay is a colorimetric method for quantifying cell membrane damage by measuring leakage of LDH into the cell culture media, and thus, is a measure of cytotoxicity. For this test, Calu-3 cells seeded in a 96 well-plate similar to the MTS assay were treated with each formulation and LDH leakage was measured using a kit (Thermo Scientific™ Pierce™ LDH Cytotoxicity Assay Kit). In brief, Calu-3 cells were incubated with 10 µL of each formulation for 24 hrs. After treatment, LDH released in the media was reacted with the LDH assay kit's components following manufacture's protocol. The plate was read on a SpectraMax plate reader at absorbance 490 and 680 nm as a reference wavelength. Cytotoxicity was calculated according the following equation where positive control was Triton X-100 and negative control (blank) was PBS:

$$Cytotoxicity(\%) = \frac{(\text{Compound treated LDH activity} - \text{Blank LDH activity})}{(\text{Maximum LDH activity (Positive control)} - \text{Blank LDH activity})} \times 100$$

Drug transport: Apical-basal investigation

The transport of flu-Na as a paracellular marker across the Calu-3 cell layer was studied as previously described (Haghi et al., 2010). Briefly, the formulations were aerosolized using a Pari nebulizer on an ALI model of Calu-3 cells. A modified glass twin stage impinger (TSI; British Pharmacopoeia Apparatus A, Copley Scientific, UK) was adapted to fit the Transwell inserts at the lower chamber, where particles with aerodynamic diameter of <6.4 µm (respirable particles) were deposited. A Transwell insert was then fixed onto the connecting jet of the lower chamber, allowing for the deposition of particles on the Calu-3 cell layer. The nebulizer solution was aerosolised at 15 l/min for 15 seconds. This short nebulization time was chosen since longer nebulization times could damage cell membranes due to drug overload. Following deposition, the Transwell insert was removed and placed into 24-well plate containing 600 µl of PBS. At pre-determined time intervals (up to 4h) the Transwell insert was transferred into a new well with fresh PBS, thus maintaining sink conditions. At the end of sampling, the Transwell insert was transferred to an empty well, and the apical side of cells were gently washed with PBS buffer to collect remaining flu-Na on the cell monolayer. Three Transwells per formulation were used to assess flu-Na transport and results presented as Mean ± StDev.

Transepithelial Electrical Resistance (TEER)

Transepithelial electrical resistance (TEER) measurements are used to assess the barrier function viability of epithelial cells. The effect of each formulation on the TEER of Calu-3 epithelial cells was measured by two techniques: Chopstick ohmmeter on Calu-3 ALI inserts and electric cell-substrate impedance sensing system (ECIS) on Calu-3 cell culture arrays.

Chopstick ohmmeter

Transepithelial electrical resistance (TEER) measurements were performed using an epithelial voltohmmeter (EVOM2, World Precision Instruments (WPI), USA) attached to STX-2 chopstick electrodes. This method has been traditionally used to measure TEER in cells grown on filter membranes. Therefore, Calu-3 cells grown on Transwells were used to measure the TEER changes after treating them with the nebulizing formulations. After nebulization, Transwells were transferred to a 24 well plate with 600 µl of PBS in the basolateral compartment, and 250 µl of PBS was added to the apical. Resistance was measured at predetermined time points (10, 30, 60, 120, 180 and 240 minutes).

Electric cell-substrate Impedance Sensing (ECIS)

Epithelial barrier function was investigated using the electric cell-substrate impedance sensing system (ECIS; Applied BioPhysics, Troy, NY, USA). This technique records TEER changes in real time. The data was analyzed using Applied BioPhysics- ECIS software (v1.2.215.0 PC). Resistance was normalized, compiled for each experiment and presented as the mean normalized resistance ± SD (n=3). Resistance was normalized by dividing the impedance values from electrodes confluent with cells by the corresponding quantities for the cell-free electrode, as previously published (Lai and Lo, 2014). Calu-3 cells were grown on collagen coated 8EW10+ arrays (Applied Biophysics, Troy, NY) at a density of 5×10^5 cell/well and were grown to confluence. Each well was treated with each nebulizer solution and control. Resistance was measured continuously every 5 minutes for up to 4 hours. Baseline values were established with culture media alone and TEER values normalized based on these values.

Membrane fluidity assay

In this experiment, the effect of Na dec, EPA and DHA on the Calu-3 cell membrane was investigated using a membrane fluidity assay kit (abcam®). This effect was measured using lipid analog probes that exhibit changes in their spectral proprieties. By measuring the ratio of fluorescence in monomer (EM max. 372nm) to excimer (EM 470 nm), a quantitative monitoring of the membrane fluidity can be measured. For this experiment, Calu-3 cells were seeded with the density of 5×10^4 cells/well in a 96 well-plate. Cells were incubated with 100 µL of each formulation for 24 hours and membrane fluidity was measured following manufacturers protocol. The fluorescence was subtracted from blank and the normalized excimer to monomer ratio was calculated.

Results and Discussion

Aerosol performance of the nebulizing formulations

The design of the NGI apparatus reflects the likely regional lung deposition of particles based on their aerodynamic diameter. The *in vitro* aerosolization of each formulation is presented in Table 1. The droplet volume and size distribution of the formulations containing fatty acids was significantly smaller than control, according to MMAD and GSD values. This finding can be due to the effect of fatty acids as a surfactant on nebulizing droplets, which results in smaller droplet sizes. Previous study confirms that adding surfactants to an aerosol formulation can reduce the droplet size due to the reduced surface tension (Hoffmann et al., 2016). Parallel to the reduction of droplet size, the aerosol performance of these formulations (FPF %) was also significantly improved. Na dec and EPA, with FPFs values of 71.55 ± 1.3 and 71.13 ± 0.4%, respectively, had the highest aerosol performance, followed by 63.38 ±

1.5 % for the DHA formulation compared to 58.30 ± 1.3% in the control formulation. Results indicated that the tested fatty acids (Na dec, EPA and DHA) could enhance lung deposition of nebulizing formulation *in vitro*.

In-vitro biological assessment of nebulizing solutions

Cytotoxicity: MTS assay

The cytotoxic effect of Na dec, EPA and DHA formulations on Calu-3 cells was assessed using the MTS calorimetric assay. Viability of cells after 24 hours of exposure to each formulation is presented in Figure 2.

DHA, EPA and Na dec treatments did not affect viability of Calu-3 significantly. Therefore, all formulations and the concentrations used in this study were shown to be nontoxic on the Calu-3 cells. It should be noted that, when cytotoxicity is tested *in vitro*, the cell layer appears to be more sensitive to the cytotoxic effects of absorption enhancers than when tested *in vivo*. For instance, Chao et al. (Chao et al., 1999) showed that when 50 mM of Na dec was delivered to Caco-2, disrupted and detached cell monolayer was observed, while up to 100 mM of a liquid formulation of Na dec delivered to the terminal ileum of rats did not result in irritation or damage of the mucosa. This *in vivo* effect can be probably due to the dilution of treatments to more tolerable concentrations to the cells due to their transient nature, compared to the *in vitro* condition where the concentration remains constant for longer period of time.

In-Vitro biological Transport of Flu-Na using the modified Twin Stage Impinger

Transport of flu-Na across Calu-3 epithelial layer, plotted as mean cumulative percentage of flu-Na over time (4 hours), is presented in Figure 3.

Results showed that Na dec significantly increased the transport of the fluorescent marker compared to the control. Literature shows that in Caco-2 cells, Na dec can act on dilating tight junctions (paracellular) and also increase cell membrane penetration (transcellular pathway) of a fluorescent marker (Hochman and Artursson, 1994; Lindmark et al., 1998). However, the effect of Na dec on drug permeation through intestinal epithelial barrier has been found to be mostly due to the tight junction's modulatory effect, suggesting that paracellular permeation is the main mechanism (Coyne et al., 2003; Lindmark et al., 1998). Nebulizing formulation of DHA also increased transport of flu-Na significantly, but only after 2 hours of treatment. EPA instead, did not enhance flu-Na transport significantly during the 4 hours of the experiment compared to the control. This finding was similar to the experiment of Beguin et al, who has shown that up to 150 μM concentration of DHA and EPA treatment did not disturb the Caco-2 epithelial barrier function (Beguin et al., 2013), despite 150 μM of DHA affected ZO-1 intensity in the immune-stained images, but not the barrier function parameters.

Transepithelial Electrical Resistance

The integrity of the Calu-3 cell barrier after nebulization of formulations containing fatty acids was investigated by measuring TEER (Figure 4) with chopstick ohmmeter. The epithelial resistance significantly decreased in the first 30 minutes after deposition of nebulized formulations: Na dec (P>0.001) and DHA (P>0.05), respectively. TEER values recovered to control values after 4 hours, indicating that the Calu-3 epithelial cell's integrity had been altered only transiently, due to the effect of the treatments on the tight junctions. This decrease in TEER was also is correlation with flu-Na transport increase, where Na dec transport after 30 minutes was significantly higher than control, as evident in our study and others (Abdayem et al., 2015; Anderberg et al., 1993a). Treatment with EPA formulation however did not significantly change the TEER.

The ECIS system was also used to monitor the effect of the nebulized treatments on the Calu-3 cells. Treatments were added to the Calu-3 cells while the resistance was measured constantly every 5 minutes up to 4 hours. Results are presented in Figure 5, where it is shown that Na dec treatment resulted in a significantly lower resistance among other treatments. Overall, resistance was altered upon treatment with all the formulations as soon as the treatment was applied, but while resistance for the DHA and EPA treated cells quickly returned back to the control values, treatment with Na dec showed a slower recovery. Calu-3 epithelial cell's integrity was altered, only temporarily, which is what is required of an absorption enhancer in order to be safe (McCartney et al., 2016).

Effect of fatty acids on Calu-3 membrane: LDH activity and membrane fluidity

LDH activity

Results from the LDH assay for DHA, EPA and Na dec treatments are presented in Figure 6-A. DHA and EPA treatments did not significantly increase LDH leakage after 24 hours exposure. This was in line with previous publications where EPA treatment (50-100 μM) did not effect on LDH leakage on human glioma cells (Antal et al., 2014). A previous study also showed that concentration range below 10 -50 μM of DHA inhibited apoptosis, without any LDH leakage on Neuro2a cells (Wu et al., 2007). However, treatment with Na dec resulted in a significant (P>0.05) altered Calu-3 cell membrane integrity and increased LDH leakage. Although the LDH leakage was increased with Na dec treatment, the MTS cytotoxicity assay did not show a substantial effect on the cell viability.

Membrane fluidity

The effect of DHA, EPA and Na dec on membrane fluidity of Calu-3 is shown in Figure 6-B. Only Na dec treatment significantly affected Calu-3 cell membrane's fluidity (*P*>0.05). This finding correlates with the LDH activity, where the membrane treated with Na dec formulation showed a significant LDH leakage. It has been showed that Na dec has non-specific interactions with the lipid bilayer, inducing changes in the morphology of the membrane and its fluidity, which has been associated with the membrane permeability, although, this effect is concentration dependent (Lapshina et al., 1995). It has also been demonstrated that Na dec can cause structural changes to the cell membrane, which results in the release of membrane phospholipids *in situ* (Aungst, 2000). The amounts of released phospholipids in the presence of Na dec were proportional to its promoting effect on drug absorption. It was concluded that Na dec interacts with the membrane lipids causing membrane perturbation (Tomita et al., 1988). Brayden et al. also demonstrated that membrane fluidity of Caco-2 cells increased at the presence of 8.5 mM Na dec (Brayden et al., 2015). The effect of Na dec on Calu-3 membrane further strengthen the intracellular absorption mechanism of Na dec, as it was mentioned in the other studies (Coyne et al., 2003; Lindmark et al., 1998).It has been proposed that membrane fluidity may play a direct role in the transcellular transport mechanisms (Reith, 1983). Therefore, increase in the membrane fluidity may enhance the transcellular transport of flu-Na.

Conclusions

Na dec showed transient and reversible alteration of Calu-3 permeability, hence increased paracellular transport and also intracellular transport of flu-Na due to its effect on Calu-3 membrane's fluidity and integrity. Similarly, nebulizing formulation of DHA decreased TEER and thereby increased paracellular transport of flu-Na, but not to the same extent. On the contrary, EPA did not show a significant effect on the transport of flu-Na across Calu-3 epithelial layer. Therefore, this study demonstrated that Na dec and DHA are potential and safe absorption enhancers and may be promising excipients for pulmonary drug delivery to improve the pulmonary absorption of therapeutic agents with poor permeability. Further study will investigate this finding *in vivo*.

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Graphical abstract:

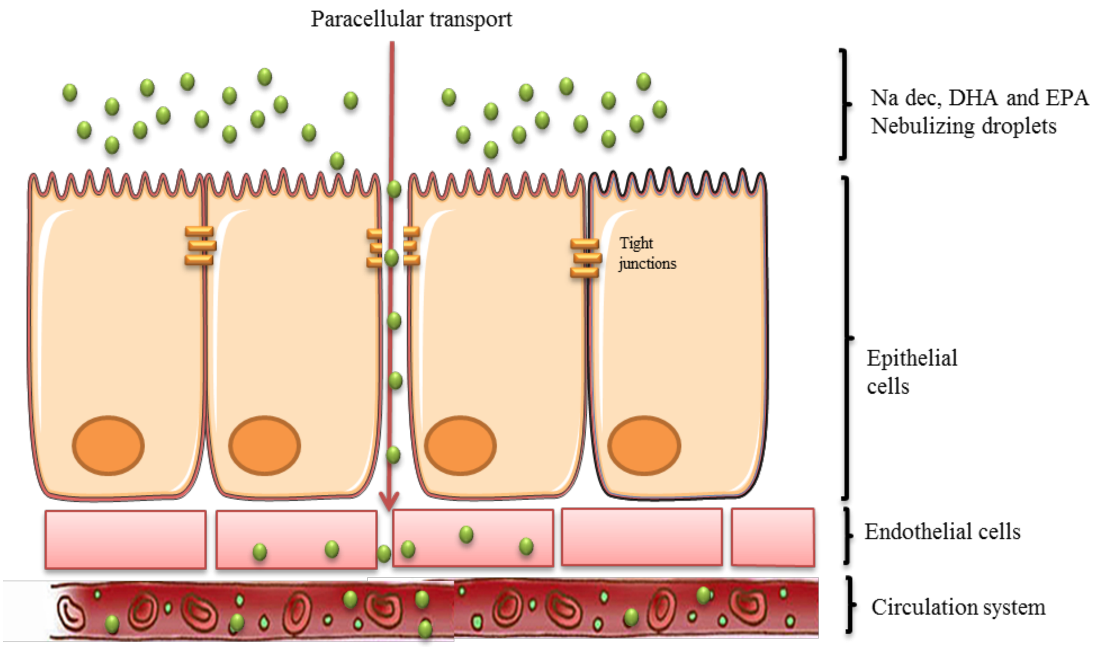

Table 1- Aerosol performance of the nebulizing formulations (n=3; ± StDev)

	FPF (%)	MMAD (µm)	GSD
Control	58.30 ± 1.3	4.90 ± 0.89	3.18 ± 0.31
Na dec	71.55 ± 1.8 [a]	3.89 ± 0.88	2.14 ± 0.23
EPA	71.13 ± 0.4[a]	2.99 ± 0.42	1.95 ± 0.30
DHA	63.38 ± 1.5[a]	3.26 ± 0.23	2.36 ± 0.21

[a] *is significantly different from the control (p> 0.001)*

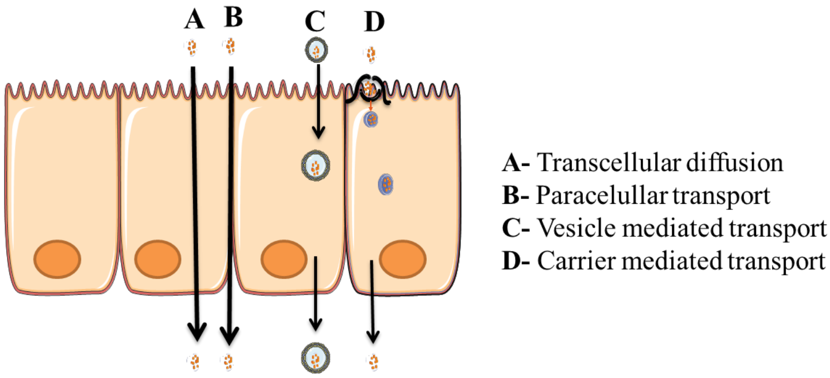

Figure 1-Schematic representation of the common route of transport across lung epithelial cells.

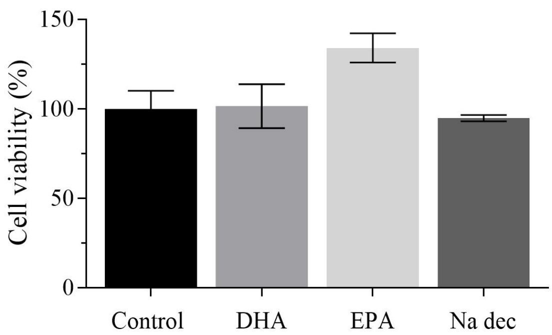

Figure 2- MTS cytotoxicity assay on Calu-3 cells treated with DHA, EPA and Na dec (n=3, ± StDev).

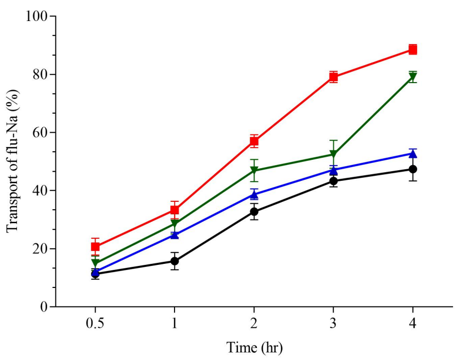

Figure 3- Apical-basal cumulative transport of flu-Na across Calu-3 epithelial cell layer after nebulizing formulations of Na dec (red line ■), DHA (green▼), EPA (blue ▲) and control (black ●) (n=3, ± StDev) using a modified Twin stage impinger.

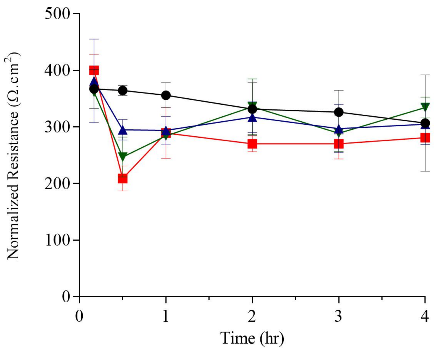

Figure 4- Average normalised resistance of Calu-3 cells after treatment with nebulizing formulations of Na dec (red line ■), DHA (green▼), EPA (blue ▲) and control (black ●) (n=3, StDev).

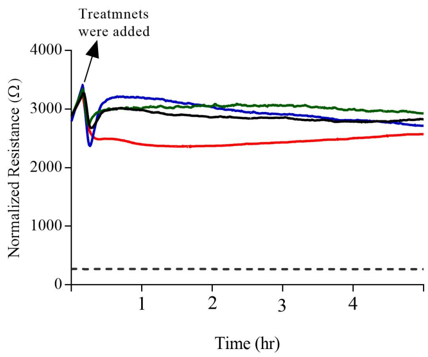

Figure 5- Electrical cell-based Impedance substrate (ECIS) analysis on Calu-3 cells following treatment with Na dec (red), DHA (green), EPA (blue) and Black line is untreated cells- dashed line is showing the resistance of a well without cell.

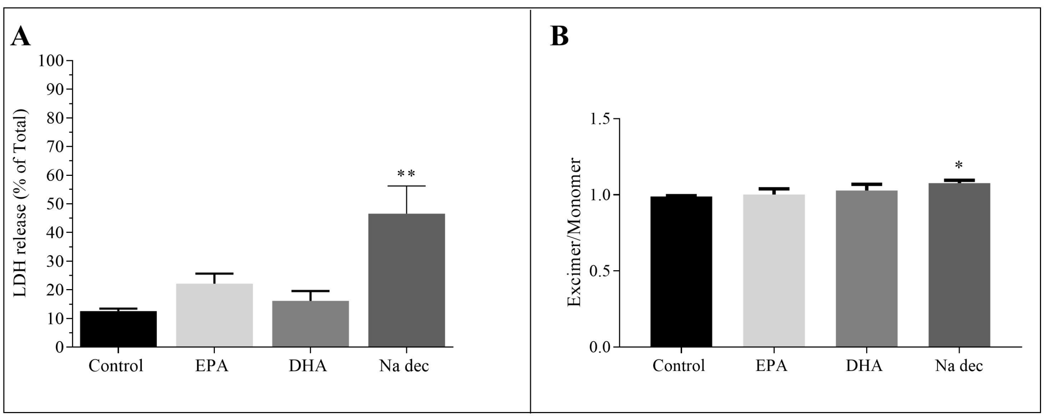

Figure 6- A) LDH activity and, B) Membrane fluidity of Calu-3 cell membrane after 24 hrs exposure to the formulations of Na dec, DHA and EPA (n=3, StDev), * indicates group is significantly different from the control group (*, *P*>0.05 and **; *P*> 0.001)