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In vitro biological activity of resveratrol using a novel inhalable resveratrol spray-dried formulation

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ABSTRACT

The aim of the study was to prepare inhalable resveratrol by spray drying for the treatment of chronic obstructive pulmonary disease (COPD). Resveratrol, with a spherical morphology and particle diameter less than 5 μm, was successfully manufactured. Fine particle fraction (FPF) and mass median aerodynamic diameter (MMAD) of spray-dried resveratrol was 39.9 ± 1.1% and 3.7 ± 0.1 μm, respectively when assessed with an Andersen cascade impactor (ACI) at 60 l/min. The cytotoxicity results of spray-dried resveratrol on Calu-3 revealed that the cells could tolerate high concentration of resveratrol (up to 160 μM). In addition, in transport experiments using Snapwells, it was observed that more than 80% of the deposited dry powder was transported across the Calu-3 cells to the basal chamber within four hours. The expression of interleukin-8 (IL-8) from Calu-3 induced with tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β1) and lipopolysaccharide (LPS) were significantly reduced after treatment with spray-dried resveratrol. The antioxidant assay (radical scavenging activity and nitric oxide production) showed spray-dried resveratrol to possess an equivalent antioxidant property as compared to vitamin C. Results presented in this investigation suggested that resveratrol could potentially be developed as a dry powder for inhalation for the treatment of inflammatory lung diseases like COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death worldwide, characterised by chronic inflammation, bronchoconstriction, airflow limitation and mucus hyper secretion (Barnes, 2007). Several factors including genetics, inhalation of noxious particles or gasses (Rahman and Adcock, 2006) and the most common factor, cigarette smoke (Barnes, 2007), have shown to contribute to the development of COPD (Barnes, 2007; Silverman et al., 1998). Current treatment for COPD is symptomatic and does not inhibit the progression of the disease or restore normal lung function (Calverley et al., 2007). The pharmacotherapy of COPD includes inhaled β_2-agonists (e.g., salmeterol, formoterol), inhaled anticholinergics (e.g., tiotropium, ipratropium) and inhaled corticosteroids (e.g., beclometasone, budesonide) (Cazzola et al., 1998; Celli et al., 2004; Dahl et al., 2010; Pauwels et al., 1999, 2001; Vogelmeier et al., 2011). In several cases, COPD airway inflammation becomes refractory to corticosteroids (Barnes et al., 2004a) and the therapy fails. Therefore, development of novel efficient therapies for the treatment of COPD is essential to improve patients' quality of life. Q2

Oxidative stress is one of the components involved in the pathogenesis of airways inflammatory diseases, such as COPD (Rahman and Adcock, 2006). Oxidative stress is the result of an imbalance between the reactive oxygen species and a biological anti-oxidant system. If the ability of biological system to detoxify and remove the toxic species is unsettled, it could result in the production of peroxides and free radicals that consequently damage cellular components (proteins, lipids, and DNA). Furthermore, oxidative stress can change normal cell signalling pathways. One instance is the reduction in expression and activity of histone deacetylases ($HDAC_2$) in COPD. These conditions are worsened in smokers, since several studies in literature have demonstrated that oxidative stress and cigarette smoke increase histone acetylation, which leads to increased expression of inflammatory genes (Barnes et al., 2004b; Lee and Yang, 2012; Rahman and Adcock, 2006). The

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inflammation caused by these agents results in the release of reactive oxygen species (Rahman and Adcock, 2006). Hence, oxidative stress can be an important pharmaceutical target for the treatment of COPD and antioxidant compounds have the potential to restore the responsiveness to corticosteroids (Rahman and Adcock, 2006).

Resveratrol (*trans*-3,5,4-trihydroxystilbene) is a polyphenolic compound synthesised in a large number of plant species and can be found in grapes, berries and legumes (Francioso et al., 2014). Resveratrol exhibits potent anti-oxidant and anti-inflammatory properties and has shown potential against cardiovascular, cancer, diabetes, and neurodegenerative diseases (Wood et al., 2010). Anti-inflammatory activity of this compound has been associated with the inhibition of cyclooxygenase (COX)-2 transcription and inhibition of COX-1 activity via peroxidase-mediate mechanism (Santangelo et al., 2007). Specifically, in lungs it has been shown that this compound is capable of scavenging oxygen-derived free radicals (Arts and Hollman, 2005) and therefore has the potential to be used as a adjunct therapy in the treatment of COPD (Knobloch et al., 2010; Wood et al., 2010). It has also been shown that polyphenolic compounds, such as resveratrol, are candidate molecules for the development of novel anti-inflammatory therapies for airway diseases, especially when patients become non-responsive to glucocorticoids, such as glucocorticoid resistant severe asthma and COPD (Donnelly et al., 2004b). Furthermore, resveratrol has been shown to inhibit the release of inflammatory cytokines from alveolar macrophages in COPD and therefore can be considered as a suitable candidate for pharmacotherapy of macrophages (Culpitt et al., 2003).

The aim of this study was to investigate the potential of resveratrol as dry powder (DPI) for inhalation and its anti-inflammatory and anti-oxidant activity in the lung. In this study, for the first time, the use of resveratrol as DPI has been presented. The physicochemical characteristics of this new formulation were investigated and the aerosol performance evaluated using Andersen cascade impactor (ACI). In addition, the deposition, transport and cell uptake of DPI resveratrol using an air interface model of Calu-3 lung epithelial cell line incorporated onto a modified ACI is also presented, together with its anti-inflammatory and anti-oxidant activities on Calu-3.

2. Materials and methods

2.1. Materials

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) was purchased from Fagron Italia (Bologna, Italy). Calu-3 cell line (HTB-55) was purchased from the American Type Cell Culture Collection (ATCC, Rockville, USA). L-Glutamine was from Invitrogen (Sydney, Australia). Dulbecco's modified eagle's medium (DMEM), CelLytic™ M reagent, α-Lipoic acid, Nitro-L-arginine methyl ester (L-NAME), L-ascorbic acid, non-essential amino acids, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,3-diaminonaphthalene (DAN) and lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) (Sigma–Aldrich, Sydney, Australia) were purchased from Sigma–Aldrich (Sydney, Australia). Hank's balanced salt solution (HBSS) and tumor necrosis factor alpha (TNF-α) were purchased from Invitrogen, (Sydney, Australia) and transforming growth factor beta (TGF-β1) was from Sapphire Bioscience (Sydney, Australia) Human IL-8 ELISA Kit II BD Opt EIATM (Becton Dickinson, Sydney, Australia). Analytical grade solvents were purchased from Sigma (Sydney, Australia).

2.2. Preparation of spray-dried resveratrol

Respiratory sized microparticles of resveratrol were produced by spray-dried using a Buchi spray dryer (Buchi B-290 Mini Spray Dryer, Buchi, Switzerland). Resveratrol (20 mg/ml) was dissolved in ethanol–water (50:50% v/v) and spray-dried at a feed rate of 40%, flow rate of 12.5 ml/min, aspiration rate 100%, inlet temperature 100 °C and a measured outlet temperature of 40 °C in a closed loop configuration.

2.3. Particle size analysis

Particle size distribution of the raw and spray-dried resveratrol was analysed using laser diffraction (Mastersizer 3000, Malvern, Worcestershire, United Kingdom). Samples (ca.10 mg) were dispersed using the Scirocco dry dispersion unit with a feed pressure of 4 bars and feed rate of 75%. Samples were analysed in triplicate, with an obscuration value between 0% and 15% and a reference refractive index of 1.762.

2.4. Scanning electron microscopy

The morphology of raw and spray-dried resveratrol was studied using a scanning electron microscope (SEM), JOEL JMC, 6000 SEM (Tokyo, Japan). Samples were dispersed onto carbon sticky tabs and sputter coated with gold at thickness of 20 nm (Smart coater, JOEL, Tokyo, Japan).

2.5. Differential scanning calorimetry

The thermal response of raw and spray-dried resveratrol was studied using differential scanning calorimetry (DSC823e; Mettler-Toledo, Schwerzenbach, Switzerland). Samples (3–5 mg) were crimp-sealed in DSC pans, with the lid pierced to ensure constant pressure, and thermal properties analysed between 25 and 320 °C using a 10 °C/min temperature ramp. Exothermic and endothermic peaks were determined using STARe software V.11.0x (Mettler Toledo, Greifensee, Switzerland).

2.6. In vitro aerosol performance

Aerosol performance and aerodynamic particle size distribution of the spray-dried resveratrol was studied in vitro using the Andersen cascade impactor (ACI). A size 3 gelatin capsule (Capsugel®, Sydney, Australia), containing spray-dried powder (5.0 ± 0.1 mg) was placed into the sample compartment of a low resistance RS01 dry powder inhalation device (Plastiare®, Osnago, Italy). The device was attached to the USP throat of the ACI and the flow was adjusted to 60 l/min using a calibrated pump (Westech Scientific Instrument, UK) and flowmeter (Serie 4000, TSS Inc., MN, USA). After actuation, the capsule, device, adaptor, throat and all ACI stages were washed separately with 50:50% v/v ethanol–water and further mixed with mobile phase (methanol–water 60:40% v/v with 0.5% acetic acid) at ratio 1:1 to improve the HPLC peak resolution. The fine particle dose (FPD) (drug recovered from stages 3 to filter, ≤4.7 μm), the fine particle fraction (FPF) (FPD/Total dose × 100), emitted dose (ED) and total mass recovery were calculated. Experiments were conducted in quadruplicate and samples were analysed using a validated high performance liquid chromatography (HPLC) method.

2.7. Chemical quantification of resveratrol using HPLC

A Shimadzu Prominence UFLC system was used equipped with: a DGU-20 A5R Prominent degasser unit, LC-20 AD liquid chromatography, SIL-20A HT Autosampler, SPD-20A UV–vis detector (Shimadzu Corporation, Japan) and Xbridge™ C18 column (5 μm, 4.6 × 150 mm) (Waters, Massachusetts, USA). The mobile phase consisted of methanol-water (60:40% v/v) with 0.5% v/v of acetic acid and run at the flow rate 0.7 ml/min. The

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content of resveratrol was quantified at wavelength 306 nm from the peak area correlated with the predetermined standard curve between 0.2 and 10 μg/ml.

2.8. Culture of Calu-3 sub-bronchial epithelial cells

Calu-3 cells were purchased from the American Type Cell Culture Collection (ATCC, Rockville, USA) and maintained in Dulbecco's modified eagle medium nutrient mixture F-12Ham (DMEM: NMF-12) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acid solution, and 1% (v/v) L-glutamine solution.

2.9. Chemical stability of resveratrol in Calu-3 culture media

The chemical stability of resveratrol was evaluated in completed DMEM:NMF-12 media. Briefly, resveratrol solution was prepared by dissolving in ethanol and diluting in the media with a final concentration of resveratrol of 100 μM. Ethanol concentration of was kept to less than 1%, in order to maintain cell viability (Scalia et al., 2013). The samples were incubated in culture condition (37 °C, humidified atmosphere and 5% CO_2) for 72 h. At different set time points (0, 6, 24, 48 and 72 h) samples were collected and the resveratrol content was quantified by HPLC. The stability of resveratrol was expressed as the remaining of resveratrol after incubation at different time points.

2.10. Cytotoxicity of resveratrol on Calu-3

The cytotoxicity of resveratrol was assessed by measuring the viability of Calu-3 cells after exposure with increasing resveratrol concentrations (from 1.25 nM to 160 μM). Following incubation with different concentrations of resveratrol and the addition of CellTiter 96® Aqueous assay (MTS reagent, Promega, USA) the absorbance was measured at 490 nm using a plate reader (Wallac 1420 VICTOR2™, Multilaber Counter, Massachusetts, USA). Cell viability was calculated with reference to the untreated cells and the absorbance values were directly proportional to cell viability.

2.11. Deposition, transport and interaction of spray-dried resveratrol with Calu-3 cells after aerosol deposition

2.11.1. Validation of the ACI deposition profiles with and without the modified plates

Aerodynamic particle size distribution of spray-dried resveratrol was assessed using the ACI as outlined in the British Pharmacopoeia. The experiments were performed in triplicate using conventional impaction plates and modified plates, containing Snapwells inserts (placed at stage 3 of ACI with cut-off diameter of 2.1–3.3 μm). Briefly, both the conventional and modified ACI was connected to pump and flow rate adjusted to 60 l/min. The DPI device containing the spray-dried resveratrol was then actuated in one shot into the ACI for 4 s. After aerosol deposition onto cell-free Snapwells, all ACI stages were washed to quantify the total amount of drug deposition. The modified ACI plate containing Snapwells was washed to determine the total amount of resveratrol deposited on stage 3 and on each Snapwell.

2.11.2. Deposition and transport of spray-dried resveratrol on Calu-3 cell lines by incorporation into modified ACI plates

Calu-3 cells were seeded on Snapwells polyester membrane (0.4 μm pore size, 1.12 cm^2 surface area) (Corning Costar, Lowell, MA, USA) and maintained in air-interface configuration for 17–19 days. Spray-dried resveratrol was deposited on Calu-3 cells grown on Snapwells using a modified ACI according to the method described by Haghi et al. (2014). Briefly, a capsule containing the spray-dried powder was placed in the RS01 device. The cells were placed on stage 3 of the ACI (cut off diameter 2.1–3.3 μm) and resveratrol was deposited at flow rate of 60 l/min for 4 s. The Snapwells were transferred to a 6-well plate containing Hank's balanced salt solution (HBSS). Sampling of the basal chamber was conducted at set time points (30, 60, 120, 180, 240 min). At the end of the experiment, the surface of the Calu-3 cells was washed to quantify the residual apical drug and intracellular content of resveratrol was analysed according the cell lysis method previously described by Haghi et al. (2010) using CelLytic™ reagent. The experiments were conducted in triplicate and all samples analysed using HPLC.

2.11.3. Anti-inflammatory effects of spray-dried resveratrol using modified ACI plates

The anti-inflammatory activity of resveratrol was studied after deposition of spray-dried resveratrol on Calu-3 cells, using the modified ACI as described by Haghi et al. (Haghi et al., 2014). The Snapwells were transferred to a 6-well plate and incubated at 37 °C, 5% CO_2 for 24 h. Tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β1) and lipopolysaccharide (LPS) were added at a concentration of 5 ng/ml and plates incubated for further 48 h to allow for the production of inflammatory cytokine, interleukine-8 (IL-8). Samples of the culture medium were analysed for IL-8 using Human IL-8 ELISA Kit II BD OptEIA™ according to the manufacturer's instructions.

2.12. Anti-oxidant effects of resveratrol

2.12.1. DPPH radical scavenging activity

The anti-oxidant activity of resveratrol was determined by measuring the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity according the method described by Basnet et al. (2012). Different concentrations of resveratrol (4 nM–4 mM) were added to the same volume of DPPH solution (60 μM). Samples were stored in the dark at room temperature for 30 min and the intensity of DPPH radical's absorbance was measured at 520 nm. The same concentrations of α-lipoic acid and nitro-L-arginine methyl ester (L-NAME) were used as negative controls, while ascorbic acid was used as the positive control. The experiment was conducted in quadruplicate.

2.12.2. DAN assay

The cells were treated with TGFβ-1 and LPS 100 ng/ml for 48 h and then with resveratrol 100 μM (final concentration) for further 24 h. The amount of nitric oxide (NO) produced from Calu-3 cells was measured with 2,3-diaminonaphthalene (DAN). Ascorbic acid and L-NAME at 100 μM (final concentration) were used as positive and negative control, respectively, according to the method described by Choi et al. (2009). Serial concentrations of nitrite (0.19–25 μM) were prepared as standard. Based on the fluorescence intensity (excitation = 360 nm and emission = 430 nm), the amount of NO detected from Calu-3 after treatment with either resveratrol, vitamin C or L-NAME was calculated against the standard curve.

2.13. Statistical analysis

One-way ANOVA or unpaired 2-tailed *t*-tests were performed to determinate significance (which was quoted at the level of $p < 0.05$) between treatment groups and control.

3. Results and discussion

Inflammation and oxidative stress are physiological factors contributing to the development of many chronic diseases, such as

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cancer (Manna et al., 2000), atherosclerosis (Wu et al., 2001) and lung diseases (i.e., COPD) (Rahman and MacNee, 2000). The overproduction of oxidants from reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly associated to DNA, RNA and protein damage, as well as lipid oxidation, contributes to significant damaging effect to cells (Rahman, 2008). Corticosteroids, such as beclomethasone dipropionate, budesonide and fluticasone, are well accepted for COPD treatment, however some patients may develop "refractory effect" after prolong treatments (Barnes and Adcock, 2009). Therefore, there has been great interest in developing new bioactive agents that could effectively control the inflammation and oxidation of this diseased state. Resveratrol could potentially be effective in preventing the progression of COPD, owing to its strong anti-oxidant and anti-inflammatory effects (Anekonda, 2006; Das and Das, 2007; Donnelly et al., 2004b; Manna et al., 2000). The delivery of resveratrol as solution is not suitable due to its instability, propensity for rapid oxidative degradation in water and to its low solubility in water (Amri et al., 2012). As reported previously, the solubility of resveratrol in water is approximately 30 mg/L which corresponds to 0.13 mM (Amri et al., 2012).

3.1. Physicochemical characterisation of raw and spray-dried resveratrol

In this study, resveratrol was manufactured as dry powder for inhalation to be delivered directly to the lung for the reduction of inflammation and oxidative stress in. Particle size distributions for raw and spray-dried resveratrol, as analysed using laser diffraction, are shown in Fig. 1A. Analysis of the data showed that the size distributions for both samples varied significantly. Median volume diameters of 13.3 ± 0.1 μm and 3.9 ± 1.0 μm ($n = 3 \pm$ SD) were observed for both raw resveratrol and spray-dried resveratrol, respectively (Fig 1A), showing spray-dried resveratrol to be suitable for pulmonary administration (Todoroff and Vanbever, 2011). The sizes of raw resveratrol did not fall within the respirable range, with approximately 90% of the spray-dried resveratrol $< 9.7 \pm 1.0$ μm. The SEM images of raw resveratrol presented a columnar shape with a volume size above 120 μm (Fig. 1B), while, the spray-dried resveratrol particles showed a corrugated plate-like morphology (Fig. 1C), with a suitable size for lung deposition (≤ 5 μm).

In addition, the thermal response of raw and spray-dried resveratrol was investigated to determine the influence of heat flow on the changes in the dry particle system. As shown in Fig. 1D, a single sharp endothermic peak at 270.0 °C was presented and was attributed to the melting of raw resveratrol (Ansari et al., 2011). The absence of any exothermic peaks prior to the melting peak suggested that any phase transition from amorphous to crystalline did not occur; thus indicating that the raw resveratrol exists as a crystalline material. Similarly, spray-dried resveratrol showed a melting peak at 267.3 °C, with no exothermic events in the low temperature region prior to this peak, suggestive the spray-dried sample was crystalline.

3.2. In vitro aerosol performance

The aerosol performance of spray-dried resveratrol dry powder was studied using the ACI cascade impaction method. Data presented are the percentage of the total drug deposited in device, throat and each stage of ACI over the emitted dose (ED) (Fig. 2). The mass median aerodynamic diameter (MMAD) of the particle was 3.7 ± 0.1 μm, with a geometric standard deviation (GSD) of 1.3. The total recovery of resveratrol was $105.9 \pm 4.7\%$ of the loaded dose, falling within the acceptable pharmacopeia range of $100 \pm 25\%$ (Commission and Britain, 2010). The FPD and FPF were calculated to be 2054.6 ± 191.0 μg and $39.9 \pm 1.1\%$, respectively (Fig. 2). These data demonstrated that spray-dried resveratrol powder had efficient aerosol performance, most likely due to the particles' corrugated surface which reduces the cohesive forces between particles, thus facilitating aerosolization of DPI (Chew et al., 2005).

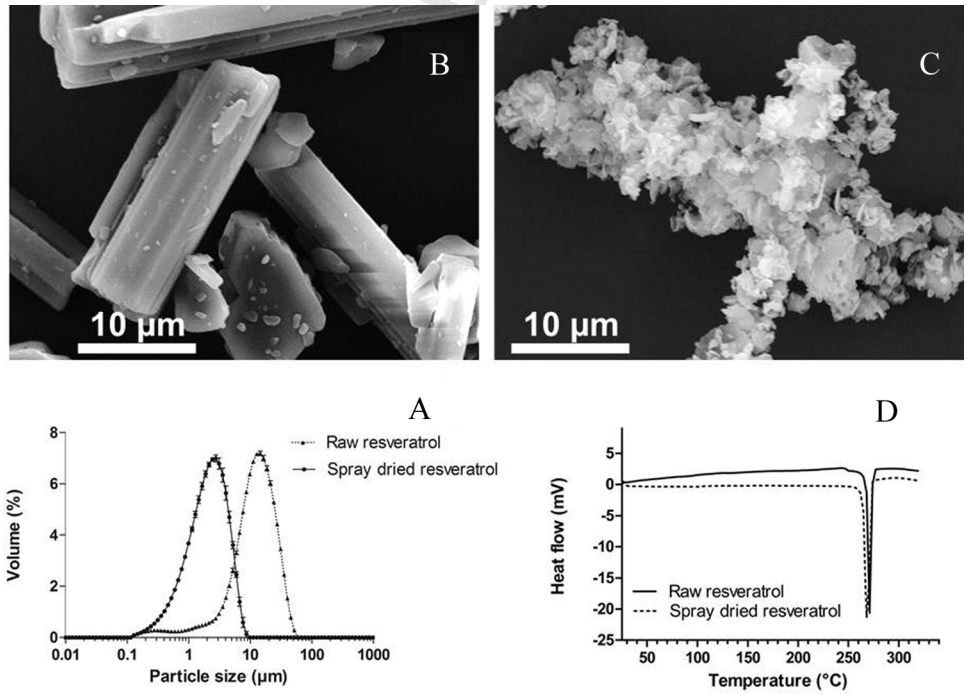

Fig. 1. (A) Particle size distribution of raw and spray-dried resveratrol. Data represents mean ± SD ($n = 3$). SEM images of (B) raw resveratrol, (C) spray-dried resveratrol. (D) Differential scanning calorimetric (DSC) thermographs of raw and spray-dried resveratrol.

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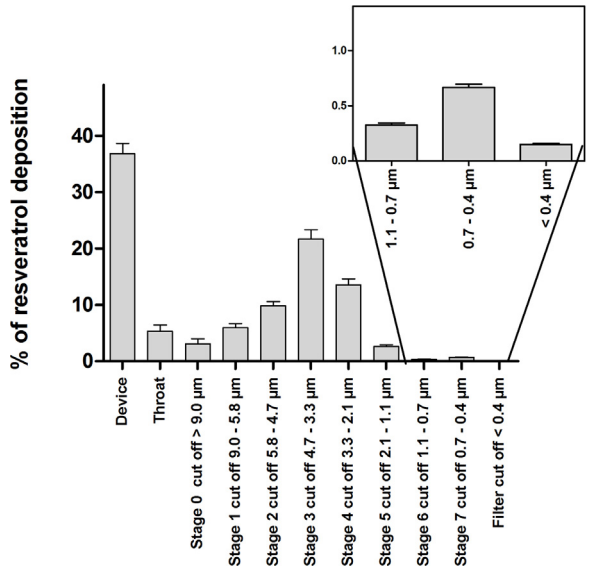

Fig. 2. Stage to stage aerosol deposition of spray-dried resveratrol across the ACI impactor. Data represents mean ± SD ($n = 3$).

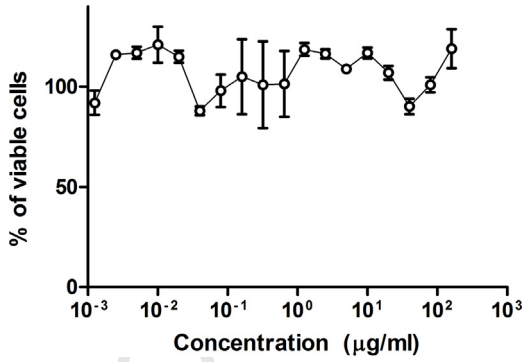

Fig. 3. Calu-3 cell viability profile after 72 h resveratrol treatment. Data represents mean ± SD ($n = 3$).

3.3. Biological responses in a representative epithelia cell line

3.3.1. Stability of resveratrol in cell culture media

In vitro cytotoxicity, transport and inflammation experiments were evaluated using spray-dried resveratrol on a Calu-3 cell line. Before performing these biological studies, resveratrol was studied with respect to its chemical stability in cell culture media in order to eliminate the possibility of resveratrol inactivation or degradation during cell experiments. Analysis of samples from the culture media revealed that resveratrol was stable during early incubation, in which the amount of resveratrol present at 24 h was 86%. Following incubation to 48 h and 72 h, the amount of remaining resveratrol was 77.19 and 57.15% of the initial concentration, respectively. This data demonstrated that the stability of resveratrol was predictable in culture medium containing serum, with 50% of degradation after three days of incubation. Our results are in agreement with previous studies in which resveratrol showed high stability in DMEM, RPMI 1640 and MEM (Long et al., 2010). In basic conditions (i.e., modified Eagle medium containing bicarbonate), the stability of resveratrol has been shown to be significantly affected, whereby 96% of resveratrol was degraded to polyphenol after 24 h of incubation at 37 °C, which could be attributable to oxidative degradation (Yang et al., 2010).

3.3.2. Cytotoxicity profiles of spray-dried resveratrol and transport across Calu-3 cell line

The dose response viability profile of resveratrol for Calu-3 cells is shown in Fig. 3. The MTS assay demonstrated that the wide range of resveratrol concentrations used, from 1.25 nM to 160 μM, were well-tolerated and non-toxic to Calu-3 cells. The viability of Calu-3 cells was maintained above 95% for this concentration range. A previous study has found that resveratrol was not toxic to A549 cell line when treated with higher concentration (100 μM) (Liu et al., 2010). However, the cytotoxicity effect was more pronounced when used on a human breast adenocarcinoma cell line (MCF-7), whereby cell viability was reduced to 60% when treated with 40 μM of resveratrol (Selvaraj et al., 2013). These data shown that resveratrol possess selective cytotoxic effect towards different cell lines. However, further experiments would be needed to be performed to investigate this aspect.

Despite the known biological effects of this compound, little is known about its in vitro transport across lung epithelial cells. Therefore, the transport of spray-dried resveratrol deposited directly onto Calu-3 cells using a in vitro lung model (modified ACI) (Haghi et al., 2014) was investigated. Table 1 shows the in vitro deposition and transport of resveratrol across Calu-3 cell monolayer. The values are presented as the percentage of total drug deposited (sum of resveratrol transported from the apical to the basal chamber, remaining on the cells surface and retained within the cells). Results shown that more than 80% of resveratrol was transported across Calu-3 cells within 4 h of deposition, and the rate of transport was independent of the initial deposited amount. The high resveratrol transport rate across cell monolayer could be correlated to the presence of fatty acids on the cell membrane that provide a lipophilic environment to improve the binding efficiency towards resveratrol. The high transport rate implies that resveratrol could reach the mesenchymal area and exert its anti-inflammatory activity in macrophages, neutrophils and lymphocytes implicated in COPD progression. In addition, resveratrol could also exert its anti-oxidant activity in the blood to scavenge the free radicals and reactive oxygen species present. In a previous study, it was noted that unusually high levels of reactive nitrogen species (RNS) and radicals were released by peripheral blood neutrophils in smokers' patients that subsequently lead to pathogenesis and development of COPD (Rahman and Adcock, 2006).

3.3.3. Anti-inflammatory effects of resveratrol

During the inflammation process in the lung, multiple inflammation markers, such as IL-8, IL-6 and TNF-α, are expressed (Rahman and MacNee, 2000). IL-8 levels were measured at 24 and 48 h, after stimulation of the Calu-3 cells with TGF-β1, TNF-α and LPS. Fig. 4 shows the anti-inflammatory activity of resveratrol in vitro. Statistically significant differences were observed between samples from the culture media after 48 h for resveratrol pre-treated cells ($p < 0.05$). IL-8 levels were measured to be 4526.2 ± 534.95 pg/ml, 5935 ± 2219.74 pg/ml and 5525.75 ± 250.06 pg/ml after 48 h of stimulation with TGF-β1, TNF-α and LPS, respectively; while the level of IL-8 after 48 h of exposure to TFG-β1, TNF-α and LPS for the cells pre-treated with resveratrol was 1229.67 ± 15.56 pg/ml, 236.33 ± 117.96 pg/ml and 3615.67 ± 676.18 pg/ml, respectively. Our results showed that resveratrol exhibited strong anti-inflammatory activity towards TGF-β1, TNF-α and LPS induced Calu-3 cells. There is also further evidence showing resveratrol to have the potential to inhibit the release of different types of inflammatory cytokines, such us granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-8 from human bronchial smooth muscle cells and macrophages in COPD patients after exposure to TNF-α and LPS, respectively (Donnelly et al., 2004b; Knobloch et al., 2010, 2011, 2014). More recently, resveratrol has shown the potential to inhibit the release of inflammatory mediators from human airway epithelial cells,

Table 1
In vitro deposition and transport of resveratrol across Calu-3 cell monolayer, using two different resveratrol doses. Data represents means ± SD ($n = 3$).

Dose of resveratrol (mg)	Amount of resveratrol (%)		
	Transported across Calu-3 monolayer	Remaining on the cell monolayer	Retained inside the cells
0.5	82.31 ± 15.49	1.86 ± 1.47	15.83 ± 14.47
1.0	87.18 ± 7.81	2.97 ± 1.94	9.85 ± 6.49

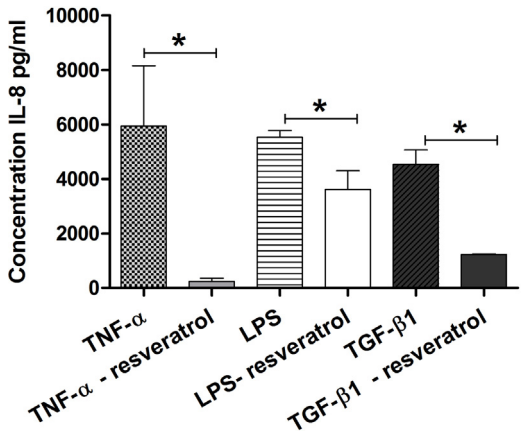

Fig. 4. Concentration of the inflammatory cytokine, IL-8, in culture media after stimulation of Calu-3 cells with TNF-α, LPS and TGF-β1 in presence and absence of resveratrol treatment. Data represents mean ± SD ($n = 4$). * $p < 0.05$.

which has been proven to be involved in inflammatory lung disease, such as COPD and asthma (Donnelly et al., 2004b). However, compared to glucocorticoids, resveratrol was found slightly less effective in reducing the expression of these markers from airway epithelial cells (Leung and Szefler, 1998). Nuclear factor kappa (NF-κB) is a key factor for activation of inflammatory proteins expression such as GM-CSF, IL-8, COX-2 and inducible nitric oxide synthase (iNOS) (Newton et al., 1997). It is known that NF-κB activity is regulated by different pathways such as IκB kinase and modification of p65 subunit of NF-κB complex (Fiebich et al., 2002). The reduction of IL-8 in our results indicated that resveratrol could potentially inhibit the activation of NF-κB. Donnelly et al., reported that the reduction of IL-8 expression from human primary epithelial cells was due to the inhibition of NF-κB activity by resveratrol (Donnelly et al., 2004b). Our results showed that resveratrol is more potent at suppressing the expression of IL-8 in cells induced with TNF-α, compared to LPS or TGF-β1. It has been reported that small amount of resveratrol (5 μM) could inhibit the activation of AP-1 involved in inflammation (Manna et al., 2000). Therefore, we speculate that resveratrol is more specific for TNF-α inhibition compared to other inducers.

3.4. Anti-oxidant activity of resveratrol

3.4.1. DPPH radical scavenging activity

The anti-oxidant activity of resveratrol was determined using in vitro DPPH assay by measuring the amount of DDPH radical after incubation for 30 min. In this assay, the free radical scavenging potential of resveratrol was measured in terms of its potential to reduce the concentrations of stable free radical DPPH. In the presence of hydrogen donating-antioxidant molecules, the odd electron of free radical DDPH was reduced and paired with hydrogen which resulting in a colour change from purple to yellow (a decrease in absorbance). The anti-oxidant activity results are shown in Fig. 5A and B. Lipoic acid, NAME and ascorbic acid were used as a negative and positive control, respectively. It was

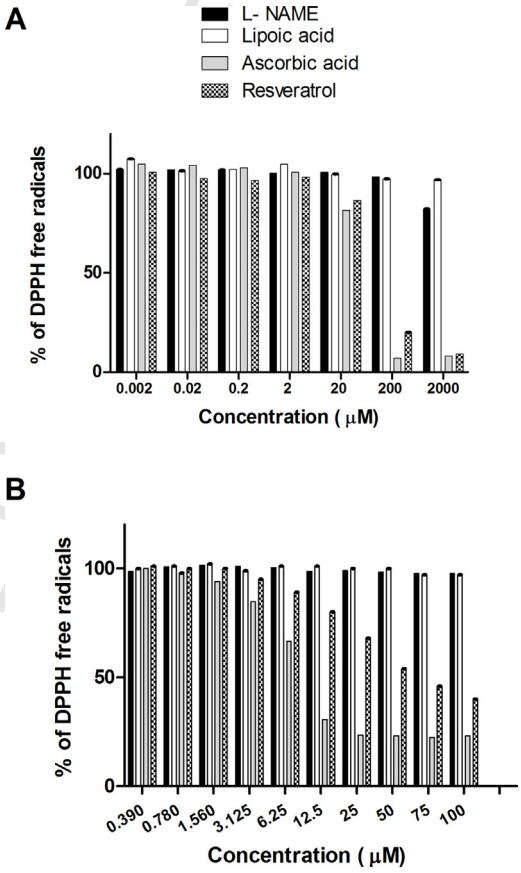

Fig. 5. Anti-oxidant activity of resveratrol. Absorbance values are directly proportional to % of DPPH free radicals. Data represents mean ± SD ($n = 4$).

demonstrated that the anti-oxidant activity of lipoic acid, NAME, ascorbic acid and resveratrol were concentration dependent. No scavenging activity was observed when up to 2 μM of these compounds reacted with the DPPH radicals. As shown in Fig. 5A, more than 18.44% and 13.49% of DPPH radicals were scavenged by ascorbic acid and resveratrol at 20 μM, respectively. However, lipoic acid and NAME demonstrated a negative effect on the anti-oxidant activity. Further increasing the concentration of ascorbic acid and resveratrol (up to 2000 μM), led to a significant reduction of the free DDPH radicals, with 8.28% and 9.13% of free radicals remaining for ascorbic acid and resveratrol, respectively. Both NAME and lipoic acid compounds did not show any significant anti-oxidant effect as the DPPH level was still maintained above 80% even at high concentrations. The scavenging effect was further investigated by reducing the concentration of the compounds from 0.39 to 100 μM. Once again, it was observed that concentration of resveratrol plays an important role in determining the level of DPPH. The reduction of DPPH radical was determined with resveratrol at relatively low concentration (3 μM). Resveratrol has been known for its potential role in preventing oxidation damage by cigarette smoke in human lung epithelial cells. Data in the literature have demonstrated that resveratrol scavenged free

radical activity through the interaction of the- *para* and *-meta* hydroxyl groups with free radicals (Stojanović et al., 2001). This amphipathic molecule is capable of scavenging lipid hydroperoxyl free radicals, as well as hydroxyl and superoxide anion radicals. The radical scavenging activity of this polyphenol was higher than commonly known anti-oxidants, such as vitamins E and C. Data demonstrated that this polyphenol molecule efficiently scavenges free radicals (about 50%) at the concentration of 100 μM, and the anti-oxidant activity was dose dependent (Fig. 5B). Our results are in good agreement with Soares et al. (2003) whereby 100 μM of resveratrol had the highest anti-oxidant activity and further increase in the concentrations of resveratrol resulted in reduced anti-oxidant activity. In addition, Acquaviva et al. have conducted a detailed anti-oxidant activity of resveratrol whereby resveratrol showed significant inhibition on xanthine oxidase, membrane lipid oxidation and DNA cleavage activities (Acquaviva et al., 2002). At a molecular level, it has been shown that resveratrol could reduce ROS production in lung epithelial cells induced by smoking via stimulation of glutamate-cysteine ligase (GCL) and glutathione (GSH) production (Kode et al., 2008). Additionally, it has been shown that the level of GCL in smoker's airway and COPD patients was considerably lower than healthy and non-smokers, which further suggests that this protein is involved in progression of lung injury via oxidative stress (Cerqueira et al., 2013; Harju et al., 2002).

3.4.2. DAN assay

Nitrosative stress (contributed by NO production) and nitration of protein in airway epithelial cells have been hypothesized to be the culprit for steroid resistances in COPD (Barnes et al., 2004b). In addition, NO is also involved in vasodilation, inhibiting platelet aggregation and smooth muscle cell proliferation, which could contribute to pulmonary hypertension (Cooper et al., 1996). Here, the effectiveness of resveratrol to inhibit the production of NO in Calu-3 induced with LPS and TGF-β1 was studied (Fig. 6A and B). In this study resveratrol was showed to effectively inhibit the expression of NO in Calu-3 cells induced by LPS and TGF-β1. As observed in the reduction of NO production, NAME and ascorbic acid showed comparatively weak inhibitory effect towards NO production, either in LPS or TNF-α induced Calu-3 cells. However, following resveratrol treatment, significant reductions ($p < 0.001$) in NO production by Calu-3induced with LPS and TGF-β1 were observed. NO was reduced by more than 75.96% and 41.09% when 100 μM of resveratrol was used to treat Calu-3 cell after induction with LPS and TGFβ-1 (final concentration of 100 ng/ml), respectively. A study has previously demonstrated that resveratrol was able to reduce the expression of NO on epithelial cells in the presence of different inducers (cigarette smoke and cytomix) (Donnelly et al., 2004a). Furthermore, a study by Bi et al. (2005) showed that the inhibition of NO release by resveratrol in microglia cells after stimulation with LPS, was concentration dependent. Resveratrol (100 μM) has also been reported to reduce by about 90% the NO production induced in macrophages with LPS, although the concentration of NO was evaluated with a different method (Man-Ying Chan et al., 2000).

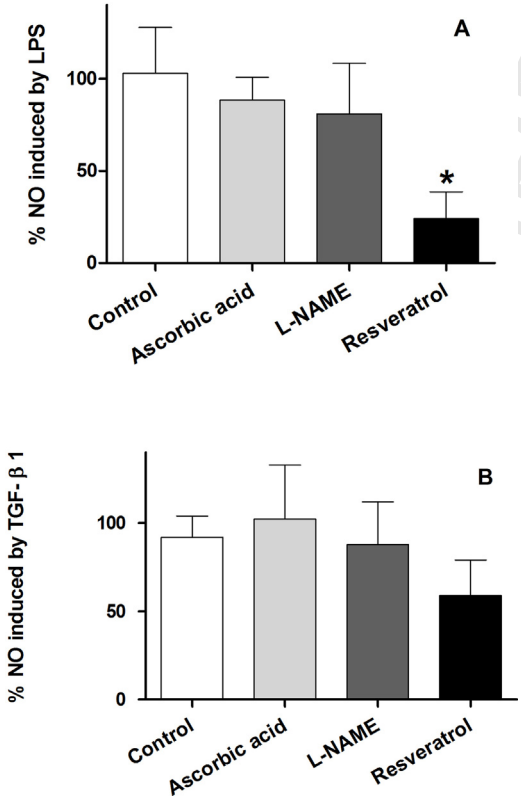

Fig. 6. NO production in Calu-3 cell after induction inflammation with (A) LPS and (B) TGF-β1. Data represents mean ± SD ($n = 9$). $^{*}p < 0.05$.

4. Conclusions

In this study, a respirable resveratrol dry powder formulation, to be used as alternative or add on-therapy for lung diseases such as COPD, was prepared and characterised. The formulation was found to be non-toxic on Calu-3 and had physico-chemical characteristics suitable for lung delivery. Moreover, resveratrol was found to have good anti-oxidant and anti-inflammatory properties on Calu-3, suggesting resveratrol could be of high therapeutic value in diseases like asthma and COPD where inflammation and oxidation is present. Future studies will be focusing on identifying the transporters protein involved in transporting resveratrol across Calu-3 and other cell lines representative of disease states like COPD or asthma.

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