International Journal of Pharmaceutics xxx (2015) xxx-xxx



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Contents lists available at ScienceDirect

International Journal of Pharmaceutics



journal homepage: www.elsevier.com/locate/ijpharm

Please cite this article in press as: Trotta, V., et al., In vitro biological activity of resveratrol using a novel inhalable resveratrol spray-dried

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

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ARTICLE INFO

Article history: Received 1 April 2015 Received in revised form 23 May 2015 Accepted 19 June 2015 Available online xxx

Keywords: Spray dried Resveratrol Calu-3 Inhalation Inflammation Oxidation

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 $39.9 \pm 1.1\%$ and $3.7 \pm 0.1 \mu$ m, respectively when assessed with an Andersen cascade impactor (ACI) at 60 l/min. The cytotoxicity results of spraydried 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- β 1) 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.

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1. Introduction

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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.,

formulation. Int J Pharmaceut (2015), http://dx.doi.org/10.1016/j.ijpharm.2015.06.033

http://dx.doi.org/10.1016/j.ijpharm.2015.06.033 0378-5173/© 2015 Published by Elsevier B.V.

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 **Q2** 28 is essential to improve patients' quality of life.

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 (HDAC₂) 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).

50 Resveratrol (trans-3,5,4-trihydroxystilbene) is a polyphenolic 51 compound synthesised in a large number of plant species and can 52 be found in grapes, berries and legumes (Francioso et al., 2014). 53 Resveratrol exhibits potent anti-oxidant and anti-inflammatory 54 properties and has shown potential against cardiovascular, cancer, 55 diabetes, and neurodegenerative diseases (Wood et al., 2010). Anti-56 inflammatory activity of this compound has been associated with 57 the inhibition of cyclooxygenase (COX)-2 transcription and 58 inhibition of COX-1 activity via peroxidase-mediate mechanism 59 (Santangelo et al., 2007). Specifically, in lungs it has been shown 60 that this compound is capable of scavenging oxygen-derived free 61 radicals (Arts and Hollman, 2005) and therefore has the potential 62 to be used as a adjunct therapy in the treatment of COPD (Knobloch 63 et al., 2010; Wood et al., 2010). It has also been shown that 64 polyphenolic compounds, such as resveratrol, are candidate 65 molecules for the development of novel anti-inflammatory 66 therapies for airway diseases, especially when patients become 67 non-responsive to glucocorticoids, such as glucocorticoid resistant 68 severe asthma and COPD (Donnelly et al., 2004b). Furthermore, 69 resveratrol has been shown to inhibit the release of inflammatory 70 cytokines from alveolar macrophages in COPD and therefore can be 71 considered as a suitable candidate for pharmacotherapy of 72 macrophages (Culpitt et al., 2003).

73 The aim of this study was to investigate the potential of 74 resveratrol as dry powder (DPI) for inhalation and its anti-75 inflammatory and anti-oxidant activity in the lung. In this study, 76 for the first time, the use of resveratrol as DPI has been presented. 77 The physicochemical characteristics of this new formulation were 78 investigated and the aerosol performance evaluated using 79 Andersen cascade impactor (ACI). In addition, the deposition, 80 transport and cell uptake of DPI resveratrol using an air interface 81 model of Calu-3 lung epithelial cell line incorporated onto a 82 modified ACI is also presented, together with its anti-inflammatory 83 and anti-oxidant activities on Calu-3.

⁸⁴ 2. Materials and methods

2.1. Materials

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86 Resveratrol (trans-3,4',5-trihydroxystilbene) was purchased 87 from Fagron Italia (Bologna, Italy). Calu-3 cell line (HTB-55) was 88 **Q3** purchased from the American Type Cell Culture Collection (ATCC, 89 Rockville, USA). L-Glutamine was from Invitrogen (Sydney, 90 Australia). Dulbecco's modified eagle's medium (DMEM), CelLy-91 ticTM M reagent, α -Lipoic acid, Nitro-L-arginine methyl ester (L-92 NAME), L-ascorbic acid, non-essential amino acids, 2,2-diphenyl-1-93 picrylhydrazyl (DPPH), 2,3-diaminonaphthalene (DAN) and lipo-94 polysaccharide from Escherichia coli 0111:B4 (LPS) (Sigma-Aldrich, 95 Sydney, Australia) were purchased from Sigma-Aldrich (Sydney, 96 Australia). Hank's balanced salt solution (HBSS) and tumor necrosis 97 factor alpha (TNF- α) were purchased from Invitrogen, (Sydney, 98 Australia) and transforming growth factor beta (TGF- β 1) was from 99 Sapphire Bioscience (Sydney, Australia) Human IL-8 ELISA Kit II BD 100 Opt EIATM (Becton Dickinson, Sydney, Australia). Analytical grade 101 solvents were purchased from Sigma (Sydney, Australia).

¹⁰² 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

formulation. Int J Pharmaceut (2015), http://dx.doi.org/10.1016/j.ijpharm.2015.06.033

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 $(5.0 \pm 0.1 \text{ mg})$ 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 601/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, $<4.7 \,\mu m$), the fine particle fraction (FPF) (FPD/ Total dose \times 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

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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 XbridgeTM C18 column (5 μ m, 4.6 \times 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 110

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161 content of resveratrol was guantified at wavelength 306 nm from 162 the peak area correlated with the predetermined standard curve 163 between 0.2 and $10 \,\mu g/ml$.

164 2.8. Culture of Calu-3 sub-bronchial epithelial cells

165 Calu-3 cells were purchased from the American Type Cell 166 Culture Collection (ATCC, Rockville, USA) and maintained in 167 Dulbecco's modified eagle medium nutrient mixture F-12Ham 168 (DMEM: NMF-12) supplemented with 10% (v/v) fetal bovine 169 serum, 1% (v/v) nonessential amino acid solution, and 1% (v/v) L-170 glutamine solution.

171 2.9. Chemical stability of resveratrol in Calu-3 culture media

172 The chemical stability of resveratrol was evaluated in complet-173 ed DMEM:NMF-12 media. Briefly, resveratrol solution was 174 prepared by dissolving in ethanol and diluting in the media with 175 a final concentration of resveratrol of 100 µM. Ethanol concentra-176 tion of was kept to less than 1%, in order to maintain cell viability 177 (Scalia et al., 2013). The samples were incubated in culture 178 condition (37 °C, humidified atmosphere and 5% CO₂) for 72 h. At 179 different set time points (0, 6, 24, 48 and 72 h) samples were 180 collected and the resveratrol content was guantified by HPLC. The 181 stability of resveratrol was expressed as the remaining of 182 resveratrol after incubation at different time points.

183 2.10. Cytotoxicity of resveratrol on Calu-3

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184 The cytotoxicity of resveratrol was assessed by measuring the 185 viability of Calu-3 cells after exposure with increasing resveratrol 186 concentrations (from 1.25 nM to 160 µM). Following incubation 187 with different concentrations of resveratrol and the addition of 188 CellTiter 96[®] Aqueous assay (MTS reagent, Promega, USA) the 189 absorbance was measured at 490 nm using a plate reader (Wallac 190 1420 VICTOR2[™], Multilaber Counter, Massachusetts, USA). Cell 191 viability was calculated with reference to the untreated cells and 192 the absorbance values were directly proportional to cell viability.

193 2.11. Deposition, transport and interaction of spray-dried resveratrol 194 with Calu-3 cells after aerosol deposition

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

197 Aerodynamic particle size distribution of spray-dried resvera-198 **Q4** trol was assessed using the ACI as outlined in the British 199 Pharmacopoeia. The experiments were performed in triplicate 200 using conventional impaction plates and modified plates, con-201 taining Snapwells inserts (placed at stage 3 of ACI with cut-off 202 diameter of 2.1–3.3 µm). Briefly, both the conventional and 203 modified ACI was connected to pump and flow rate adjusted to 204 601/min. The DPI device containing the spray-dried resveratrol was 205 then actuated in one shot into the ACI for 4s. After aerosol 206 deposition onto cell-free Snapwells, all ACI stages were washed to 207 quantify the total amount of drug deposition. The modified ACI 208 plate containing Snapwells was washed to determine the total 209 amount of resveratrol deposited on stage 3 and on each Snapwell.

210 2.11.2. Deposition and transport of spray-dried resveratrol on Calu-211 3 cell lines by incorporation into modified ACI plates

212 Calu-3 cells were seeded on Snapwells polyester membrane 213 (0.4 µm pore size, 1.12 cm² surface area) (Corning Costar, Lowell, 214 MA, USA) and maintained in air-interface configuration for 17-215 19 days. Spray-dried resveratrol was deposited on Calu-3 cells 216 grown on Snapwells using a modified ACI according to the method 217 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 $2.1-3.3 \,\mu\text{m}$) and resveratrol was deposited at flow rate of 601/min for 4s. 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% CO₂ for 24 h. Tumor necrosis factor alpha (TNF- α), transforming growth factor beta (TGF- β 1) 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 OptEIATM 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 ($60 \mu M$). 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-Larginine 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

257 The cells were treated with TGFB-1 and LPS 100 ng/ml for 48 h 258 and then with resveratrol 100 µM (final concentration) for further 259 24 h. The amount of nitric oxide (NO) produced from Calu-3 cells 260 was measured with 2,3-diaminonaphthalene (DAN). Ascorbic acid 261 and L-NAME at 100 µM (final concentration) were used as positive 262 and negative control, respectively, according to the method 263 described by Choi et al. (2009). Serial concentrations of nitrite 264 $(0.19-25 \,\mu\text{M})$ were prepared as standard. Based on the fluores-265 cence intensity (excitation = 360 nm and emission = 430 nm), the 266 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

270 One-way ANOVA or unpaired 2-tailed t-tests were performed to 271 determinate significance (which was quoted at the level of 272 p < 0.05) between treatment groups and control.

3. Results and discussion

274 Inflammation and oxidative stress are physiological factors 275 contributing to the development of many chronic diseases, such as

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276 cancer (Manna et al., 2000), atherosclerosis (Wu et al., 2001) and 277 lung diseases (i.e., COPD) (Rahman and MacNee, 2000). The 278 overproduction of oxidants from reactive oxygen species (ROS) and 279 reactive nitrogen species (RNS) are highly associated to DNA, RNA 280 and protein damage, as well as lipid oxidation, contributes to 281 significant damaging effect to cells (Rahman, 2008). Corticoste-282 roids, such as beclomethasone dipropionate, budesonide and 283 fluticasone, are well accepted for COPD treatment, however some 284 patients may develop "refractory effect" after prolong treatments 285 (Barnes and Adcock, 2009). Therefore, there has been great interest 286 in developing new bioactive agents that could effectively control 287 the inflammation and oxidation of this diseased state. Resveratrol 288 could potentially be effective in preventing the progression of 289 COPD, owing to its strong anti-oxidant and anti-inflammatory 290 effects (Anekonda, 2006; Das and Das, 2007; Donnelly et al., 291 2004b; Manna et al., 2000). The delivery of resveratrol as solution 292 is not suitable due to its instability, propensity for rapid oxidative 293 degradation in water and to its low solubility in water (Amri et al., 294 2012). As reported previously, the solubility of resveratrol in water 295 is approximately 30 mg/L which corresponds to 0.13 mM (Amri 296 et al., 2012).

297 3.1. Physicochemical characterisation of raw and spray-dried 298 resveratrol

299 In this study, resveratrol was manufactured as dry powder for 300 inhalation to be delivered directly to the lung for the reduction of 301 inflammation and oxidative stress in. Particle size distributions for 302 raw and spray-dried resveratrol, as analysed using laser diffraction, 303 are shown in Fig. 1A. Analysis of the data showed that the size 304 distributions for both samples varied significantly. Median volume 305 diameters of $13.3 \pm 0.1 \,\mu\text{m}$ and $3.9 \pm 1.0 \,\mu\text{m}$ (*n* = 3 ± SD) were 306 observed for both raw resveratrol and spray-dried resveratrol, 307 respectively (Fig 1A), showing spray-dried resveratrol to be 308 suitable for pulmonary administration (Todoroff and Vanbever, 309 2011). The sizes of raw resveratrol did not fall within the respirable 310 range, with approximately 90% of the spray-dried resveratrol

 $<9.7 \pm 1.0 \,\mu$ m. The SEM images of raw resveratrol presented a columnar shape with a volume size above 120 μ m (Fig. 1B), while, the spray-dried resveratrol particles showed a corrugated plate-like morphology (Fig. 1C), with a suitable size for lung deposition ($\leq 5 \,\mu$ m).

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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 $3.7 \pm 0.1 \,\mu$ m, 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 $2054.6 \pm 191.0 \,\mu$ g 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 \pm 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 \pm 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 24h 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 24h of incubation at 37°C, which could be attributable to oxidative degradation (Yang et al., 2010).

365 3.3.2. Cytotoxicity profiles of spray-dried resveratrol and transport across Calu-3 cell line 367 The does response viability profile of resveratrol for Calu 2 cell

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 μ M, 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 μ M) (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 μ M 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.



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

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- β 1, 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 48h for resveratrol pretreated cells (p < 0.05). IL-8 levels were measured to be $4526.2 \pm 534.95 \text{ pg/ml}, 5935 \pm 2219.74 \text{ pg/ml}$ and $5525.75 \pm$ 250.06 pg/ml after 48 h of stimulation with TGF- β 1, TNF- α and LPS, respectively; while the level of IL-8 after 48 h of exposure to TFG- β 1, 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 $3615.67 \pm$ 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 granulocytemacrophage 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,

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Please cite this article in press as: Trotta, V., et al., In vitro biological activity of resveratrol using a novel inhalable resveratrol spray-dried formulation. Int J Pharmaceut (2015), http://dx.doi.org/10.1016/j.ijpharm.2015.06.033

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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- β 1 in presence and absence of resveratrol treatment. Data represents mean \pm SD (n=4). * p < 0.05.

431 which has been proven to be involved in inflammatory lung 432 disease, such as COPD and asthma (Donnelly et al., 2004b). 433 However, compared to glucocorticoids, resveratrol was found 434 slightly less effective in reducing the expression of these markers 435 from airway epithelial cells (Leung and Szefler, 1998). Nuclear 436 factor kappa (NF-κB) is a key factor for activation of inflammatory 437 proteins expression such as GM-CSF, IL-8, COX-2 and inducible 438 nitric oxide synthase (iNOS) (Newton et al., 1997). It is known that 439 NF-KB activity is regulated by different pathways such as IKB 440 kinase and modification of p65 subunit of NF-кB complex (Fiebich 441 et al., 2002). The reduction of IL-8 in our results indicated that 442 resveratrol could potentially inhibit the activation of NF-KB. 443 Donnelly et al., reported that the reduction of IL-8 expression from 444 human primary epithelial cells was due to the inhibition of NF-KB 445 activity by resveratrol (Donnelly et al., 2004b). Our results showed 446 that resveratrol is more potent at suppressing the expression of IL-447 8 in cells induced with TNF- α , compared to LPS or TGF- β 1. It has 448 been reported that small amount of resveratrol (5 µM) could 449 inhibit the activation of AP-1 involved in inflammation (Manna 450 et al., 2000). Therefore, we speculate that resveratrol is more 451 specific for TNF- α inhibition compared to other inducers.

⁴⁵² 3.4. Anti-oxidant activity of resveratrol

⁴⁵³ 3.4.1. DPPH radical scavenging activity

454 The anti-oxidant activity of resveratrol was determined using in 455 vitro DPPH assay by measuring the amount of DDPH radical after 456 incubation for 30 min. In this assay, the free radical scavenging 457 potential of resveratrol was measured in terms of its potential to 458 reduce the concentrations of stable free radical DPPH. In the 459 presence of hydrogen donating-antioxidant molecules, the odd 460 electron of free radical DDPH was reduced and paired with 461 hydrogen which resulting in a colour change from purple to yellow 462 (a decrease in absorbance). The anti-oxidant activity results are 463 shown in Fig. 5A and B. Lipoic acid, NAME and ascorbic acid were 464 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 \pm 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 $2\,\mu$ M 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 antioxidant activity. Further increasing the concentration of ascorbic acid and resveratrol (up to 2000 μ M), 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

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487 radical activity through the interaction of the- para and -meta 488 hydroxyl groups with free radicals (Stojanović et al., 2001). This 489 amphipathic molecule is capable of scavenging lipid hydroperoxyl 490 free radicals, as well as hydroxyl and superoxide anion radicals. The 491 radical scavenging activity of this polyphenol was higher than 492 commonly known anti-oxidants, such as vitamins E and C. Data 493 demonstrated that this polyphenol molecule efficiently scavenges 494 free radicals (about 50%) at the concentration of 100 μ M, and the 495 anti-oxidant activity was dose dependent (Fig. 5B). Our results are in 496 good agreement with Soares et al. (2003) whereby 100 µM of 497 resveratrol had the highest anti-oxidant activity and further 498 increase in the concentrations of resveratrol resulted in reduced 499 anti-oxidant activity. In addition, Acquaviva et al. have conducted a 500 detailed anti-oxidant activity of resveratrol whereby resveratrol 501 showed significant inhibition on xanthine oxidase, membrane lipid 502 oxidation and DNA cleavage activities (Acquaviva et al., 2002). At a 503 molecular level, it has been shown that resveratrol could reduce ROS 504 production in lung epithelial cells induced by smoking via 505 stimulation of glutamate-cysteine ligase (GCL) and glutathione 506 (GSH) production (Kode et al., 2008). Additionally, it has been 507 shown that the level of GCL in smoker's airway and COPD patients 508 was considerably lower than healthy and non-smokers, which 509 further suggests that this protein is involved in progression of lung 510 injury via oxidative stress (Cerqueira et al., 2013; Harju et al., 2002).

3.4.2. DAN assay

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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-B1 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-B1 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 μ M) 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).

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.

Acknowledgements

A/Professor Traini is the recipient of an Australian Research Council Future Fellowship (project number FT12010063). Professor Young is the recipient of an Australian Research Council Future Fellowship (project number FT110100996).

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