Co-spray dried resveratrol and budesonide inhalation formulation for reducing inflammation and oxidative stress in rat alveolar macrophages

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Abstract

Oxidative stress is instrumental in the pathogenesis and progression of chronic obstructive pulmonary disease (COPD). Novel therapeutic strategies that target macrophages, based on the use of antioxidant compounds, could be explored to improve corticosteroids responses in COPD patients. In this study, inhalable microparticles containing budesonide (BD) and resveratrol (RES) were prepared and characterized. This approach was undertaken to develop a multi-drug inhalable formulation with antioxidant and anti-inflammatory activities for treatment of chronic lung diseases. The inhalable microparticles containing different ratio of BD and RES were prepared by spray drying. The physico-chemical properties of the formulations were characterized in terms of surface morphology, particle size, physical and thermal stability. Additionally, in vitro aerosol performances of these formulations were evaluated with the multi-stage liquid impinger (MSLI) at 60 and 90 l/min, respectively. The cytotoxicity effect of the formulations was evaluated using rat alveolar macrophages. The biological responses of alveolar macrophages in terms of cytokine expressions, nitric oxide (NO) production and free radical scavenging activities were also tested. The co-spray dried (Co-SD) microparticles of all formulations exhibited morphologies appropriate for inhalation administration. Analysis of the deposition profiles showed an increase in aerosol performance proportional to BD concentration. Cell viability assay demonstrated that alveolar macrophages could tolerate a wide range of RES and BD concentrations. In addition, RES and BD were able to decrease the levels of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in lipopolysaccharide (LPS) induced alveolar macrophages.
This study has successfully established the manufacture of Co-SD formulations of RES and BD with morphology and aerosol properties suitable for inhalation drug delivery, negligible \textit{in vitro} toxicity and enhanced efficacy to control inflammation and oxidative stress in LPS-induced alveolar macrophages.

\textbf{Keywords:} Alveolar macrophage; Anti-inflammatory; Anti-oxidant; Budesonide; Chronic obstructive pulmonary disease; Resveratrol, Dry powder inhalation.
1. Introduction

Chronic obstructive pulmonary disease (COPD) represents one of the leading causes of morbidity and mortality worldwide (Viegi, Scognamiglio et al. 2001). COPD is a lung disease characterized by chronic inflammation, airflow limitation, hyper mucous production, emphysema, bronchoconstriction, a decline of respiratory activity and eventual death (Barnes 2007). The pathogenesis of COPD is multi-factorial which includes genetic predisposition, age, inhaled pollution and cigarette smoke. Previous studies have shown that cigarette smoke (CS) is the main risk factor for the development and progression of COPD (Rabe, Hurd et al. 2007). This is because CS causes a production of reactive oxygen species (ROS) that increase the oxidative stress and for this reason it is implicated in the pathogenesis and in irreversible airway inflammation. Oxidative stress causes airway inflammation by stimulating the release of inflammatory mediators such as IL-6, IL-8 and TNF-α. These inflammatory mediators result in an increase of ROS and hence an increase in oxidative stress in the lungs (Rahman and Adcock 2006). Furthermore, COPD exacerbations caused by chronic bacterial infection can result in additional airway inflammation owing to the further release of pro-inflammatory mediators (Khair, Davies et al. 1996).

Alveolar macrophages are one of the first lines of defence of the respiratory tract against inhaled noxious agents. Although airway epithelial cells as a whole are involved in COPD, several studies have demonstrated that alveolar macrophages play an important role in the pathogenesis of the disease (Tetley 2002; Hodge, Hodge et al. 2007), mainly in smokers, by regulating the release of inflammatory mediators that attract neutrophils into the airway (Kent, Smyth et al. 2008). Corticosteroid molecules are able to suppress
the release of these inflammatory mediators in alveolar macrophages but these drugs are relatively ineffective in COPD patients (Barnes, Ito et al. 2004, Bhavsar, Hew et al. 2008). For this reason a novel therapeutic strategy is needed.

The current first-line therapy for COPD involves the use of bronchodilators such as long acting beta agonists (LABA) in combination with inhaled corticosteroids. However, unlike other inflammation-based diseases, such as asthma, corticosteroids are less effective in improving lung function of COPD patients, and have limited effect in reversing the progression of tissue damage (Pauwels, Löfdahl et al. 1999; Pauwels, Buist et al. 2001; Dahl, Chung et al. 2010; Vogelmeier, Hederer et al. 2011). Furthermore, previous studies have shown that ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors such as nuclear factor-kappa B (NF-κB) (Rahman and MacNee 1998). This results in vicious cycle of oxidative stress by ROS and airway inflammation. Histone deacetylases activities are required for NF-κB blockade by corticosteroid receptors (Barnes, Ito et al. 2004; Barnes 2009). In several cases COPD patients became non-responsive to corticosteroid treatment as histone deacetylases (HDAC2) activities can become inhibited in presence of oxidative stress (Barnes, Ito et al. 2004).

For these reasons, the use of anti-oxidant compounds in association with one of the corticosteroids drugs could provide a new therapeutic approach for the treatment and management of COPD.
Polyphenolic compounds are potential candidate molecules since these compounds naturally exhibit potent anti-oxidant and anti-inflammatory activities (Biswas, Hwang et al. 2013). Resveratrol (3, 5, 4'-trihydroxystilbene) (RES) is a naturally occurring polyphenolic compound found in a large number of plant species (e.g. grapes, berries and legumes) and in red wine. Resveratrol is a light sensitive molecule with two isoforms, cis-resveratrol and trans-resveratrol, the trans form being more stable and also the more biologically activity form (Neves, Lucio et al. 2012). The anti-oxidant activity of this polyphenol is due to its ability to scavenge free radicals (Arts and Hollman 2005). Furthermore, different studies have shown the differential properties of resveratrol as anti-inflammatory, anti-allergic, antiviral, anti-carcinogenic and anti-asthmatic (Cheong, Ryu et al. 1999; Docherty, Fu et al. 1999; Manna, Mukhopadhyay et al. 2000; Alarcón de la Lastra and Villegas 2005; Faith, Sweet et al. 2006; Athar, Back et al. 2009; Lee, Kim et al. 2009). Specifically in the lungs, in vitro and in vivo experiments have shown that RES can reduce inflammation in lung cells, scavenging oxygen-derived free radicals; subsequently, RES maybe a potential adjunct therapy in the treatment of COPD (Trotta, Lee et al. 2015). In addition, RES has been shown to inhibit the release of inflammatory cytokines from alveolar macrophages in COPD and therefore can be considered a suitable candidate for pharmacotherapy of macrophages (Culpitt, Rogers et al. 2003).

The aim of this study was to develop inhalable microparticles containing RES and budesonide (BD), a common anti-inflammatory corticosteroid. To the authors’ knowledge, this is the first attempt to deliver a combination formulation containing anti-oxidant and anti-inflammatory compounds for improvement of COPD. Different series
of co-spray dried (Co-SD) formulations were prepared and the physico-chemical characteristics and *in vitro* aerosol performance were investigated. Importantly, the biological responses of alveolar macrophages cell lines in terms of cell viability, anti-inflammatory and anti-oxidant activities were evaluated with the prepared spray dried formulations.

2. Experimental methods

2.1. Materials

Resveratrol, *trans*-3,4′,5-trihydroxystilbene, (RES) was purchased from Fagron Italia (Bologna, Italy). Budesonide (BD) used in this study was supplied by Yicheng Chemical Corp, Jiangsu, China. Nitro-L-arginine methyl ester (L-NAME), α-Lipoic acid, L-ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipopolysaccharide (LPS) from *Escherichia coli* and 2,3-diaminonaphthalene (DAN) were purchased from Sigma-Aldrich (Sydney, Australia). Other cell culture reagents inculding phosphate buffer saline (PBS), fetal bovine serum (FBS) and Ham’s F-12 nutrient mix media were purchased from Invitrogen, (Sydney, Australia). Elisa kit for determination of inflammation markers such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were supplied from BD Bioscience (Sydney, Australia). All solvents used were analytical grade and purchased from Biolab (Victoria, Australia).

2.2. Preparation of spray dried (SD) microparticles

Single and combination microparticles were produced by spray drying RES and/or BD using a Buchi B-290 Mini spray dryer (Buchi, Switzerland) under conditions listed in Table 1. Both RES and BD, either alone or in combination, were dissolved in ethanol-
water (80:20% v/v) and spray dried using a nozzle of 1.4 mm at feed rate of 40% and
aspiration of 100% in a close loop configuration. Single or combinations of RES and
BD with final dry weight percentages (%w/w) were labelled as follow: 100% RES, 75%:25% RES-BD, 50%:50% RES-BD, 25%:75% RES-BD and 100% BD.

2.3. Physico-chemical characterization of SD formulations

2.3.1. Scanning electron microscopy (SEM)

Scanning electron microscopy was used to study the morphology of the SD formulations. Briefly, SD-RES, SD-BD and Co-SD RES-BD formulations were dispersed on carbon tapes, placed onto aluminium stubs and coated with gold at 15 nm thickness (JEOL USA Smart Coater). A bench top SEM (JMC, 6000 JEOL, Japan) operating at 15KV was used for imaging samples.

2.3.2. Laser diffractive of Co-SD microparticles

Particle size distribution of Co-SD microparticles were determine by laser diffraction (Mastersizer 3000, Malvern, Worcestershire, United Kingdom). Approximately 10 mg of microparticles were dispersed in air with a feed pressure and feed rate of 4 bars and 35%, respectively. Co-SD formulations were analysed in triplicate with an obscuration value between 0% and 15%. Moreover, a refractive index of 1.67 was used for all measurements and was calculated by the average of the refractive index of the two single components (Salama, Young et al. 2014, Trotta, Lee et al. 2015).

2.3.3. Thermal analysis of SD formulations
The thermal responses of the raw RES and BD and the single and Co-SD formulations were investigated using differential scanning calorimetry (DSC; DSC823e, Mettler-Toldeo, Switzerland). Approximately 3 to 5 mg of powder were weighed and crimp-sealed in DSC aluminium pans and heated at 10°C/min over a temperature range of 25–320 °C. The exothermic and endothermic responses of the SD microparticles, raw RES and raw BD were determined using STARE software V.11.0x (MettlerToledo). In addition, the temperature stability and solvent evaporation of each formulation was assessed using the thermal gravimetric analysis (TGA; Mettler-Toledo, Switzerland). Approximately 13 mg of SD powders were placed onto aluminium crucible pans. The weight loss of the samples was evaluated by heating the samples from 25–200°C with scanning rate of 10°C/min under constant nitrogen gas at flow 110 ml/min. Data were analysed using STARE software V.11.0x (MettlerToledo, Switzerland) and expressed as the percentage of weight loss with respect to initial sample weight.

2.3.4. *In vitro aerosol performance*

The multi stage liquid impinger (MSLI; CopleyScientific Ltd., Nottingham, UK) was used to evaluate the aerosol performance of SD microparticles, as per the methodology outlined in the British Pharmacopoeia(Pharmacopoeia 2009). Approximately 10 mg of SD powders was loaded in a size 3 gelatin capsules (Capsugel®, Sydney, Australia) and placed into the dosage chamber of standard-resistance RS01 dry powder inhalation device (Plastiape®, Osnago, Italy). The device was connect to a custom-built mouthpiece addpator connected to a US Pharmacopeia induction port (throat), connected to the MSLI. The aerosol performance was assessed at two different flow rates, 60 l/min and 90 l/min (calibrated using a suitable flow meter). After actuation, the
device, capsule, adaptor, throat, all MSLI stages and filter were washed separately with methanol:water (80:20 %v/v) into suitable volumetric flasks. The samples were analysed using high performance liquid chromatography (HPLC). All experiments were conducted in triplicate and the aerosolization efficiency was evaluated in terms of fine particle dose (FPD) (amount of RES and BD recovered from stage 3 to filter, corresponding to the amount of microparticles with a diameter < 6.8 μm), fine particle fraction of the loaded dose (FPF_{LD}) (percentage ratio of FPD respect the total amount of RES and BD collected from device, capsule, adaptor, throat and all MSLI stages and filter).

2.4. HPLC quantification of RES and BD

Resveratol and BD were quantified using a validated HPLC method. A Shimadzu Prominence UFLC system equipped with a DGU-20 A5R Prominent degasser unit, LC-20 AD Liquid chromatography, SIL-20A HT Autosampler and SPD-20A UV-Vis detector was used (all Shimadzu Corporation, Japan). For both, RES and BD, the volume of sample injection was 100 μl and the detection wavelength for BD and RES were 243 nm and 306 nm, respectively. Quantification of BD was conducted using methanol:water 80:20 (%v/v) as the mobile phase at flow rate of 1 ml/min in isocratic mode with a Luna C18 column (3 μm, 4.6 × 150 mm) (Phenomenex, Sydney, Australia). The linearity was confirmed between 0.2 and 50 μg/ml ($R^2 = 0.999$). For quantification of RES, the mobile phase consisted of methanol:water 60:40 (%v/v) with 0.5% acetic acid (%v/v). Samples were analysed using a XbridgeTM column (5 μm, 4.6 × 150 mm), (Waters, Massachusetts, USA) at a flow rate of 0.7 ml/min. The content of
resveratrol was quantified from the peak area correlated with the predetermined standard curve between 0.2 and 50 μg/ml ($R^2 = 0.999$).

2.5. In vitro biological responses

2.5.1. Alveolar macrophage NR8383 cell line and culture conditions

Rat alveolar macrophage NR8383 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were tested for mycoplasma contamination (mycoplasma PCR detection Kit) and found to be negative. Cells were maintained in complete Ham’s F-12 nutrient mix medium with 10% (v/v) heat-inactivated FBS in a humidified atmosphere containing 5% CO$_2$ at 37 °C. Medium was changed every 3 days and passaged (passage number 20-30) according to ATCC recommended guidelines.

2.5.2. MTS cell cytotoxicity assay in response to SD formulations

To evaluate the suitability of delivering RES and BD into lungs, a in vitro MTS cytotoxicity assay was conducted. Alveolar NR8383 macrophages were seeded onto 96-well plates at a density of 50,000 cells/well and incubated at 37°C in a humidified atmosphere at 5% CO$_2$ overnight. This was followed by the addition of drugs (RES and BD) with increasing concentrations, from 0.612 μM to 50 μM, either as single or combined formulations. After 72 h of incubation, 20 µl of CellTiter 96® Aqueous assay (MTS reagent, Promega, USA) was added into each well and incubated for 4 h. The absorbance of the samples were measured at 490 nm with microplate reader (Wallac 1420 VICTOR$^{TM}$, Multilaber Counter, Massachusetts, USA). Experiments were
performed in triplicate and data expressed as % cell viability relative to untreated control.

2.6. In vitro anti-oxidant activities of SD formulations

2.6.1. DPPH radical scavenging activity

The anti-oxidant activity of the SD formulations were evaluated using a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay according the method described by Basnet et al. (Basnet, Hussain et al. 2012). Different concentrations (1 µM to 1 mM) of RES and BD either in single or combination formulation were reacted with 60 µM DPPH ethanolic solution. After 30 min of incubation in the dark, the absorbance of the reactive mixture was measured at 520 nm. The same concentrations of Nitro-L-arginine methyl ester (L-NAME) and α-Lipoic acid as well as ascorbic acid were used as negative and positive controls, respectively. All experiments were conducted in triplicate.

2.6.2. NO production using 2,3-diaminonaphthalene (DAN) assay

The ability of SD formulations to reduce the production of nitric oxide (NO) in LPS-induced alveolar macrophages were evaluated using DAN assay in accordance to the method described previously (Choi, Zhang et al. 2009, Trotta, Lee et al. 2015). Briefly, alveolar macrophage cells were treated with 5 ng/ml LPS for 24 h. This was followed by treatment with SD formulations with different concentrations. Samples were collected at different time points (12 h to 72 h). Ascorbic acid and L-NAME were used as positive and negative control, respectively. Serial concentrations of nitrite (0.19 to 25 µM) were prepared as standard. The concentration of NO was determined by measuring the fluorescence intensity (excitation=360 nm and emission= 430 nm).
2.7. Anti-inflammatory activity of SD formulations against LPS-induced alveolar macrophages NR8383 cells

The anti-inflammatory effects of the SD formulations were evaluated using a LPS-induced alveolar macrophages cell study. Approximately 500,000 cells/well were seeded into 6 well plates with 5 ng/ml of LPS before incubation for 24 h at 37°C, with 5% CO₂. The cells were subsequently treated with the drugs at different concentrations, ranging from 3.125 μM to 50 μM, at different incubation time points. It should be noted that the LPS concentration used in this experiment was found not toxic to the cell (cell viability above 95%) as the cell viability was pre-determined before the study. At different time points, the samples were harvested by centrifugation at 13,000 rpm at 4°C for 5 min. A clear supernatant was kept in –80°C. Cytokine expression (IL-6 and TNF-α) was measured using Rat IL-6 and TNF-α ELISA kits according to the manufacturer instructions.

2.8. Statistical analysis

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

3. Results and discussion

3.1. Physical-chemical characterization of SD formulations

3.1.1. Assessment of particle size and morphology
In this study, inhalable microparticles containing anti-oxidant RES and anti-inflammatory BD compounds were formulated using SD. Particle sizes with uniform distributions within inhalable range were obtained via manipulation of the spray-drying parameters, which included inlet temperature, outlet temperature, atomisation, and feed rate of solution. Additional factors such as ratios of co-solvent and concentrations feed solutions led to significant changes in particle distribution as well as yield. The inlet temperature range was chosen so that the outlet temperature would be below the glass transition temperature ($T_g$) of BD and RES, which are reported as ~90°C and >340 °C, respectively (Nolan, Tajber et al. 2009; Cash, Davis et al. 2013). Through the optimization of spray-drying conditions, five formulations with RES to BD concentration were successfully prepared. These included: 100% BD, 75%BD/25%RES, 50%BD/50%RES, 25%BD/75%RES and 100% RES. Throughout the manuscript, these formulations were denoted as formulation 0%, 25%, 50%, 75% and 100% respectively, in accordance to the percentage w/w RES in the final SD particles.

As seen from the SEM images presented in Figure 1, the microparticles of single spray dried and Co-SD combinations at different concentrations showed spherical geometries; morphological characteristic typical of a spray-drying process. Qualitative measurement indicated that the particles were between 1 to 5 µm in size. Uniform spherical microparticles with smooth surface morphology were characteristics of SD BD alone (Figure 1A). In comparison, spray dried RES alone (100%) exhibited spherical particles with rough surfaces (Figure 1E). When Co-SD, the particles exhibited a change in morphology between the two single drug formulations based on the ratio of BD to RES.
Interestingly, overall Co-SD microparticles exhibited increase in smoothness of particles’ surface proportional to the increase of BD concentration (Figure 1B, C and D). It is speculated that, as BD is more hydrophobic and with higher molecular mass, in the binary system it would preferentially accumulate on the air-liquid interface of the droplet during the spray drying process (Parlati, Colombo et al. 2009).

Particle size analysis of Co-SD microparticles were measured using laser diffraction. The volume weighted median diameter ($D_{50}$) of SD BD was $1.0 \pm 0.01 \mu m$, significantly smaller ($p < 0.05$) than SD RES ($7.74 \pm 1.66 \mu m$). A gradual increase in particle size for Co-SD formulations with increasing RES concentration was observed. As such, the diameter for Co-SD containing 25%, 50% and 75% RES were $1.2 \pm 0.02$, $2.32 \pm 1.01$ and $6.23 \pm 1.32 \mu m$, respectively. Moreover the presence of BD on the surface reduced the cohesiveness between particles of RES, thus resulting in decreased particles’ agglomeration and increased aerosol performance, as reported below (Adi, Adi et al. 2008).

3.1.2 Thermal characteristics of spray dried formulations

The thermal response of spray dried formulations containing either RES and/or BD is shown in Figure 2. Both thermograms of crystalline raw BD and RES demonstrated only a single endothermic peak indicative of melting at 260°C and 270°C, respectively. Spray drying processes did not change RES crystallinity, as seen in the presence of single endothermic melting at 269°C for SD RES alone (Trotta, Lee et al. 2015). The DSC thermogram for SD BD alone however showed a broad exothermic peak at 130°C, followed by a sharp endothermic peak at 260°C. The presence of exothermic peak prior to melting suggests that SD BD particles were amorphous and underwent phase
transition from amorphous to crystalline when heated to 130°C. In general, the melting peaks of Co-SD formulations were shifted to lower temperature. In addition, irrespective of the concentrations of RES and BD used, the Co-SD formulations exhibited a ‘new’ endothermic peak at 190°C, which was not thermodynamically related to raw RES or BD compounds (Figure 2). The presence of this peak suggested the chemical interaction between the two compounds which resulted in the shifting of the melting peak proportional to the strong solid–solid interaction (Budavári, Zelko et al. 1999). For instance, for Co-SD containing 75% RES, two endothermic peaks were observed at 190°C and 257°C, respectively. In the Co-SD formulations containing 75% BD, an exothermic peak at 167 °C and one endothermic peak at 206 °C were also observed.

Further, spray dried formulations were stable up to 200 °C with TGA data reporting a weigh lost ≤ 0.1% w/w.

3.2. Aerosolization efficiency of spray dried formulations

The evaluation of aerosol efficiency for dry powder inhalers is an important tool for predicting the amount of the inhalable microparticles that could reach the lungs. The aerosol performance of single SD and the Co-SD formulations were tested using the MSLI at two different flow rates (60 l/min and 90 l/min). Data are expressed as the percentage deposition of BD and RES recovered in each stage of MSLI, throat, and device over the total mass calculated (Figure 3 and 4). The FPF\textsubscript{LD} values of SD BD were significantly higher than for SD RES at both flow rates ($p < 0.001$). At 60 l/min, the FPF for SD BD and SD RES were $39.4 \pm 2.8\%$ and $26.3 \pm 1.5\%$, respectively. Meanwhile, the FPF of SD BD ($45.7 \pm 1.4\%$) was 2-fold higher than SD RES ($25.8 \pm$
Furthermore, SD RES had a higher throat deposition (11.5 ± 1.6% and 17.8 ± 0.9%) compared to SD BD with values of deposition of 3.6 ± 0.9% and 5.0 ± 0.3% at 60 l/min and 90 l/min, respectively. This could be attributed to the adhesive/cohesive nature of RES and high surface area of the microparticles, which promoted agglomerates; hence the poor dispersion at set flow rates (Figure 3 and 4). It was also observed that deposited doses of each component (RES and BD) in the throat, devices and all stages of the MSLI had no significant differences, irrespective of the Co-SD combinations. These results indicate that the Co-SD particles were homogeneous in composition, rather than binary system containing individual components. Analysis of the deposition profiles of Co-SD formulations showed an increase in aerosol performance proportional to RES decreasing concentration. The FPF of RES for Co-SD formulations containing 25%, 50% and 75% RES were 42.5 ± 1.7%, 38.8 ± 2.9%, and 23.8 ± 3.7%, respectively at 60 l/min (Figure 3). Similarly, the FPF calculated for BD were 42.5 ± 1.6%, 38.5 ± 3.1%, and 23.0 ± 1.2%, respectively (Figure 3). At 90 l/min, the FPF of RES for Co-SD formulations containing 25%, 50% and 75% RES were 46.7 ± 2.8%, 41.7 ± 4.4%, and 25.9 ± 4.2%, respectively (Figure 4). Moreover the FPF of BD for Co-SD formulations containing 25%, 50% and 75% RES were 46.5 ± 2.8%, 41.2 ± 4.3% and 26.4 ± 2.7% at 90 l/min.

3.3. In vitro activity of RES and BD using alveolar macrophages cells

3.3.1. Viability of alveolar macrophages cells to RES and BD

It is widely accepted that inflammation and oxidative stress is the central pathogenesis contributing to the development of chronic lung diseases such as asthma and COPD (MacNee 2001). Continuous exposure to noxious agents such as cigarette smoke,
viruses, bacteria and foreign particulates promotes inflammation and oxidative stress in lung cells and alveolar macrophages. Budesonide is the treatment of choice to control inflammation but some COPD patients are unresponsive towards corticosteroids. Therefore, combining BD with RES, which possesses strong anti-oxidant and anti-inflammatory properties, could be beneficial for COPD treatment. Prior to anti-inflammatory and anti-oxidant studies, any potential cytotoxicity effects of RES and BD towards alveolar macrophage cells need to be evaluated. Different SD formulations, both as combination or single compounds, were added to rat alveolar macrophages with increasing concentrations (0.612 to 50 µM) and evaluated using the MTS cytotoxicity assays and the data are shown in Figure 5. In general, the viability of alveolar macrophage cells did not decrease significantly with RES and/or BD increase regardless of in single (Fig. 5 A) or combination formulations (Fig. 5 B). As shown in Figure 5A, alveolar macrophage cells demonstrated satisfactory tolerance towards RES and BD in the range of concentrations investigated. These results are in accordance with published studies indicating that RES was well tolerated by airway lung cells, such as Calu-3, A549 and murine macrophages cells (Billack, Radkar et al. 2008; Liu, Tsai et al. 2010; Trotta, Lee et al. 2015). Moreover BD has been shown to be relatively safe for alveolar macrophages even at micromolar concentration (Zetterlund, Larsson et al. 1998).

3.3.2. Anti-oxidant activities of Co-SD formulations

The anti-oxidant activity of RES and BD were investigated by measuring their ability to scavenge free reactive radicals, using the DPPH assay (Figure 6A). The hydrogen donating molecules reduce the free radical DPPH by pairing its odd electron to hydrogen, thus causing a colour change from purple to yellow (a decrease in
absorbance). Lipoic acid and L-NAME were used as negative controls while vitamin C, a potent free radical scavenger, was used as positive control in this study (Figure 6A). As expected, no scavenging activities were observed for lipoic acid and L-NAME even at high concentration (500 µM). Vitamin C demonstrated the highest anti-oxidant potential by reducing up to 80% DPPH free radicals at 40 µM which was consistent with previous study (Trotta, Lee et al. 2015). As for BD, no detectable radical scavenging activities were found in the ranges of concentrations studied (25 to 500 µM). Meanwhile, it was observed that the anti-oxidant activities of RES were dose-dependent. The increase of RES from 2 to 500 µM led to significant reduction of free DPPH radicals, whereby only 87.8% and 25.2% of DPPH remained, respectively (Figure 6A). The dose-response curve of RES showed a gradual increase in DPPH scavenging activity while dramatic elevation of anti-oxidant activity was observed for vitamin C. For instance, approximately 70% and 31% of DPPH free radicals remained after treatment with 3.9 and 7.8 µM vitamin C, respectively (Figure 6A).

Oxidative damage in lung tissues caused by stimuli such as cigarette smoke is well known. The introduction of an anti-oxidant molecule such as resveratrol to existing therapies to potentially treat oxidative-stress related lung injury (i.e. COPD) could consequently be advantageous. In both smokers and COPD patients it was shown that the expression of glutamate-cysteine ligase (GCL) and glutathione (GSH) was reduced compared to healthy subjects, hence an indication of lung injury caused by oxidative stress (Harju, Kaarteenaho-Wiik et al. 2002; Cerqueira, Khaper et al. 2013), while resveratrol can stimulate lung repair by enhancing GCL and GSH productions (Kode, Rajendrasozhan et al. 2008).
Steroid resistance in COPD patients has been linked to nitrosative stress which is contributed by NO production and protein nitration in airway cells (Barnes, Ito et al. 2004). In this study, the inhibitory effect of spray dried formulations on NO production was investigated using LPS-induced alveolar macrophage cells (Figure 6B and 6C). It has been demonstrated that LPS stimulated iNOS expression in macrophages cells, which in turn led to enhanced production of NO via conversion of L-arginine to L-citrulline (Jørholmen, Škalko-Basnet et al. 2015). When high-output of NO levels is non-attenuated, it could facilitate synthesis of peroxynitrite intermediates, which react to form 3-nitrotyrosine that causes severe lung epithelial damage (Peng, Abdulnour et al. 2005, Crosswhite and Sun 2010). In a study by Peng et al, the increase of NO production was correlated to 3-nitrotyrosine concentration in lungs (Peng, Abdulnour et al. 2005). It is therefore likely that during COPD exacerbation caused by bacterial infection, activated alveolar macrophages resulting in elevated NO levels and inflammatory cytokines, which lead to lung injury and fibrosis. These unstable NO radicals are rapidly converted to NO$_2^-$ or NO$_3^-$. Therefore the amount of NO$_2^-$ measured using Griess reagent could indirectly determine NO production by alveolar macrophages. Results demonstrated that the inhibition of NO was dose-dependent, irrespective to the drugs used (Figure 6B). The concentration of NO decreased from 38.3 ± 2.5 µM (untreated control) to 23.8 ± 4.7 µM, 12.5 ± 1.1 µM and 31.5 ± 3.1 µM when treated with 50 µM BD, RES and L-NAME, respectively. Resveratrol demonstrated the strongest activity, while NAME showed weak inhibitory effect across all concentrations assayed ($p < 0.05$). Only 25% inhibition of NO production could be observed even at high concentration (100 µM) of NAME used.
Comparatively, at the same concentration, RES and BD inhibited 75% and 60% of NO production from alveolar macrophages, respectively (Figure 6B). The combination of RES and BD was also studied to evaluate possible additive/synergistic inhibitory effects of combination therapies towards NO productions in rat alveolar macrophage cells (Figure 6C). Interestingly, the reduction of NO was more pronounced when RES and BD were used in combination. The reduction was directly correlated to the increase of RES in the formulation. The amount of NO produced showed the following trend: 23.8 ± 4.7 µM (0% RES or 100% BD) > 14.6 ± 0.7 µM (25% RES + 75% BD) > 12.5 ± 1.1 µM (100% RES or 0% BD) > 11.5 ± 1.1 µM (50% RES + 50% BD) > 9.9 ± 0.7 µM (75% RES + 25% BD) (Figure 6C).

As mentioned, NO synthesis in alveolar macrophages is regulated by iNOS expression, which in turn is controlled by NF-κB. Inhibiting high-output of NO by down-regulating the iNOS expression at the transcription level, through inactivation of the NF-κB signalling pathway, could be a treatment strategy (Cho, Koo et al. 2002; Li, Yan et al. 2002). In work by Li et al, the inhibition of NO and iNOS was achieved in LPS-stimulated alveolar macrophages by pre-treating the cells with low concentrations of BD (10⁻¹⁰ M) (Li, Yan et al. 2002). In contrast, higher concentrations of BD or RES were needed in order to inhibit iNOS expression, as well as translocating NF-κB compared to NO production in LPS-induced alveolar macrophages, consistent with our results (Cho, Koo et al. 2002). In addition, RES was not effective to totally inhibit NO productions once cells were stimulated with LPS. This suggests that both inhibition of iNOS protein transcription and NF-κB inactivation is not the primary RES target towards NO regulation. Cho et al hypothesized that RES interferes with LPS-induced
expression of genes responsible for NO expression, independent of the NF-kB signalling pathway (Cho, Koo et al. 2002).

3.3.3. Anti-inflammatory activity

The anti-inflammatory properties of RES and BD have been widely documented. Results showed that RES and BD alone inhibited cytokine expressions (IL-6 and TNF-α) in LPS-stimulated alveolar macrophages as shown in Figure 7. These data are consistent with other findings using either RES or BD to inhibit cytokine expressions in different pulmonary cells line such as RAW 264.7, A549 and Calu-3 (Lilly, Nakamura et al. 1997; Donnelly, Newton et al. 2004; Trotta, Lee et al. 2015). Based on these results, RES is more effective than BD to reduce IL-6 and TNF-α (p < 0.05). Approximately 60% and 20% of IL-6 was inhibited after 72 h of treatment with RES and BD alone, respectively (Figure 7A). A similar inhibitory profile was observed for TNF-α production. After 72 h treatment with RES and BD, only 45% and 32% of TNF-α markers remained, respectively (Figure 7B). Our data also demonstrated that BD preferentially inhibited the expression of TNF-α compared to IL-6 in LPS-stimulated alveolar macrophage cells. This was supported by earlier findings whereby BD almost completely inhibited TNF-α release, while only partially inhibited IL-6 expression from macrophages (Linden and Brattsand 1994; Ek, Larsson et al. 1999). Our study clearly demonstrated the time-dependent response for both RES and BD in inhibiting cytokines (Figure 7). For BD, the percentages of TNF-α remained after 12 h, 24 h, 48 h and 72 h were 83 ± 4%, 76 ± 3%, 70 ± 2%, and 55 ± 4%, respectively. As for RES, the amount of
IL-6 remained after 12 h, 24 h, 48 h and 72 h were 80.0 ± 1.9%, 62.0 ± 2.0%, 55.0 ± 2.2%, and 40.0 ± 1.8%, respectively (Figure 7).

The effectiveness of corticosteroids in bacterial-induced COPD exacerbations remains debatable. In a previous study, IL-8, MMP-9 and MCP-1 expressions from LPS-stimulated alveolar macrophages were resistant to corticosteroids while IL-6 releases were effectively inhibited (Knobloch, Hag et al. 2011). The authors also demonstrated that the provision of RES alone reduced IL-8, MMP-9, IL-6 and MCP-1 expressions down to baseline readings in alveolar macrophage isolated from COPD patients (Knobloch, Hag et al. 2011). Therefore it was hypothesized that treatment of alveolar macrophages with inhalable formulations containing BD and RES might enhance the anti-inflammatory effect in COPD. As shown in Figure 7, cytokine releases from alveolar macrophages were significantly inhibited with the presence of RES in the Co-SD formulations. By increasing the ratio of RES from 0% to 75%, a reduction of IL-6 expression from 75% to 52% after 72h of treatment was observed. Similarly, significant reductions of TNF-α production were observed by increasing RES (Figure 7). The exact reason underlying stronger attenuation effect of RES towards cytokine compared to BD is still unclear, but could be due to the antioxidant properties of RES in addition to its anti-inflammatory effect.

The lack of corticosteroid efficacy could be the result of increased steroid-resistance in pulmonary macrophages. The disparities in steroid sensitivity towards inflammatory genes have been postulated to be attributable to impacts of corticosteroid-sensitive and corticosteroid-resistant transcription mechanisms (Armstrong, Sargent et al. 2009,
Knobloch, Sibbing et al. 2010). In other words, the corticosteroids’ efficacy towards specific cytokine is highly dependent on the ratio of corticosteroid-sensitive and corticosteroid-resistant signalling genes for transcriptions of cytokine proteins. For instance, LPS-induced cytokine genes transcription in alveolar macrophages are dependent on NF-κB signalling and mitogen-activated protein kinases (MAPK)/AP-1 pathway, whereby the former is sensitive to corticosteroids while the latter is non-responsive to corticosteroids (Armstrong, Sargent et al. 2009). In contrast, RES is known to be effective against both NF-κB and MAPK signalling (Cottart, Nivet-Antoine et al. 2010), which could explain the higher efficacy of RES to reduce IL-6 from alveolar macrophages. Mechanistically, corticosteroid-resistance in COPD is also augmented by reduced HDAC activities in alveolar macrophages caused by oxidative and nitrosative stress (Barnes 2009). As RES scavenges ROS and RNS radicals readily, RES could restore HDAC functions. This could also be a contributing factor for the enhanced anti-inflammatory activity showed when combination of BD and RES was administered.

4. Conclusions

In this study novel co-spray dried formulations of an anti-oxidant (RES) and anti-inflammatory (BD) compounds were produced. The spray dried powders showed appropriate morphologies and suitable aerosol properties for inhalation drug delivery. In vitro studies showed that alveolar macrophages could tolerate RES and BD in the range of concentrations investigated (0.612 to 50 µM). Moreover, RES and BD showed to have anti-inflammatory activities due to their ability to reduce the levels of TNF-α and IL-6. The data presented provide preliminary evidence that these compounds if
delivered in combination could be suitable for the treatment of chronic inflammatory lung diseases such as asthma and COPD, where both inflammation and oxidative stress are present.

Declaration of interest

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). WH Lee is the recipient of Early Career Fellowship Cancer Institute New South Wales.

References


Figure captions

Figure 1. Scanning electron microscopy images (SEM) of single and Co-SD formulations containing RES and BD at different ratios. (A) SD BD alone, (B) Co-SD containing 25% RES, (C) Co-SD containing 50% RES, (D) Co-SD containing 75% RES and (E) SD RES alone.

Figure 2. DSC thermograms of (A) raw BD, (B) SD BD alone, (C) Co-SD containing 25% RES, (D) Co-SD containing 50% RES, (E) Co-SD containing 75% RES, (F) SD RES alone and (G) raw RES.

Figure 3. In vitro aerosol deposition of the single spray dried and Co-SD formulation using multi stage liquid impinger (MSLI) at flow rate of 60 l/min. Data represents mean ±SD (n=3). Black bar denotes resveratrol and white bar denotes budesonide.

Figure 4. In vitro aerosol deposition of the single spray dried and Co-SD formulation using multi stage liquid impinger (MSLI) at flow rate of 90 l/min. Data represents mean ±SD (n=3). Black bar denotes resveratrol and white bar denotes budesonide.

Figure 5. Viabilities of alveolar macrophages cells evaluated using MTS cytotoxicity assay after 72 h of treatment with (A) single or (B) combination SD formulations. The percentages referred to the resveratrol concentration present in SD formulations. Data represents mean ±SD (n=3).
Figure 6. (A) The DPPH free radical scavenging activities. NO production in rat alveolar macrophages (B) in the presence of lipoic acid, ascorbic acid, L-NAME, BD and RES and (C) the effect of combination SD formulations. The percentages referred to the resveratrol concentration present in SD formulations. Data represents mean ±SD (n=3).

Figure 7. Cytokines expression in culture media of LPS pre-stimulated rat alveolar macrophage cells after treatment with SD formulations at different time points. (A) IL-6 and (B) TNF-α. The percentages referred to the resveratrol concentration present in SD formulations. Data represents mean ±SD (n=3).