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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 corticosteroid 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 anti-oxidant and anti-inflammatory activities for treatment of chronic lung diseases. The inhalable microparticles containing different ratios 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 tumour 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 *in vitro* toxicity and enhanced efficacy to control inflammation and oxidative stress in LPS-induced alveolar macrophages.

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 multifactorial 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 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

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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 the lung function of COPD patients, and a 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 a vicious cycle of oxidative stress by ROS and airway inflammation. Histone deacetylase 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 deacetylase ($HDAC_2$) activities can become inhibited in the presence of oxidative stress (Barnes, Ito et al., 2004).

For these reasons, the use of anti-oxidant compounds in association with one of the corticosteroid 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 active 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 resveratrol as anti-inflammatory, anti-allergic, anti-viral, 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 the 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 macrophage 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 including phosphate buffer saline (PBS), foetal bovine serum (FBS) and Ham's F-12 nutrient mix media were purchased from Invitrogen (Sydney, Australia). ELISA kits for determination of inflammation markers such as interleukin-6 (IL-6) and tumour 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 a 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 follows: 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 15 kV was used for imaging samples.

2.3.2. Laser diffraction of Co-SD microparticles

Particle size distribution of Co-SD microparticles were determined 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 Tolledo, 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 (Mettler Toledo). 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

Table 1
Spray drying conditions. Component, ratio (% *w*/*w*), feed concentration, inlet temperature, outlet temperature and flow rate used to produce inhalable microparticles.

Component	Ratio (% *w*/*w*)	Feed concentration (mg/ml)	Inlet temperature (°C)	Outlet temperature (°C)	Flow rate (ml/min)
RES	100	50	100	52	12.5
RES:BD	75:25	50	100	49	12.5
RES:BD	50:50	48	100	48	12.5
RES:BD	25:75	48	100	46	12.5
BD	100	30	100	46	12.5

RES, resveratrol; BD, budesonide.