Co-spray dried resveratrol and budesonide microparticles: Preparation, characterization and anti-inflammatory activity on alveolar macrophages

Valentina Trotta[1,2], Wing-Hin Lee[1], Ching-Yee Loo[1] , Santo Scalia [2], Paul M. Young[1], Daniela Traini [1]

[1]Respiratory Technology, The Woolcock Institute of Medical Research, and Discipline of Pharmacology, Sydney Medical School, The University of Sydney, NSW 2006, Australia

[2]Department of Chemical and Pharmaceutical Sciences, University of Ferrara, 44121 Ferrara, Italy Summary

Summary

Background:

Oxidative stress is instrumental in the pathogenesis and progression of chronic obstructive pulmonary disease (COPD). Patients with severe COPD are sometime afflicted by corticosteroid' unresponsiveness due to inhibited histone deacetylase (HADC2) activity in alveolar macrophages. Therefore, 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, an inhalable microparticle formulation containing resveratrol (RES) and budesonide (BD) was developed.

Methods:

The co-spray dried (co-SD) RES and BD microparticles were produced using a Buchi B-290 Spray dryer and their morphologies and aerosol performances characterized using scanning electron microscopy (SEM) and multi stage liquid impinger (MSLI), respectively. The effect of spray-dried RES and BD, alone and in combination, on cell viability were investigated against a NR8383 alveolar macrophages cell line. The extent of anti-inflammatory activity of RES and BD, alone and in combination was also studied on lipopolysaccharides (LPS) induced NR8383 cells.

Results:

The co-SD microparticles of all formulations exhibited morphologies appropriate for inhalation administration, as observed by SEM. 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 LPS induced alveolar macrophages.

Conclusions:

Single, co-SD RES and BD microparticles exhibited morphology and aerosol properties suitable for inhalation drug delivery. *In vitro* studies showed that alveolar macrophages could tolerate concentrations of RES and BD from 1.25 to 80μM and both were able to reduce the levels of TNF-α and IL-6 in a time dependent manner.

Introduction

Alveolar macrophages are the first line lung defence against inhaled noxious agents. In chronic obstructive pulmonary disease (COPD), alveolar macrophages have been implicated in COPD pathogenesis, as the number of activated macrophages are markedly increased in the lung of COPD patients and have higher stimulated reactive oxygen species (ROS) and pro-inflammatory cytokines secretion [1-2]. Corticosteroids molecules are able to suppress the release of these pro-inflammatory mediators in alveolar macrophages, but these drugs become ineffective as COPD patients become non-responsive to corticosteroids' therapy [3]. Recently, it was reported that oxidative stress is also involved in COPD pathogenesis and responsible for corticosteroids' unresponsiveness [4-5]. Therefore, novel therapeutic strategies , which incorporate both anti-oxidant and anti-inflammatory characteristics, aimed to alveolar macrophages could be expected to increase corticosteroids responses. Resveratrol, RES (3, 5, 4- trihydroxystilbene) is a naturally polyphenolic compound with anti-oxidant and anti-inflammatory activities, thus could be considered as a potential compound for COPD treatment [6]. Moreover, RES has anti-inflammatory and anti-oxidant activity on Calu-3 cells, as reported in a previously study [7].

In this study, inhalable microparticles containing RES and budesonide (BD), a common anti-inflammatory corticosteroid, were formulated using spray drying. This formulation could potentially be used as anti-oxidant and anti-inflammatory combination therapy for chronic inflammatory lung diseases to restore corticosteroid responsiveness and consequently improve clinical outcomes in these patients.

Experimental methods

Materials

Resveratrol was purchased from Fagron Italia (Bologna, Italy) and budesonide (EP grade, Yicheng Chemical Corp., Jiangsu, China). Rat alveolar macrophage NR8383 cell line (HTB-55) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Tumor necrosis factor alpha (TNFα) was purchased from Invitrogen, (Sydney, Australia), while the ELISA kit was provided by BD Biosciences (Sydney, Australia). Analytical grade solvents were purchased from Sigma (Sydney, Australia).

Spray dried formulations

Resveratrol and BD, either alone or in combination, at different ratios were dissolved in ethanol-water (80:20% v/v) and spray dried using a Buchi B-290 Mini Spray Dryer (Buchi, Switzerland) at flow rate of 12.5 ml/min. The relative concentrations of each drug in the feed solution were adjusted so that the final dry weight percentages (%, w/w) were: 100% RES, 75% RES, 50% RES, 25% RES and 100% BD, respectively. Unless otherwise stated, the spray dried formulations are referred as RES concentration (%, w/w).

Scanning electron microscopy (SEM)
The morphologies and distribution of SD particles were observed using SEM (JMC, 6000 JEOL, Japan). SD-RES, SD-BD and co-SD RES-BD formulations were dispersed onto carbon sticky tapes, placed on aluminium stubs and sputter-coated with gold (JEOL USA Smart Coater) prior to SEM observation at 15KV.

***In vitro* aerosolization performance of spray dried formulations using Multi stage liquid impinger (MSLI)**
Aerosol performance and particle size distribution of spray dried formulations were studied using the MSLI (Copley Scientific Ltd., Nottingham, UK) at 60 l/min for 4 s. Spray dried powders (10 ± 0.2 mg) were loaded into a size 3 gelatin capsules (Capsugel®, Sydney, Australia), placed into the dosage chamber of a low-resistance RS01 dry powder inhalation device (Plastiape®, Osnago, Italy). After actuation, the device containing the capsule, adaptor, throat, stages and filter were washed separately with methanol:water (80:20 %v/v). The experiments were conducted in triplicate and samples analysed using high performance liquid chromatography (HPLC).
The fine particle dose (FPD) (drug recovered from stages 3 to filter, ≤ 6.8μm), the fine particle fraction (FPF) (FPD/Total dose x100) and total mass recovery were calculated.
Resveratrol and BD were quantified using a validated HPLC method. Chemical analysis of BD was performed using methanol-water 80:20 (%v/v) as mobile phase at flow rate of 1 ml/min, with an isocratic pump and a Luna C18 (3 μm 4.6 × 150 mm) (Phenomenex, Sydney, AUS) column. The HPLC method for RES used as mobile phase of methanol-water 60:40 (%v/v) with 0.5% Acetic acid (%v/v) and XbridgeTM column (5 μm, 4.6 × 150 mm) (Waters, Massachusetts, USA).

Cytotoxicity of RES and BD against alveolar macrophages
The alveolar macrophages viability to RES and BD was assed using a MTS cell proliferation assay. Briefly, the alveolar macrophages were exposed to increasing concentrations (from 1.25 to 80 μM) of RES, BD and a combination of RES-BD and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 72h. After this time point, CellTiter 96® Aqueous assay (MTS reagent, Promega, USA) was added to each well and incubated at 37°C in a humidified atmosphere with 5% CO_2 for 4h. Absorbance was measured at 490 nm using a plate reader (Wallac 1420 VICTOR^{2TM}, Multilaber Counter, Massachusetts, USA). The alveolar macrophage viability was calculated with reference to the untreated cells (control).
Experiments were performed in triplicate and data expressed as % cell viability [(average absorbance of treated wells/average absorbance of control wells) x100].

Anti-inflammatory activities of RES and BD against alveolar macrophages
The anti-inflammatory effects of RES, BD and RES-BD (at different ratios) on a rat derived alveolar macrophage cell line NR8383 was assessed by measuring TNF-α and IL-6 levels using an ELISA kit according to the manufacturer's instructions. Prior to treatment, the alveolar macrophages cells were induced with 5 μg/ml LPS for 24 h at 37°C. Then, the cells were treated with RES and BD, alone and in combinations in different ratios, using as final concentration of 50 μM of RES, BD and RES-BD.

Results and discussion

The SEM images in Figure 1 showed that SD microparticles in all formulations had a size suitable for inhalation. Single SD and Co-SD at different RES concentrations, showed spherical geometry with the average diameter <5μm. Interestingly, the SD RES alone (Figure 1A) exhibited rough corrugated surface, while spray dried BD alone (Figure 1E) was smooth. It is also noted that co-SD particles became smoother with increasing BD concentration (Figure 1B to Figure 1D).

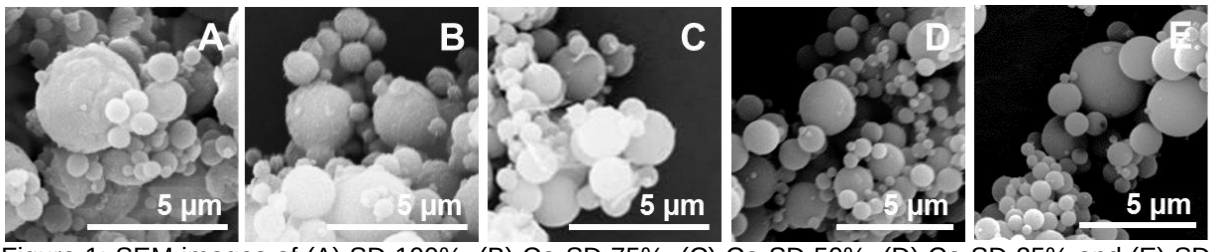

Figure 1: SEM images of (A) SD 100%, (B) Co-SD 75%, (C) Co-SD 50%, (D) Co-SD 25% and (E) SD 0%. The particle formulations are referred to by resveratrol concentration (% w/w).

The FPF for SD-RES and SD-BD were 26.3 ± 1.5 % and 39.4 ± 2.8%, respectively. From the aerosol deposition data, it is found that SD-RES alone had higher throat deposition, probably due to the 'sticky' nature of RES, enhancing cohesion between particles and thus promoting agglomeration and reducing dispersion. Analysis of the deposition profiles of Co-SD formulations shows an increased FPF, proportionally to BD concentration. The FPF values for co-SD containing 25% (w/w), 50% (w/w) and 75% (w/w) of RES were 42.5 ± 1.7%, 38.8 ± 2.9%, 23.8 ± 3.7 %, respectively.

The dose response cytotoxicity profiles of RES and BD, alone and in combination, on alveolar macrophages are presented in Figure 2. The cell viability assay demonstrated that alveolar macrophages could tolerate a wide range of RES and BD concentrations.

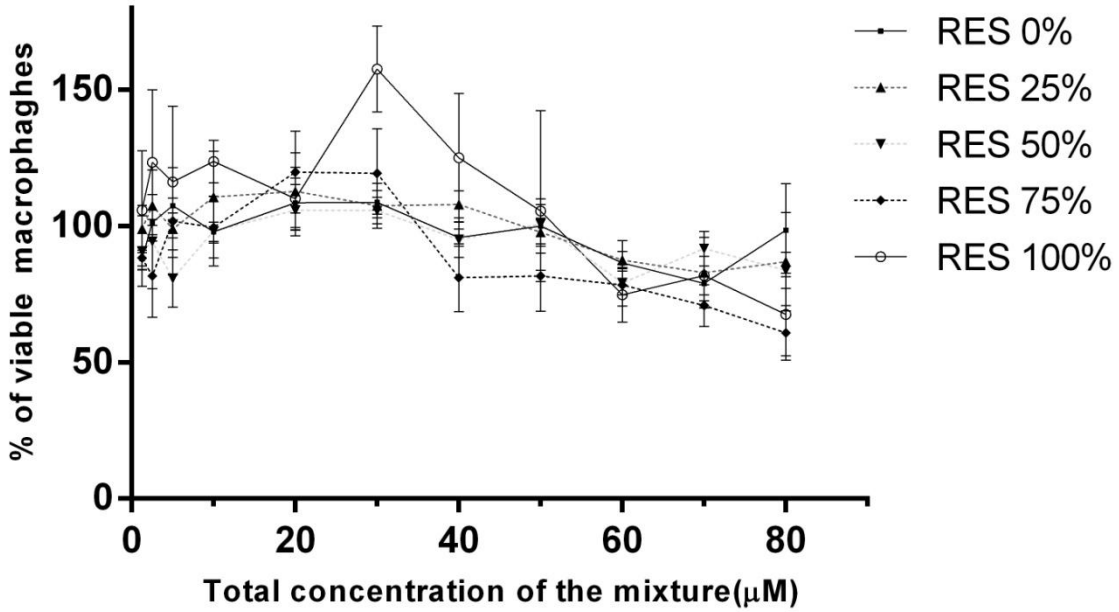

Figure 2: The effect of RES and BD on alveolar macrophages viability after 72h drug treatment (n=3, data represents mean ± standard deviation). The percentages reported in the legend are referred to the RES concentration.

The anti-inflammatory studies showed that the combination of RES and BD increased the anti-inflammatory and that in general the anti-inflammatory activities of all formulation were time-dependent (Figure 3). As shown in Figure 3A, the reduction of TNF-α was 22% and 17%, after 12 h of RES and BD treatment, respectively. This increased to more than 45% and 68% after 72 h treatment with BD and RES, respectively. In contrast, the reduction of IL-6 expression was more than 20% and 60% after 72 h of treatment with BD and RES, respectively (Figure 3B).

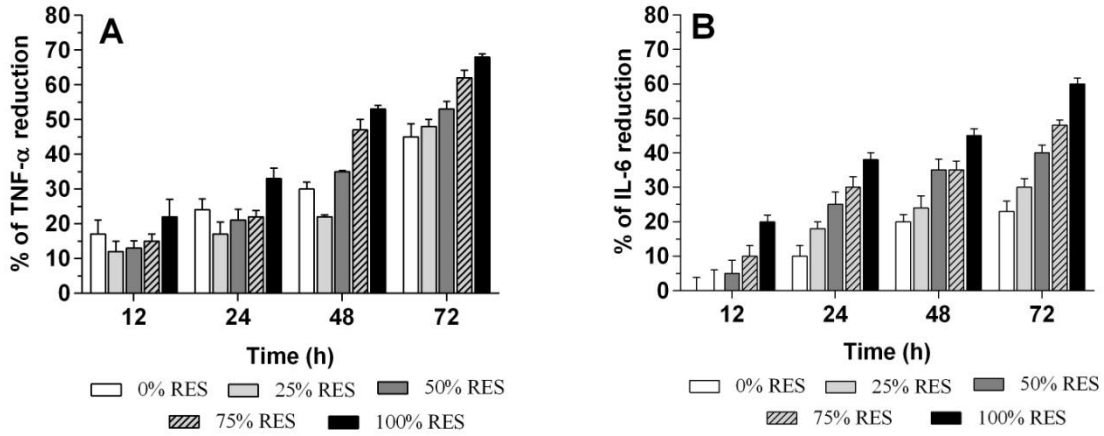

Figure 3: % of (A) TNF-α and (B) IL-6 reduction on alveolar macrophages after induced with LPS. Data represents mean ± SD (n= 3).

Conclusions

In this study new co-spray dried formulations of an anti-oxidant (RES) and anti-inflammatory (BD) compounds were produced. The spray dried powders showed appropriate morphology 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 (1.25 to 80 μM) investigated. 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 in the study provided preliminary evidence that these combination compounds would be desirable for treatment of chronic inflammatory lung diseases such asthma and COPD where both inflammation and oxidative stress are the hallmark in the pathogenesis and the development of the diseases.

References

1. Tetley, Teresa D. "Macrophages and the pathogenesis of COPD." *CHEST Journal* 121.5_suppl (2002): 156S-159S.
2. Kent, Lauren, et al. "Cigarette smoke extract induced cytokine and chemokine gene expression changes in COPD macrophages." *Cytokine* 42.2 (2008): 205-216.
3. Bhavsar, Pankaj, et al. "Relative corticosteroid insensitivity of alveolar macrophages in severe asthma compared with non-severe asthma." *Thorax*63.9 (2008): 784-790.
4. Barnes, Peter J., Kazuhiro Ito, and Ian M. Adcock. "Corticosteroid resistance in chronic obstructive pulmonary disease: inactivation of histone deacetylase."The Lancet 363.9410 (2004): 731-733.
5. Wood LG, Wark PAB, Garg ML. Antioxidant and Anti-Inflammatory Effects of Resveratrol in Airway Disease. Antioxidants & redox signaling 2010;13:1535-48.
6. Knobloch J, Sibbing B, Jungck D, Lin Y, Urban K, Stoelben E, Strauch J, Koch A. Resveratrol impairs the release of steroid-resistant inflammatory cytokines from human airway smooth muscle cells in chronic obstructive pulmonary disease. The Journal of pharmacology and experimental therapeutics 2010;335:788-98.
7. Trotta, V., Lee, W. H., Loo, C. Y., Haghi, M., Young, P. M., Scalia, S., & Traini, D. (2015). In vitro biological activity of resveratrol using a novel inhalable resveratrol spray-dried formulation. International journal of pharmaceutics, 2015; 491(1), 190-197.