Dry Powder formulation of Simvastatin for Pulmonary Inflammatory Diseases

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Summary

Background: Simvastatin (SV) is an inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, used for lowering cholesterol levels in myocardial infarction prevention. Recently, there has been increasing evidence that SV possess anti-inflammatory properties and that these could be useful in decreasing the inflammation processes in pulmonary disease. Hence, this study focuses on the development of SV dry powder inhalation (DPI) formulation, as a novel therapy for inflammation and mucus over production in chronic respiratory diseases. **Methods:** Micronized SV powders were prepared by dry jet milling and physico-chemical properties of the formulations were characterised in terms of particles size. Also, the *in vitro* aerosol deposition of the formulation was studied. In addition, SV was evaluated for its effects on cilia beat frequency using primary nasal epithelial cells. Furthermore, the formulation was assessed for its muco-inhibitory effect on an established air interface Calu-3 cell model. **Results**: SV particle size of the formulation was suitable for inhalation therapy, with a fine particle fraction (FPF, defined as percentage of SV mass deposited from stage three to filter, as a function of the ex-valve dose (ED), corresponding to the cut-off size ≤ 5 μm) of 44.62 ± 5.77 %. Additionally, the SV was found to be non-toxic on the nasal cilia up to a concentration of 10^{-6}M, and to decrease mucus production in Calu-3 after 4 days exposure to a single dose of SV DPI. **Conclusion:** This therapy could potentially be used for the local treatment of respiratory diseases, where hyper mucus production and inflammation is present.

Introduction

Simvastatin (SV) is used to treat cardiovascular diseases and hypercholesterolemia as an oral anti-cholesterol prodrug via complete inhibition of the HMG-CoA reductase, an essential enzyme for cholesterol biosynthesis [1]. Recently, SV has been found to have anti-inflammatory properties unrelated to its lipid lowering activity, making it potentially useful in the management pulmonary diseases, all of which have an inflammatory component [2]. A study by Blamoun et al. showed a decrease in the number of exacerbations and intubations occurring in a group of 185 chronic obstructive pulmonary disease patients on SV treatment [3]. Furthermore, Rezaie Majd et al., found that hyper cholesterolemic patients treated with simvastatin over 6 weeks exhibited a decrease in systemic cytokine levels, including IL-8, IL-6 and monocyte chemo-attractant protein-1 [4]. More recently, SV has been shown to have a muco-inhibitory effect on *in vitro* Calu-3 human bronchial epithelial cell and Marin et al. discovered that chronic dosing of 1 or 10 μM solution of SV from the basolateral chamber caused a significant inhibition in mucus production on air interface Calu-3 epithelial cell model [5]. Chen YJ et al., found that SV may decrease acrolein-induced mucin protein synthesis in the airway and airway inflammation, maybe by blocking ERK activation mediated by Ras protein isoprenylation and this indication of simvastatin can treat mucus over production of airway [6].

Aim: The aim of the current study was to investigate the formulation of SV as a dry powder inhaler (DPI) for direct lung delivery and examination of its *in vitro* aerosol depositions, its effect on cilia beat activity using ciliated nasal epithelial cells *in vitro* and its ability to reduce mucus secretion.

Material and Methods

Particle production: Milled SV was produced by air jet milling (Comhas, Cinisello Balsamo, Italy) at grinding gas pressure of 6 bar and feed pressure of 2.8 bar.

Particle Size Analysis: The particle size distribution of micronized SV was determined by laser diffraction using a dry feed cell (Malvern Mastersizer 3000, Instruments Ltd., UK) the sample was dispersed in air using 4 bar pressure and measured when an obscuration of 5-15% was achieved, with a refractive index of 1.53. The d0.5, d0.1 and d0.9 diameters were determined. This is to certify that the micronized particles were within the respirable range

***In vitro* aerosol dispersion characterisation:** The *in vitro* aerosol performance was determined using a multi-stage liquid impinger (MSLI, Apparatus A, European Pharmacopoeia, Chapter 2.9.18; Copley Scientific, Nottingham, UK). 5 mg of milled SV powder were loaded into size 3 hard gelatin capsules (Capsugel, Sydney, Australia) and placed within the chamber of an Aerolizer® device (Norvartis Surrey, UK) at flow rate 60 L/min for 4 s (Westech Scientific Instruments, Bedfordshire, UK). Acetonitrile: water (65:35 v/v) was used to wash capsule, device, mouthpiece adapter, throat, all stages of MSLI and filter and samples analysed using High Pressure Liquid Chromatography (HPLC) with a validated method.

Cilia toxicity assay: The toxicity of SV on cilia beat frequency (CBF), using primary nasal epithelial cells from healthy volunteers was performed [7]. Cell viability was maintained using 5 mL of Medium 199 to suspend the nasal cells. The cell suspensions were treated with $1x10^{-6}$ M of SV for 15–30 min. Then, under a light microscope, CBF of nasal cells was analysed.

Mucus Inhibition study: The effect of SV DPI on mucus production was studied; a single dose of 0.3 mg powder was deposited on Calu-3 at day 11 using a modified twin stage impinge (TSI). At day 14, mucus glycoproteins were stained with alcian blue as previously described [5, 8] to allow the mucus visualisation on Calu-3 cells after SV deposition and compared with untreated cells (control). Images of each sample were taken using Olympus BX60 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP71 camera (Wetzlar, Germany).

RESULTS AND DISCUSSION

Geometric particle size distributions of micronized SV determined by laser diffraction and the d0.5, d0.1 and d0.9 diameters were 1.18 ± 0.07, 2.2 ± 0.03 and 4.03 ± 0.2 µm, respectively. The result recommends micronized SV was appropriate for inhalation applications.

In vitro aerosolisation performance of the SV DPI formulation using the MSLI at 60 L/min and samples collected from the device, throat, stages and the FPF were calculated (Table I). The total dose delivered was 4790.83 ± 115.36 µg (target dose = 5000 µg). Results showed that the formulation is appropriate for inhalation therapy.

Toxicity of SV drugs on ciliary activity was assessed using primary nasal epithelial cells with functioning cilia. These cells displayed identical morphologies to lung epithelium cells. SV at a concentration of $1x10^{-6}$M did show not to have any toxicity on CBF (SV: 7.48 ±1.4 Hz), compared to baseline (control: 7.03 ±1.3 Hz). Results showed SV could be used as a treatment for chronic hyper secretory mucus diseases.

SV DPI was also showed to reduce mucus production; with a significant mucus inhibition showed when deposited on Calu-3 in comparison with untreated controls at day 14 (Figure 1) Further studies to confirm cell membrane integrity, during and after the course of the mucus inhibition experiments, are ongoing.

Table I. The percentage of SV deposited on device, throat and Fine particle fraction (FPF) after *In vitro* aerosolisation performance of SV DPI formulation using an MSLI at 60 L/min.

Days	Device (%)	Throat (%)	Stage 1 (%)	Stage 2 (%)	Stage 3 (%)	Stage 4 (%)	FPF (%)
Day0	26.48 ± 4.4	7.89 ± 4.5	4.21 ± 4.7	4.66 ± 0.2	20.95 ± 1.9	19.37 ± 2.7	44.62 ± 5.7

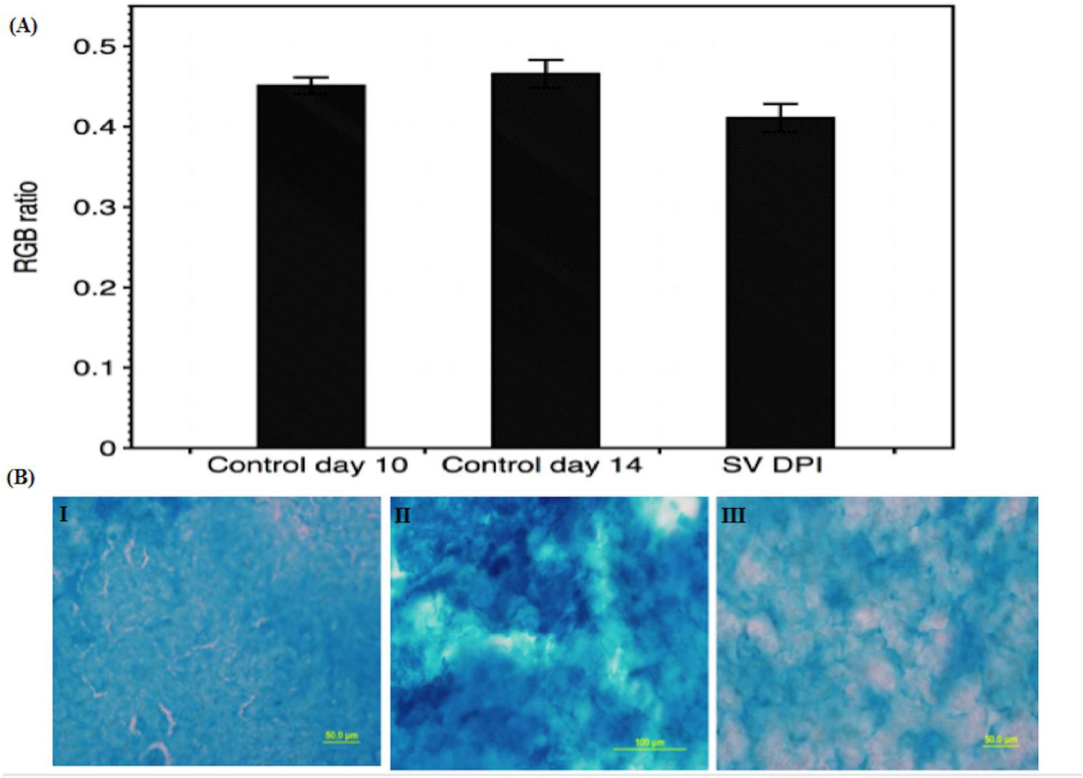

Figure 1: (A) RGB ratio* of alcian blue intensity at day 10 (initial point), after deposition of 0.3 mg of DPI SV on air interface Calu-3 and untreated cells at 14. Data represent mean ± SD. (B) Microscopic images of stained Calu-3 monolayers on (I) day 10 (initial point); (II) day 14 (control) and (III) DPI SV treated monolayers at day 14.

* The ratio of red, green, blue (RGB ratio) was calculated by dividing the mean RGBB by the sum of the RGB values for each image (RGBR + RGBG + RGBB). The mean RGBB was used to quantify mucus production, in both the control and the SV treated monolayers.

Conclusions

Dry powder SV was prepared for inhalation drug delivery and showed no toxic effect on ciliary activity *in vitro*. Results also confirmed the ability of SV DPI to significantly decrease mucus production on Calu-3 cels. This novel formulation has the potential to provide a promising dry powder therapy for the treatment of hypersecretory pulmonary diseases.

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