Simvastatin Nanoparticles Reduce Inflammation in LPS-Stimulated Alveolar Macrophages

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1.0 Abstract

Simvastatin (SV) is widely used as lipid- lowering medication that has also been found to have beneficial immuno-modulatory effects for treatment of chronic lung diseases. Although its anti-inflammatory activity has been investigated, its underlying mechanisms have not yet been clearly elucidated. In this study, the anti-inflammatory and anti-oxidant effects and mechanism of simvastatin nanoparticles (SV-NPs) on lipopolysaccharide (LPS)-stimulated alveolar macrophages (AM) NR8383 cells were investigated. Quantitative cellular uptake of SV-NPs, the production of inflammatory mediators (interleukin (IL)-6, tumour necrosis factor (TNF) and monocyte chemoattractant protein-1 (MCP-1)), and oxidative stress (nitric oxide, NO) were tested. Furthermore, the involvement of the Nuclear factor KB (NF-KB) signaling pathway in activation of inflammation in AM and the efficacy of SV were visualized using immunofluorescence. Results indicated that SV-NPs exhibit a potent inhibitory effect on NO production and secretion of inflammatory cytokine in inflamed AM, without affecting cell viability. The enhanced anti-inflammatory activity of SV-NPs is likely due to SV improved chemical- physical stability and higher cellular uptake into AM. The study also indicates that SV targets the inflammatory and oxidative response of AM, through inactivation of the NF-KB signalling pathway, supporting the pharmacological basis of SV for treatment of chronic inflammatory lung diseases.

KEYWORDS: Simvastatin nanoparticles, NF-Kb, NR8383 cells, inhalation, anti-inflammation.

2.0 INTRODUCTION

It is predicted that chronic obstructive pulmonary disease (COPD) will be one of the most common cause of morbidity and mortality worldwide by 2020 (1). Chronic lung diseases are characterized by chronic inflammation, pulmonary oedema, mucus production, bronchitis, decline in respiratory function and eventual death (2-4). Inflammation is an essential aspect of the lung's response to any stimuli to maintain a healthy state (5). However, chronic perpetual inflammation causes the release of pro-inflammatory cytokines and enzymes, including interleukin (IL)-1, IL-6, IL-8, IL-1ß, tumour necrosis factor-a (TNF- α) (6, 7), nitric oxide synthase (NOS) and cyclooxygenase (COX), respectively, as a defense mechanism against infections (8, 9). Particularly, TNF-α has an important role in stimulating the production of other inflammatory cytokines secretion in many chronic inflammation diseases, perpetuating the condition (10, 11), via nuclear factor-kappaB (NF-κB) activation. NF-κB regulates many genes expression that are involved in inflammatory responses (12, 13). Inflammation also leads to the up-regulation of alveolar macrophages (AM) recruitment into the airways. Under normal physiological conditions, they are the first line of defense against inhaled noxious agents and pathogens in the alveolar region (14-16). However, these AM play an important role in the pathogenesis of COPD that amplifies tissues injury and chronic inflammation by generating proinflammatory responses including increased oxidative stress, increased production of proinflammatory cytokines, upregulation of antigen presentation and costimulatory molecules (17).

Currently, corticosteroids are used as mainstay therapy for the treatment of chronic lung diseases including beclomethasone dipropionate, budesonide and fluticasone.

However, most COPD patients respond poorly to high doses of inhaled corticosteroids with no effect on inflammation, disease progression and mortality, which is in sharp contrast with asthma patients (18). Therefore, developing new therapies that target lung inflammation by inhibiting pro-inflammatory mediators production through different mechanisms (19, 20) could be a potential new alternative. Simvastatin (SV) is an oral anti-cholesterol drug for the treatment of hyperlipidemia that acts as an inhibitor of the 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) reductase enzyme. Many previous studies have indicated that SV has a variety of immuno-modulatory effects such as anti-inflammatory, anti-oxidant and muco-inhibitory unrelated to its anti-cholesterol effect (21-23). Studies have shown that SV is able to reduce airway inflammation in murine asthmatic models, however published clinical data are conflicting, which may be due to it systemic (24)(25), rather than topical delivery. Administration by inhalation could offer several advantages over other oral administrations including rapid onset of action, first-pass metabolism avoidance, high local concentration and decrease systemic side effects.

Nanotechnology systems offer an exciting opportunity to make significant advances in inhalation therapy due to their large surface area, small particle size and surface charge properties that could help enhancing therapeutic outcomes (26). These systems have been used for drugs delivery to control the pharmacokinetics, pharmacodynamics, toxicity and efficacy of drugs (27) by using drug carriers (i.e polymers, liposomes, micelles, dendrimers and lipid nanoparticles). Drug carriers have been used due to their significant advantages and have the ability to reduce drug degradation, prevent harmful side effects and target the delivery when administered via inhalation (28)(29)(30)(31). SV has been formulated as nanoparticles for inhalation to overcome its low water

solubility, overcome its stability issues in the presence of water, enhance its bioavailability, pharmacokinetic profiles, and toxicity (32).

In this study, as part of an on-going screening program to evaluate the anti-inflammatory potential of simvastatin, the anti-inflammatory properties of inhalable polymeric simvastatin nanoparticles (SV-NPs) and the responsible underlying molecular mechanisms involved in LPS-stimulated AM NR8383 cell model were investigated. It is hypothesised that the beneficial immunomodulatory effects of SV is mediated via the NF-κB pathway and that these effects will be significantly improved by formulating SV as a nano-formulation using polymeric materials such as poly(lactic-co-glycolic acid) (PLGA) and pluronic. This is due to improvements in drug's stability, solubility (33), and bioavailability, that will in turn increases its efficacy at the target site (34-38). In addition, SV is a lipophilic prodrug, easily hydrolyzed into its active metabolite, simvastatin hydroxy acid (SVA), that is hydrophilic (7). However, conversion into SVA prior to entering the cells will render it ineffective as it is unable to pass through the cells' membrane bilayer. Consequently, the polymeric NP shell will protect SV from hydrolysis to SVA prior to entering into the cells for its therapeutic efficacy.

3.0 MATERIALS AND METHODS

3.1 Materials

Simvastatin was used as supplied from Jayco Chemical Industries (Thane, India). Simvastatin hydroxy acid (SVA) was manufactured as previous described (39) (40). Pluronic F-127, Dimethylsulfoxide (DMSO), Poly (Lactic-Co-Glycolic) acid (PLGA)

75:25 (average MW = 66,000–107,000), Griess reagent and Lipopolysaccharide (LPS, from Escherichia coli) were obtained from Sigma Aldrich, Australia. All other solvents used were of analytical grade and supplied by Bio-lab (Victoria, Australia). Alveolar macrophage NR8383 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), MTS [3-(4,5-dimeth- ylthiazol-2-yl)-5-(3-carboxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolim] reagent was purchased from Promega, USA. Transwell cell culture inserts (0.33 cm^2 polyester, 0.4 μm pore size) were purchased from Corning Costar (Lowell, MA, USA), and all other sterile culture plastic wares were from Sarstedt (Adelaide, Australia). DAPI (4,6-diamidino-2-phenylindole), proLong® Diamond Antifade reagent, serum, Ham's F-12 nutrient mix media, Antibodies against NF-κB p65, inhibitor kappaB (IκB) were purchased from Invitrogen, (Sydney, Australia).

3.2 Methods

3.2.1 Preparation of PLGA encapsulated SV Nanoparticles (SV-NPs)

Briefly, SV-NPs were prepared via a solvent and anti-solvent precipitation method as previously described (28). First, dichloromethane (DCM, 5% w/v) was used to dissolve SV. Subsequently, the SV solution was added to chilled Pluronic F-127 (3 mg/mL) aqueous solution dropwise to a concentration of 2% v/v and homogenized (Silver- son L4RT, East Longmeadow, MA, USA) at 6,000 rpm for 2 min on ice. Then, the solution was added into 50 mg/mL of PLGA in DCM solution and homogenised at 6,000 rpm for another 2-3 min to encapsulate the nanoparticles with PLGA. After that, the NPs were collected and washed 3 times with distilled water by centrifugation using the Amicon® Ultra-15 Centrifugal filter unit (10 kDa) at 2750 rpm; 4ºC for ~ 45-60 min.

The resultant particles were stored at -80 ºC for 1 h before lyophilisation at -50 ºC for 24 hours (B. Braun, Melsungen, Germany).

3.2.2 Particle size and charge analysis: Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) (Nano Series ZS Zetasizer, Malvern Instruments, Worcestershire, UK) was used to determine the particle size, surface charge (zeta potential) and polydispersity index (PDI) of SV-NPs. Measurements were performed before freeze drying, after freeze drying, and after 60 days in 4 °C storage. About 1 mg/mL of SV-NPs were examined after re-suspended the particles in deionized water followed by sonication. Size measurements were performed in triplicate at 25 ºC.

3.2.3 High- Performance Liquid Chromatography (HPLC)

To quantify SV and its metabolite SVA, a Shimadzu HPLC system that consisted of an SPD-20A UV–VIS detector (Shimadzu, Sydney, NSW, Australia), a LC20AT pump and a SIL20AHT autosampler was used. The mobile phase was a mixture of acetonitrile and 0.025M sodium dihydrogen phosphate at a ratio of 65:35 (v/v), with pH adjusted to 4.5 using phosphoric acid. Analysis of SV and SVA were achieved using a reverse phase C-18 column (Phenomenex ODS hypersclone, 250×4.6 mm, 5-µm particle size) with the following conditions: UV detector wavelength of 238 nm, 100 µL injection volume and flow rate of 1.5 mL/min. The retention time was 9.1 and 5.5 min for SV and SVA respectively, and linearity was obtained between 0.01 and 50 µg/mL (R2=0.99) for both SV and SVA.

3.2.4 Cell culture

Alveolar macrophage NR8383 cells were used and cultured between passages 37-47 in pre-warmed Dulbecco modified eagle medium (DMEM) maintained in complete Ham's F-12 nutrient mix medium with 10% (v/v) heat inactivated Fetal bovine serum in humidified atmosphere containing 5% CO_2 at 37°C until confluency was reached. The medium was exchanged twice a week and cells were passaged weekly.

3.2.5 Cytotoxicity of SV-NPs, SV solution and lipopolysaccharide using Alveolar Macrophage NR8383 cells

The cytotoxicity of SV-NPs, SV solution and LPS was evaluated using alveolar macrophage NR8383 cells, as previously described (40, 41). Briefly, the cells were centrifuged at 1,000 rpm for 5 min before seeding (500,000 cells/ml) onto 96 well plates (100ul/well) and then were incubated in 5% CO_2 at 37°C for 24 h. After 24 h, the cells were treated with different concentrations (from 0.004 to 500 μM) of SV-NPs and SV solution and (100 to 0.00076 ug/ml) for LPS. LPS is an endotoxin molecule that is found on the outer membrane bacteria (42, 43) such as *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* (44) used to simulate the inflammatory response following an infection. SV solution was prepared by dissolving SV into 100% ethanol to prepare the stock solution and then diluted in DMEM medium to a final ethanol concentration of <2%, ensuring negligible toxicity to the cells. The SV-NPs were suspended in DMEM medium alone to the same concentration as SV solution. Subsequently, cells were treated and incubated for 72 h. After this incubation period, 20 μL of MTS reagent was added to the cells and incubated for another 3 h. The cells were also treated with cell media as negative controls as previously described (45, 46).

All absorbance readings were measured at 490 nm using a fluorescence microplate reader (SpectraMax M2; Molecular devices, USA). The cell viability was calculated as a percentage of untreated cells and was plotted against the treatment concentrations (ng/mL) on a logarithmic scale. The concentration that produced a 50% decrease in cell viability following 72 h of treatment is defined as the IC_{50} values, which was calculated by fitting the data to the Inhibitory sigmoid Emax model onto the plot using GraphPad Prism.

3.2.6 Cellular Uptake

The cellular uptake of SV-NPs and SV solution were carried out using alveolar macrophage NR8383 cells. Using 6 well plates, cells were seeded at 100,000 per well and incubated for 24 h at 37 °C. The cells were then stimulated for 24 h with 1μg/ml of LPS and the plate incubated at 37 °C. After 24 h, the cells were treated with 1.25 μg/mL of SV-NPs and SV solution and incubated for 6 h at 37 °C to check the cellular uptake. To achieve this, the cells were lysed at fixed time points. Phosphate Buffer Solution (PBS) was used to wash the cells and cell lytic reagent (Sigma, Australia) was added to improve the cell membrane lysis. The cell lysates were then centrifuged at 13,000 rpm at 4 °C for 10 minutes. The resultant supernatant was then analysed using the validated HPLC method to quantify the amount of SV that was internalized. The cellular uptake measurements were normalized with total drug and expressed as % of drug concentration (μg/μL).

3.2.7 NO Production in LPS-Induced Alveolar Macrophages NR8383 cells

The amount of nitric oxide (NO) production was measured in the supernatant from LPS-induced alveolar macrophages cells after treatment with 1.25 μg/mL SV-NPs and SV solution. First, alveolar macrophage cells were induced with 1 μg/ml LPS for 24 h (to simulate physiological inflammation) and then treated with 1.25 μg/mL of SV-NPs and SV solution concentrations for 6 and 24 h. Positive control cells were LPS only. Cell-free supernatant (centrifuged at 13,000 rpm, at 4°C for 10 min) was mixed with equal amount of Griess reagent and incubated at room temperature for 10 min and measured at UV 550 nm.

3.2.8 Anti-inflammatory activity of SV-NPs formulation and SV solution against LPS induced alveolar macrophages NR8383 cells

The anti-inflammatory effects of SV-NPs and SV solution were evaluated on LPS-induced alveolar macrophage cells. 100,000 cells/well were seeded in 6 well plates with 1 ng/ml of LPS before incubation for 24 h at 37 °C. The cells were subsequently treated with 1.25 μg/mL of SV-NPs formulation and SV solution, at different incubation time points: 6 h and 24 h. At these time points, the samples were harvested by centrifugation at 13,000 rpm at 4 °C for 5 min. Then, the supernatant was stored at − 80 °C until analysis. Cytokine expressions of IL-6, MCP-1 and TNF-α were measured using Rat IL-6, MCP-1 and TNF-α ELISA kits (BD OptEIA^{TM}, BD Biosciences, San Diego, California, USA) according to manufacturer's instructions.

3.2.9 Immunofluorescence analysis by fluorescence microscopy

The NF-κB p65 nuclear localization was detected by immunofluorescence assays using a fluorescence microscope. For this study, alveolar macrophage NR8383 cells were

seeded (1×10^6/ml) directly on Lab-Tek II 8 well glass bottom cell culture chamber slide (Thermo Fisher Scientific, Australia) for 24 h. After that, cells were stimulated with 1 ng/mL of LPS and incubated overnight. Then, 1.25 μg/mL of SV-NPs and SV solution were added and incubated for 24 h. Un-treated cells and LPS were used as reference control.

The cells were then fixed with 4% paraformaldehyde in PBS, permeabilised with 0.3% triton X-100 (Sigma Aldrich, Australia) in PBS containing 4% bovine serum albumin (BSA) for 30 min and blocked with 1.5% normal donkey serum. Polyclonal antibodies against anti-NF-κB p65 (1:50 dilution phospho-NF-κB p65) were applied on the cells for 24 h, at 4°C. This was then followed by an additional 1 h incubation with fluorescein isothiocyanate - conjugated anti-rabbit IgG antibody (1:150 dilution) at room temperature. Cell nucleus was visualised with 4′,6-diamidino-2-phenylindole (DAPI). After washing with PBS, the coverslips were mounted in Fluoromount-G, and images were captured using fluorescence microscope (Nikon Elipse TI time-lapse microscope with NIS-Elements, Tokyo, Japan) for a qualitative assessment of immunofluorescence. Apple Automator (v 2.0.4 Apple, Inc., CA, USA) was then used to convert the images into TIFF files for quantitative analysis using ImageJ (v1.42q, NIH, MD, USA) with the Colour Profile plugin (Dimiter Prodanov; Leiden University Medical Center, Leiden, The Netherlands). The green colour intensity was calculated by diving the RGB_G by the total cell surface coverage area. The mean of the green colour intensity was used to quantify the induction of NF-κB p65 in the control and SV treated AM. At least 10 images from 3 independent replicates were analysed.

3.3 Statistical Analysis

All results are expressed as mean ± standard deviation (S.D.) of at least three separate determinants. The significance was determined between groups and control, unpaired two-tailed t-tests and one-way ANOVA were performed (quoted at the level of $p<0.05$).

4.0 Results and Discussion

Alveolar macrophages in the airway are the first-line of defence against inhaled particulates. It has been well documented that any micro-environment alteration in alveolar macrophages can result in airway inflammation and oxidative stress in the lungs, leading to the development of diseases such as COPD, pulmonary fibrosis and other lung diseases (47). Consequently, this study focused on the biological activities of SV-NPs using LPS stimulated alveolar macrophage NR8383 cells in terms of inflammation.

4.1 Particle characterisation

The size distribution of SV-NPs after freeze drying was measured by DLS, as shown in Figure 1. The mean diameter of majority of the SV-NPs particles was found to be 213.8± 6.69 nm, with a polydispersity index of 0.45. Another smaller population of the SV-NPs with a diameter of 19.28 ± 5.7nm was also observed, which correspond to smaller components formed during the manufacturing process. Importantly after freeze-drying, the NPs particle size did not change and remained stable after 60 days in storage. In addition, the SV-NPs presented a negative zeta potential charge of -9.25 ± 1.17 mV, and an encapsulation efficacy of approximately 99.5%. As NPs have large surface area to volume ratio, they are prone to agglomerate to minimize their surface

energy. In this study, the agglomeration effect was counteracted by the use of stabilizers, such as pluronic, present on the NPs surface, that provided a stability effect on the colloidal suspension (48, 49). Hence, it is expected that lower concentrations of these SV-NPs will not have an impact on the aggregation of the NPs used in subsequent biological experiments. Although the composition of SV in the NPs is 5% with a high proportion of polymer, evidence from the current study has clearly showed that the benefits of SV-NPs over the same concentration of SV includes better cytotoxicity profile, enhanced anti-inflammatory activity and anti-oxidant effect, and chemical stability up to 9 months (28). These features could potentially result in lower requirements of SV dosage in the lungs.

4.2 Cytotoxicity of SV-NPs, SV solution and LPS

Manufacture of SV-NPs could lead to changes in the cytotoxicity responses of alveolar macrophage cells, compared to SV solution. To observe this effect, MTS cytotoxicity assays were performed on alveolar macrophage cell lines treated with SV-NPs and SV solution to understand the range of concentrations suitable for pulmonary drug delivery. Alveolar macrophages NR8383 were treated for 72 h with different concentrations of SV-NPs, SV solution and LPS, in order to define the IC_{50} value. As shown in Figure 2, the IC_{50} value was calculated and found to be 0.399 ug/mL and 0.0270 ug/mL for SV-NP and SV solution, respectively (Figure 2A). This data demonstrate that the AM were able to tolerate approximately 15 times higher SV concentration of the SV-NP compared to the free SV solution. The polymeric nanoparticle shell that encapsulates SV protects not only the drug from degradation but also control drug release from the NPs for an extended period of time and from coming in direct contact with the AMs, making it less toxic and safer compared to the free drug. This study highlights the

differential cytotoxicity profiles of the different formulations and provides the basis for safe SV concentration selection for subsequent experiments. In addition, the AMs were also treated with increasing concentrations of LPS and was found to be safe over the wide range of concentrations (Figure 2B).

4.3 Uptake of SV-NPs and free SV in inflamed alveolar macrophages

The ability and extent of SV to suppress inflammation in LPS-induced macrophages will most likely be dependent on the internalization rate of nanoparticles by these cells. To investigate this, the cellular uptake of SV-NPs and free SV by the stimulated AM as a function of time were assessed. Previous studies have shown that the AM are adversely affected in an inflammatory environment such as in chronic respiratory disease in terms of their phagocytic activity, morphology and motility (50, 51). Hence, the AM were first stimulated with LPS to simulate inflammation in the airways and were subsequently treated with SV-NPs or free SV solution at 1.25 μg/mL of simvastatin concentration. As demonstrated in Figure 3, SV-NPs had higher cellular internalisation efficacy after 6 h and 24 h of incubation (1.67 ± 0.5 % and 2.16 % ± 0.14, respectively) compared to the free SV solution, where cellular internalisation was significantly lower (0.29 % ± 0.01 and 1.41% ± 0.25, respectively). This data confirms that SV-NPs had a significant enhanced drug internalisation by inflamed AM over the free SV solution after 6 h and 24 h of treatment, respectively. The increased in cellular internalization of the SV-NPs could be related to the electrostatic interactions between cell membrane. Previous studies have shown that charged particles are internalised better compared to their uncharged counterparts. However, positively charged NPs are generally internalised better than negatively charged ones, due to association to the negatively charged sialic acid groups on macrophages (41, 52). Nevertheless, the

increase in drug internationalization of NPs is most likely mediated by both phagocytosis and endocytic pathways, unlike microparticles which are only mediated by phagocytosis in macrophages (41, 53); whereas free drugs typically passes through the membrane but are often subjected to efflux pump mechanisms that efflux drugs out of the cells (54). The increased uptake of charged polymeric NPs provides a means to enhance delivery of drug into macrophages during an inflammatory, a phenomenon that could result in an increased therapeutic efficacy, as well as possibly reducing the side effects of free drugs.

4.4 Antioxidant Properties of SV-NPs on inflamed AM

In several airway diseases including asthma, COPD and lung fibrosis it has been found that NO is key factor for airway deteriorations (55). NO generation by AM is part of the normal human immune response. However, activated AM following a bacterial infection or inflammatory responses generate high levels of NO that helps drive inflammatory disease progression. NO over-secretion in the lungs will subsequently recruit more macrophages to the airway and shift the balance towards a pro-inflammatory state, thus initiating an exacerbation of inflammation in airways (55).

Hence, NO is often used as a signal of inflammation in macrophages. In this study, we considered the potential of SV as NO inhibitor for curbing airway inflammation. One of the possible routes to activate macrophages to produce NO include interactions of nanoparticles with cell receptors and intracellular activation of NF-κB (56). Specifically, NF-κB activation in macrophages is usually accompanied by an increase in production of nitric oxide synthase (enzyme involved in high amount NO production) (56).

NO production from AM cell line NR8383 was subsequently induced with LPS for 24 h prior to treatment, and was subsequently followed by treatment using 1.25 μg/mL of SV-NPs and SV solution for 6 and 24 h. After that, cell culture media were collected and NO levels in the supernatant were quantified using Griess reaction and compared with LPS stimulated but untreated control cells, as shown in Figure 4. LPS was able to significantly increase the production of NO after 6 and 24 h by 3.7 (p = 0.000035) and 11 (p = 0.00056) folds, respectively. After 6 h incubation with the SV treatments, it was found that both treatments were able to slightly reduce NO production to approximately 3 folds the NO concentrations from baseline, compared to untreated cells. No significant difference between the treatments ($p < 0.05$) was observed. After 24 h of treatment instead, SV-NPs was able to significantly reduce the production of NO levels from a fold change from baseline of 11.1 ± 1.75 in untreated macrophages to 6.3 ± 0.3 (p = 0.0098). However, the antioxidant effect was not apparent with AM treated with free encapsulated SV at 24 h ($p > 0.05$). This is most likely due to the combined factor of improved physico-chemical stability of SV within the polymeric NPs preventing the conversion to SVA in solution and the sustained release of the drug from SV-NPs (28).

This data is in agreement with previous work from Tong et al., which reported that pretreatment with simvastatin-loaded poly(ethylene glycol)-*b*-poly(gamma-benzyl L-glutamate) (PEG-*b*-$PBLG_{50}$) nanoparticles was more effective than free simvastatin in reducing intestinal oxidative stress and inflammation in rats for the potential treatment of intestinal ischemia and reperfusion injury (57). It was proposed that 2 mechanisms were involved in NO scavenging activities of SV, which include the inhibition of iNOS enzyme activity and suppression of NF-κB expression (56), specifically Rac1, to reduce

generation of ROS through inactivation of the NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) system (58).

4.5 Anti-inflammatory activities of SV-NPs against inflamed AM

Over-secretion of pro-inflammatory cytokines such as IL-6, MCP-1, IL-8 and TNF- α are considered markers for various chronic lung diseases, which then leads to the activation of neutrophils migration to the lungs causing irreversible damage and irritation in lung tissue (59-61). In a murine asthmatic study, Kim Dy *et al*. found that airway inflammation was reduced after simvastatin treatment, but other published clinical studies have found conflicting outcomes (7, 24, 25). Therefore, in this study the potential of SV-NPs for inhalation therapy was compared to free SV solution in its ability to reduce pro-inflammatory chemokines production, specifically IL-6, TNF-α and MCP-1. IL-6 and TNF-α are proinflammatory cytokines responsible for the continued lung irritation, leading to uncontrolled inflammation and irreversible lung tissue damage; while MCP-1chemokine plays an important role in the recruitment of inflammatory cells into the lungs (62-65).

In this study the anti-inflammatory properties have been evaluated following stimulation of AM with 1ng/mL LPS 24-h prior to drug treatment at 6 and 24 h time point. Results showed that SV-NPs could significantly (p<0.05) downregulate the expression of all IL-6, TNF-α and MCP-1 secreted by inflamed AM stimulated by LPS after 6 and 24 h, compared to untreated control cells, as shown in Figure 5A, B and C. However, free SV solution was only able to reduce the production of TNF-α mediator level compared to untreated control (Figure 5B). There were no significant effects on

pro-inflammatory chemokines production of both the IL-6 and MCP-1 after 6 and 24 h of the treatment (Figure 5A and C). Similar results were also found by Tulbah *et al*. where nebulised SV nanoparticles were shown to reduce inflammation markers, including IL-6, 8 and TNF-α on inflamed Calu-3 epithelial cells 24 h following drug deposition (28, 66). The main mechanism involved in the inhibitory activities of simvastatin against pro-inflammation marker expression is envisaged to be via the NF-κB de-activation in alveolar macrophages. The same mechanism was proposed in other studies that have also demonstrated the SV potential to reduce airway inflammation in a mouse allergic asthma model (24). In addition, the antioxidant activity of SV could be synergic to its innate anti-inflammatory effect, as oxidative stress enhanced lung airway inflammation through stress kinases and redox-sensitive transcription factors (67).

4.6 Effects on nuclear translocation of NF- κB in inflamed AM

To further characterize the mechanism underlying the anti-inflammatory and antioxidant effects of SV, fluorescence microscopy was used to assess the NF-κB signalling pathway, which is critical in the activation or pro-inflammatory enzymes and cytokines. Under normal physiological conditions, NF-κB are sequestered in the cell's cytoplasm (Figure 6A). In the majority of the cells, in the absence of stimuli, NF-κB is associated with an inhibitor protein, IκBs, and as a result, NF-κB is retained within the cytoplasm. However, upon stimulation with LPS, the nuclear translocation of NF-κB p65 in the NR8383 AM was significantly induced, with translocation of the protein from the cytoplasm and accumulation into the nuclei (Figure 6B). The green fluorescence intensity in the AM NR8383 cells was quantified (Figure 7) and it was found to be significantly enhanced after stimulation with LPS, compared to control

unstimulated macrophages (p = 0.018). When the cells are stimulated with pro-inflammatory stimuli such as LPS, IκBs are rapidly phosphorylated, degraded, and thereby dissociated from NF-κB. The resulting free NF-κB is then able to be translocated into the nucleus, where it binds to the κB elements and induces the transcription of genes encoding pro-inflammatory mediators and cytokines (68, 69).

While, both the SV treatments demonstrated a significant reduction in the NF-κB intensity compared to untreated inflamed AM ($p < 0.05$), treatment with SV-NPs were able to produce further reductions in the fluorescence intensity of NF-κB staining throughout the cells (p = 0.0075). It could also be observed that with SV-NPs treatment, there was a redistribution of the protein back into the cytoplasm compared to the free SV solution, whereby the majority of the NF-κB p65 protein remained in the nucleus. These data further support the enhanced anti-inflammatory and anti-oxidant efficacy of SV-NPs on LPS-stimulated AM NR8383 compared to the free drug, which was mediated by inactivation of the NF-κB signaling pathway. Furthermore, in combination with the cytotoxicity profiles, these anti-inflammatory and anti-oxidant effects were not due to the cytotoxicity of SV or the NPs and were mediated through the inhibition of the nuclear translocation of NF- κB. The efficacy of SV to reduce inflammation could potentially have future therapeutic use as an inhalation localized therapy (19, 70).

5.0 Conclusions

This study has showed that SV- NPs are safe and non-toxic at the concentrations studied on alveolar macrophage cells, *in vitro*. Additionally, the cellular uptake of SV in inflamed AM stimulated with LPS was enhanced with SV-NPs compared to the free

drug. Consequently, these SV-NPs had an enhanced anti-oxidant and anti-inflammatory efficacy against inflamed macrophages. These activities are most likely mediated by the inhibition of pro-inflammatory mediators and cytokines in an LPS-induced nuclear translocation of NF-κB p65 in AM, via the down-regulation of the NF-κB signalling pathway to attenuate their corresponding gene activations. Future study will focus on comparing the anti-oxidant and anti-inflammatory activities of SV-NPs to other currently marketed treatments such as corticosteroids and the potential use of SV-NPs formulations in animal models to assess the toxicities, efficacies and pharmacokinetics.

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DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

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