Liposomal curcumin inhibits cigarette smoke induced senescence and inflammation in human bronchial epithelial cells

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\textbf{A B S T R A C T}

Curcumin, the principal curcuminoid of turmeric (Curcuma longa extract), is very well known for its multiple biological therapeutic activities, particularly its anti-inflammatory and antioxidant potential. However, due to its low water solubility, it exhibits poor bioavailability. In order to overcome this problem, in the current study, we have employed liposomal technology to encapsulate curcumin with the aim of enhancing its therapeutic efficacy. The curcumin-loaded liposomes (PlexoZome®) were tested on a cigarette smoke extract-induced Chronic Obstructive Pulmonary Disease (COPD) in vitro model using minimally immortalized human bronchial epithelial cells (BCIN1.1). The anti-senescent and anti-inflammatory properties of PlexoZome® were explored. 5 \(\mu\)M PlexoZome® curcumin demonstrated anti-senescent activity by decrease in X-gal positive cells, and reduction in the expression of p16 and p21 in immunofluorescence staining. Moreover, PlexoZome® curcumin also demonstrated a reduction in proteins related to senescence (osteonectin, FGF basic and uPAR) and inflammation (GM-CSF, EGF and ST2). Overall, the results clearly demonstrate the therapeutic potential of curcumin encapsulated liposomes in managing CSE induced COPD, providing a new direction to respiratory clinics.

\section{1. Introduction}

Chronic respiratory conditions have shown to impact a third of all Australians and can encompass range of symptoms from hay fever to asthma to chronic obstructive pulmonary disease (COPD) [1]. In 2018, COPD was found to impact 4.8 \% of Australians aged 45 and older highlighting it the most common chronic respiratory disease that year. Unfortunately this statistic has increased over time with it now being ranked as the fifth leading cause of total disease burden in Australia [1]. Similarly, COPD impacts a large portion of society with its global burden affecting 10.6 \% in 2020 and is ranked as the third leading cause of death worldwide [2]. COPD is known to be one of the most common diseases caused by cigarette smoke (CS) [3–5]. However, it has also been seen to be the result of various environmental, lifestyle and genetic factors [6]. Goblet cells are primarily located within the epithelial lining, in particular the trachea and bronchi. They are vitally important cells that are rapidly responsive in producing vast quantities of mucus during acute airway insult and thus, can prove as a front-line defender of the airways in normal homeostasis [7]. In COPD patients, the number and size of goblet cells are increased [8,9] also acknowledged as mucus hyperplasia (MCH). The increased amount of MCH and fibrosis restrict the airways obstructing airflow. The inflammation results in the...
Inhaled corticosteroids (ICS) are typically used in conjunction with common treatment used for asthmatic people and is a type of bronchodilators. This combination treatment is one of the airways whilst also helping widen the bronchi. Salbutamol is a shortness of breath, wheezing, chest tightness or coughing this is mainly due to the obstruction of airways with the mucus accumulation and inflammation [6].

The current therapies available for COPD have limited efficacy and typically focus on the relieving of symptoms such as coughing, shortness of breath and inflammation. Bronchodilators, corticosteroids and mucolytics are some of the most common therapies offered to COPD patients [14-16]. These treatments aid in reducing mucus preventing the airways from becoming obstructed resulting in adverse reactions. Bronchodilators help relieve bronchoconstriction and bronchospasms, the two common side effects of COPD. Bronchodilators utilise β-agonists such as salbutamol and formoterol to relax the surrounding muscles of the airways whilst also helping widen the bronchi. Salbutamol is a common treatment used for asthma-like airflow when experiencing an asthma episode. Corticosteroids are another common treatment used to manage airway inflammation, lower exacerbations, and improve lung function. Inhaled corticosteroids (ICS) are typically used in conjunction with long-acting beta agonists (LABA). This combination treatment is one of the most common for COPD patients. Despite the development of these therapies, they aid in relieving symptoms thus, improving the quality of patients’ life. Unfortunately, these treatments are limited, some drugs can have varying efficacy on patients, hence many variables need to be considered such as tolerance, allergic or adverse reactions and drug resistance. Some of the most common adverse reaction experienced by patients include pneumonia, poor oral conditions and easy bruising [17, 18]. Additionally, these treatments are not cost effective for prolonged use making it difficult for patients to maintain an essential cost long term, further highlighting the need for alternative therapies.

Phytochemicals have been used for centuries in traditional medicine, however, recently have been gaining traction for their pharmacological activities with minimal to no side effects other than gastrointestinal symptoms at high doses [19-23]. Curcumin has become increasingly popular amongst society due to its antioxidant and anti-inflammatory properties and its natural benefits are highly acknowledged in literature [24-27]. Curcumin offers several benefits including the inhibition of alveolar epithelial thickness and proliferation, diminution of inflammatory response, airway remodelling and ROS production [25]. Unfortunately, due to the hydrophobic nature of curcumin, it has a very poor solubility and bioavailability making it susceptible to degradation [28]. Notably, various studies have assessed the advantages of using advanced delivery techniques to enhance the natural properties of phytochemicals such as curcumin whilst simultaneously improving the bioavailability of the active [29,30].

Advanced delivery techniques such as micelles, liposome to nano-tubes have become increasingly popular to accelerate systemic drug delivery to specific target site [19, 31-33]. The nano-delivery systems allow for the natural properties of an active pharmaceutical ingredient (API) to be enhanced whilst simultaneously improving the bioavailability [34-36]. Additionally, nanoparticles enable the slow release of drugs allowing for larger doses to be administered. By using passive loading, poorly soluble APIs can be encapsulated within the phospholipid bilayer and protected against the harsh gastric conditions in the stomach. This allows for an increased bioavailability to be achieved whilst also offering less invasive ways of administering an array of drugs to patients. To overcome this issue, we used curcumin-loaded vesicular systems in comparison to free powder curcumin and assessed its ability in inhibiting proliferation, migration, and associated signalling pathways in an in vitro COPD model using human broncho epithelial cell line (BCN51.1).

The objective of this research is to investigate how the utilisation of nano-delivery using liposomes enhances the natural benefits of curcumin on human bronchial epithelial cells and its ability to mitigate COPD features. Furthermore, the use of liposomes will reflect how the bioavailability of curcumin is increased enabling prosperous results. We therefore have performed functional and mechanistic assessments on the proteins that play a key role in COPD and importantly, assessed the anti-senescent and anti-inflammatory properties of curcumin liposomes. From this study it was made evident that liposomal curcumin significantly counteracts the effects of cigarette smoke extract by reducing the expression of proinflammatory proteins including GM-CSF, EGF and ST2, which were induced by the cigarette smoke extract. Similarly, key cigarette smoke extract-induced markers contributing to cell senescence were identified (p16, p21, osteopontin, FGF basic and uPar) and to reduce with exposure to liposomal curcumin.

2. Methodology

2.1. Preparation of Curcumin Liposomes

PlexoZome® is a registered technology owned by Pharmako Biotechnologies, which utilises phosphatidylcholine to encapsulate active ingredients within the lipid bilayer of a liposome protecting it from degradation and increasing its stability. The liposomes were characterised by Pharmako Biotechnologies Pty Ltd through zeta potential, particle size and Polydispersability Index (PDI). Additional on-going stability studies are also being conducted. The sample of PlexoZome® curcumin used throughout this study consisted of a 0.1% concentration of 95% Curcuma longa extract. The free powder used was also from the same batch of curcumin used for the PlexoZome®.

2.2. Cell culture and treatment

Minimally immortalised human bronchial epithelial cells (BCN51.1) were cultured using bronchial epithelial basal media with growth supplement (Lonza). The cells were routinely checked for mycoplasma contamination, and only mycoplasma free cells were used.

The 5% cigarette smoke extract (CSE) was prepared as per K.R Paudel, et al. [37]. A research grade cigarette from University of Kentucky and smoked/extracted into 10 mL of cell culture media. This was considered as a 100% CSE solution which was filtered (0.22 µm) and diluted to a final concentration of 5% CSE.

2.3. Cell Viability - MTT

By utilising 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) assay, the cell viability of BCN51.1 was determined. The protocol was followed as per K.R. Paudel, et al. [13]. 10,000 cells per well were seeded in a 96 well plate. Various concentrations (2.5, 5, 10 and 20 µM) of both the free curcumin powder and liposomal curcumin treated for 24 hrs were tested on the cells. 10 µL of 5 mg/mL stock of MTT was dispensed into each well and then left to incubate at 37°C for 4 hours. After incubation, the media was removed and 100 µL of DMSO was added to dissolve the formazan formed at the bottom of each well. A plate reader was used to obtain an absorbance reading at a wavelength of 570 nm.

2.4. X-gal

The method used was followed as per K.R. Paudel, et al. [13] using an Abcam X-gal kit (cat. No. Ab102534). BCN51.1 cells were cultured on a cover slip inside a 6 well plate and pre-treated with the curcumin powder and liposomal curcumin. The cells were pretreated for one hour with the curcumin powder and liposomal curcumin and continue for next 24hrs together with the 5% CSE solution. After the period of exposure, the cells were washed using PBS and fixed using a fixative solution for 10 minutes at room temperature. The cells were stained using X-gal solution and incubated over night at 37°C. After
After exposure the cells were washed using PBS and fixed with 4 % curcumin and continue for next 24 hrs together with 5 % CSE solution. and pre-treated for one hour with the curcumin powder and liposomal et al. [37] various concentrations. Values have been expressed as a percentage. ns

2.5. Immunofluorescence microscopy - p16 and p21 staining

Immunofluorescence method used was followed as per K.R. Paudel, et al. [37]. The cells were cultured on a cover slip inside a 6 well plate and pre-treated for one hour with the curcumin powder and liposomal curcumin and continue for next 24 hrs together with 5 % CSE solution. After exposure the cells were washed using PBS and fixed with 4 % paraformaldehyde for 10 minutes, permeabilised for 30 minutes with 0.5 % Triton X-100 and finally blocking with 1 % bovine serum albumin for 30 minutes. Cells were incubated overnight with a 1:800 dilution of anti-p21 and anti-p16 antibody at 4°C followed by washing and incubation again with Alexa 488 (for p16) and Alexa 647 (for p21) at 1:1000 dilution for 1 hour. The cover slips were mounted using mounting media containing DAPI which stains the nucleus. The images of the cells were taken at 40X magnification using Zeiss Axio Imager Z2 microscope and then quantified by using Image J.

2.6. Human cytokine protein array

The kit was purchased from In Vitro Technology Australia and contains various proteins which are analysed in duplicate. The cells were seeded in 6 well plates and pre-treated with curcumin liposome (PlexoZome® Curcumin) or free curcumin, followed by 5 %CSE as per previous studies [37]. Protein were extracted from each group, quantified and equal amount of protein was used to quantify the expression of various protein following manufacturer instruction.

2.7. Statistical analysis

All experiments have been completed in triplicate (at the minimum) to confirm repeatability and accuracy. By utilising the software program Image J, the data obtained from the protein array and immunofluorescence microscopy was quantified allowing for it to then be graphed in the software prism. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison test.

3. Results

3.1. Identification of ideal concentration of curcumin for BCiNS1.1 cells treated with CSE

When conducting the MTT assay both free powdered curcumin and liposomal curcumin were required to be tested to proceed with further mechanistic experiments. Four different concentrations were tested in addition to the only vehicle (solution mixture used to prepare curcumin liposomes) to find the maximum safe concentration of curcumin and curcumin liposome for further experiments. From Fig. 1, it is evident that 5 μM of free curcumin powder and 2.5 μM of liposomal curcumin yielded the highest viability percentage before becoming toxic to the cells at higher concentration. The empty vehicle showed no significant impact on the cells viability as portrayed by Fig. 1. Therefore, further functional, and mechanistic experiment were conducted in BCiNS1.1 cells with concentration of free and liposomal curcumin not exceeding 2.5 μM.

3.2. X-gal

The rapid test, X-gal staining, is used on cells measuring the presence of β-galactosidase. An increased production of β-galactosidase results in an increase of blue staining, reflecting cells undergoing senescence. In Fig. 2(A), the 5 %CSE induced senescence of BCiNS1.1 cells as shown by blue staining of X-gal positive cells. A pre-treatment of 2.5 μM of PlexoZome® curcumin for 24 hours before stimulation with 5 % CSE was found to significantly reduce X-gal positive cells compared to 5 % CSE alone (Fig. 2B).

3.3. Mean fluorescence quantification of p-16 staining

Fluorescence microscopy is used for identifying the expressions of key senescence marker proteins (p16 and p21) in cells. The data obtained for p16 fluorescence staining confirmed the anti-senescence activity of both curcumin powder and liposomal curcumin. From Fig. 3 (A), 5 %CSE treatment induced increase in the p16 positive cells as shown by p16-Alexa488 positive green cells.

Both treatments (pure powder curcumin and liposomal PlexoZome® curcumin) showed positive effects in reducing the fluorescence signal. The MFI of p16 was significantly increased by approximately 3-fold by 5 % CSE while reduced with treatment of PlexoZome® curcumin and this reduction was even higher compared to free powder curcumin (Fig. 3B).

3.4. Mean fluorescence quantification of p-21 staining

Similarly, in Fig. 4A, there was an increased in intensity of red fluorescence staining of p21-Alexa647 in 5 %CSE exposed cells, while reduced fluorescence intensity in the liposomal curcumin. After fluorescence quantification, the MFI of p21 was found to be decreased drastically in both free powder curcumin and liposomal curcumin.

![Fig. 1. Cell viability of BCiNS1.1 bronchial epithelial cells with treatment (A) free powder curcumin at various concentrations (B) liposomal curcumin formulation at various concentrations. Values have been expressed as a percentage. ns = non-significant, **** p < 0.0001 vs control (only media). Veh = vehicle control. Statistical analysis was performed with one-way ANOVA followed by Dunnett’s multiple comparison test.](image-url)
However, there was no significant different between the effect of free curcumin powder and liposomal curcumin.

3.5. Human cytokine protein array

From the cytokine protein array conducted, eight proteins upregulated by 5% CSE were significantly reduced by curcumin free powder and curcumin liposomes. The protein expression of osteopontin, FGF basic, and uPAR was significantly upregulated by 5% CSE exposure while PlexoZome® curcumin (but not pure curcumin powder) showed significant decrease of osteopontin by 63.38% (Fig. 5A), FGF basic by 18.06% (Fig. 5B), and uPAR (Fig. 5C) by 37.2% in comparison to the CSE alone group. This clearly suggests the potency of PlexoZome® curcumin is superior to pure powder curcumin.

With regards to granulocyte macrophage colony stimulating factor (GM-CSF), CSE significantly increases its expression. Free powder curcumin had a decrease of 61.4% while PlexoZome® curcumin was slightly higher with a decreased of 65.67% (Fig. 5D). Similarly powder curcumin reduced the expression of epidermal growth factor (EGF) caused by CSE by 84.7% while PlexoZome® curcumin further reduced it by 86.29% (Fig. 5E). The final protein seen to contribute to inflammatory markers was ST2, a similar trend was evident with an upregulation by CSE which was reduced by powder curcumin by 85.89% and further reduced by 92.96% from treatment with PlexoZome® curcumin (Fig. 5G).

4. Discussion

COPD has an increased prevalence amongst society over the years. There are a limited number of studies that investigate the effectiveness of curcumin in COPD. Reviewed studies show improvement in COPD samples that received curcumin supplements [38]. Most studies suggest decreasing airway inflammation as the reason for the alleviation effect of curcumin [25,39]. Though curcumin possesses several advantages, the solubility or its formulation is an issue. Therefore, various studies have investigated an alternative way to deliver the curcumin to its target...
Osteopontin has crucial role in airway inflammation through FGF is considered as an important immunomodulatory factor (a mediator between immune cells and airway structural cell) in asthma and COPD and associated with airway remodelling [56]. In Fig. 5B, a comparable trend was seen with a 33.99% overexpression of FGF basic after 5% CSE exposure supporting its contribution to senescence. Furthermore, the reduction of the FGF basic expression cause by PlexoZome® curcumin revealed how the advanced delivery system enhances the activity of curcumin reducing cell senescence.

The increase level of soluble urokinase-type plasminogen activator receptor (uPAR) in COPD patients is an indication of the risk of acute exacerbation increases suggesting soluble uPAR as a novel biomarker has potential for early diagnosis of COPD and prediction of acute exacerbation of COPD [57]. In this study when exposed to CSE an increase of uPAR was seen (Fig. 5C) which then significantly decreased post-treatment with liposomal curcumin by 37.2%, uPAR (Fig. 5B) similarly was found in the study by our team [13] reflecting how it is associated with the pathogenesis of COPD. A clinical trial using 60 patients with varying degrees of COPD found similar results that uPAR, P1-1 and CAP18 were associated with airflow limitations [58], the findings from our study support these findings and reflect the link an upregulation of uPAR has with COPD.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a cytokine which plays a role in regulating macrophage, neutrophil activation, and survival as well as the progression of pulmonary fibrosis [59, 60]. Furthermore GM-CSF has been found to contribute to inflammatory markers and have pro-inflammatory features [61]. Our study supports this finding, as the protein array showed GM-CSF was drastically decreased when the BCNS1.1 cells exposed to CSE was pre-treated with PlexoZome® curcumin (Fig. 5D) signifying that a reduction of expression GM-CSF results in reduced airway inflammation. The epidermal growth factor (EGF) protein has been seen to typically increase in COPD patients [62,63] supporting our findings from Fig. 5E as the expression EGF by BCNS1.1 cells exposed to CSE dramatically increased by 8 fold, compared to the control, and similarly was seen to drastically decrease when exposed to either of the tested curcumin samples.

ST2 the receptor of IL-33 was found, in Fig. 5F to yield one of the best results with a significance value of p < 0.0001 (PlexoZome formulation versus CSE alone). Multiple papers have found a similar expression caused by ST2 when exposed to cigarette smoke [64,65] suggesting that ST2 induces inflammation in a CSE model. The collective findings of the study with PlexoZome® curcumin with CSE induced COPD is shown in Fig. 6.

Even with the encouraging data gathered, further mechanistic testing would be beneficial for the broadening of breadth and scope of the discussed results. For a more extensive understanding of airway remodelling and the impact of curcumin in reducing COPD features additional cell lines could be used. By utilising more complex cell-based models such as microfluidics, 3D organoids, air-lung interface or organ-
on-a-chip, a deeper understanding of the mechanisms in action would be made. Furthermore, similarly replicating the complex nature of the diseases when treated with liposomal curcumin.

5. Conclusion

Several studies have shown that liposomal technology have been beneficial in the delivery of curcumin for chronic diseases due to an improvement in the molecule’s stability, and the possibility to achieve controlled release [66]. Our findings have provided an inclination to the use of liposomes containing curcumin (PlexoZome®) in CSE-induced COPD by targeting various senescence (SA-β-Gal, p16, p21, osteopontin, FGF Basic, uPAR) and inflammatory (GM-CSF, EGF, ST2) markers in vitro, reducing the characteristic features of COPD. This not only reduces the pathophysiology of COPD but also attenuates its progression, making PlexoZome® curcumin a viable option for the treatment and management of COPD. Despite the promising data collected from this study, further investigation can be conducted to probe the in-depth cellular mechanism along with in vivo studies to bring this novel curcumin loaded liposomal technology to the pulmonary clinics.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors have no conflict of interest to declare.

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