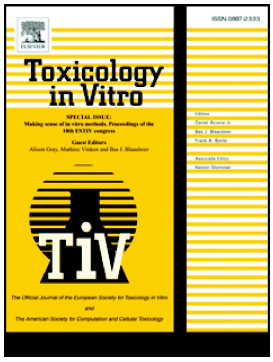

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Berberine-loaded engineered nanoparticles attenuate TGF-β-induced remodelling in human bronchial epithelial cells

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Abstract

Airway remodelling occurs in chronic respiratory diseases (CRDs) such as asthma and chronic obstructive pulmonary disease (COPD). It is characterized by aberrant activation of epithelial reparation, excessive extracellular matrix (ECM) deposition, epithelial-to-mesenchymal transition (EMT), and airway obstruction. The master regulator is Transforming Growth Factor-β (TGF-β), which activates tissue repair, release of growth factors, EMT, increased cell proliferation, and reduced nitric oxide (NO) secretion. Due to its fundamental role in remodelling, TGF-β is an emerging target in the treatment of CRDs. Berberine is a benzylisoquinoline alkaloid with antioxidant, anti-inflammatory, and anti-fibrotic activities whose clinical application is hampered by poor permeability. To overcome these limitations, in this study, berberine was encapsulated in monoolein-based liquid crystalline nanoparticles (BM-LCNs). The potential of BM-LCNs in inhibiting TGF-β-induced remodelling features in human bronchial epithelial cells (BEAS-2B) was tested. BM-LCNs significantly inhibited TGF-β-induced migration, reducing the levels of proteins upregulated by TGF-β including endoglin, thrombospondin-1, basic fibroblast growth factor, vascular-endothelial growth factor, and myeloperoxidase, and increasing the levels of cystatin C, a protein whose expression was downregulated by TGF-β. Furthermore, BM-LCNs restored baseline NO levels downregulated by TGF-β. The results prove the *in vitro* therapeutic efficacy of BM-LCNs in counteracting TGF-β-induced remodelling features. This study supports the suitability of berberine-loaded drug delivery systems to counteract airway remodelling, with potential application as a treatment strategy against CRDs.

Keywords

Asthma, COPD, lung cancer, airway remodelling, TGF-β, berberine, phytoceuticals, liquid crystalline nanoparticles

1. Introduction

Chronic respiratory diseases (CRDs) are a heterogeneous group of diseases affecting the airways and other lung structures and including asthma, chronic obstructive pulmonary disease (COPD), occupational lung diseases, pulmonary hypertension, idiopathic pulmonary fibrosis, and others [1-3]. Lung cancer (LC), particularly non-small cell lung cancer (NSCLC), is often considered a CRD as well [4]. The main aetiologic factor associated with the development of these diseases is cigarette smoking through the exposure to the thousands of toxic chemicals in tobacco smoke, which promote a chronic pro-inflammatory and pro-oxidant state that further prompts disease progression [5-7]. CRDs, particularly asthma, COPD, and LC, are among the leading causes of mortality and morbidity worldwide, and their global burden is significant [8]. According to recent data, in 2017-2019, COPD caused about 3.3 million deaths annually [9], while about 500 000 yearly deaths were associated with asthma [8]. Overall, LC is one of the deadliest types of cancer, with 1.7 million deaths attributed to LC globally in 2020, a trend that is currently increasing [12]. Although less frequent compared to other CRDs, idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disease characterized by the irreversible scarring of the lung's interstitial framework with a median survival of 2-5 years if untreated [16].

Current therapeutic approaches for CRDs include pharmacological and non-pharmacological strategies. These are severely limited and, in the case of asthma, IPF and COPD, aim at improving and managing disease symptoms rather than tackling the underlying disease mechanisms [16-18]. Despite showing some efficacy, the currently available pharmacotherapies for CRDs are limited by severe side effects [16, 21, 22]. With regards to LC, therapeutic approaches include various combinations of surgical removal, radiotherapy, chemotherapy, and immunotherapy, depending on the tumor stage [23]. Chemotherapies are all limited by severe organ toxicity, adverse effects, as well as by the eventual development of cancer multidrug resistance [23-25]. This underlines the need to develop novel therapeutics with improved treatment outcomes and reduced adverse effects. In this context, developing treatment strategies tackling one or more cellular and molecular mechanisms shared by different diseases would be advantageous.

A fundamental feature shared between all these CRDs is the progressive radical deterioration and alteration of the structure of the respiratory tract, also termed airway remodelling [27, 29,

30]. Structural transformations in airway remodelling include subepithelial fibrosis, infiltration of immune cells, disruption of epithelial cell layers, excessive mucus secretion, excessive production of matrix metalloproteinases (MMPs), and thickening of the basement membrane due to excessive collagen deposition [31]. This leads to severe airway obstruction [27, 30, 32]. The thickening of the basement membrane is caused by the presence of an excessive number of highly synthetic myofibroblasts, which express alpha-smooth muscle actin (α-SMA) [32]. Epithelial-to-mesenchymal transition (EMT) of lung epithelial cells, which acquire increased proliferative and migratory capacity, is considered an important source of myofibroblasts contributing to airway remodelling. [32-34].

One of the master regulators of tissue remodelling, particularly in the airways, is transforming growth factor-beta (TGF-β) [34-36]. TGF-β is a multifunctional cytokine present in three different isoforms: TGF-β1, TGF-β2, and TGF-β3, with partially overlapping biological activities [37, 38]. TGF-β is secreted in an inactive form called large latent complex (LLC), in which the functional protein is bound to the latency-associated peptide (LAP) and other proteins [36, 39]. The LLC is primarily localized in the ECM and it functions as a reservoir of inactive TGF-β [36]. The active form of TGF-β is released following different stimuli, including temperature spikes, acidification of the microenvironment, oxidative stress, proteolysis, and integrin binding [36]. Thrombospondin-1 and MMPs are among the main proteins activating TGF-β, and the fact that these proteins are upregulated by TGF-β itself represents an important positive feedback mechanism enhancing TGF-β activation [40, 41]. Upon binding to its receptor, TGF-β regulates the expression of a plethora of target genes mainly through the canonical TGF-β/Smad pathway, in which Smad proteins are phosphorylated and translocated into the nucleus, where they act as transcription factors [42]. Many other proteins are involved in the regulation and action of TGF-β signalling in airway remodelling and EMT. These include growth factors such as the vascular endothelial growth factor (VEGF), which is a known inducer of lung remodelling [43] and whose secretion is enhanced by TGF-β in airway smooth muscle cells [44], as well as the basic fibroblast growth factor (bFGF), which is co-expressed with TGF-β in the lung of ovalbumin (OVA)-induced mice [45] and induces angiogenesis associated to remodelling in asthma and COPD [46]. Other proteins involved in TGF-β-induced remodelling and EMT include myeloperoxidase [47], and endoglin, which is associated with the TGF-β receptors and affects TGF-β responses [49]. TGF-β signalling is also controlled by negative regulators such as cystatin C [51].

An important mechanism through which TGF-β induces EMT in alveolar epithelial cells is through the reduction of baseline nitric oxide (NO) levels [52]. NO, in fact, is a critical factor that attenuates EMT, and TGF-β reduces its baseline production in the injured lung through the inhibition of the endothelial NO synthase (eNOS) [52] and other enzymes involved in NO production such as soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase I (PKGI) [53].

Physiologically, TGF-β plays a pleiotropic role in lung health and development. Its activity is fundamental in lung organogenesis, and in the regulation of homeostatic alveolar epithelial growth, differentiation, and EMT [54]. Due to its fundamental role in lung homeostasis, dysregulation of TGF-β signalling is common in many diseases where tissue remodelling plays a relevant role, including CRDs [54, 55]. Considering its function as a promoter of EMT in healthy cells, dysregulation of TGF-β signaling is also an important factor contributing to increased cell migration and invasion in many types of cancer, including LC, where it is recognised as the most potent inducer of EMT [54]. Interestingly, increased levels of TGF-β have been detected in the airways of COPD, asthma, and LC patients, as well as tobacco smokers [54, 55].

The multifaceted role played by TGF-β in CRDs makes it a valuable pharmacological target [35]. In the quest for novel pharmacological strategies to treat respiratory disorders, plant-derived molecules, also known as phytoceuticals, are an endless source of inspiration [56]. In this context, one promising phytoceutical is berberine, an isoquinoline alkaloid found in barberry, tree turmeric, and other plants [57, 58]. Berberine is widely known for its potent antioxidant, anti-inflammatory, and anticancer properties [58-62], and it also exerts antifibrotic activity in the lungs, heart, liver, pancreas and kidneys of rodents [63]. In the lung, in particular, the antifibrotic activity of berberine is exerted through the suppression of nuclear factor-κB (NF-κB)-induced TGF-β activation [64]. Furthermore, berberine was shown to counteract TGF-β-induced EMT in vitro on A549 human NSCLC cells [65].

Despite its promising biological activity, the clinical use of berberine, similarly to that of other phytoceuticals, is currently limited by its poor solubility and permeability which, together with a high rate of hepatobiliary excretion, translates into poor oral bioavailability and unfavourable pharmacokinetics [66, 67]. To overcome these limitations, encapsulation of phytoceuticals within advanced, nanoparticle (NP)-based drug delivery systems is an advantageous strategy [56, 68, 69]. This allows to drastically increase the solubility,

permeability, and stability of the encapsulated molecules, improving their bioavailability and pharmacokinetic properties [35, 56, 70, 71]. Among the many available classes of nanoformulations, liquid crystalline nanoparticles (LCNs) are particularly versatile in the treatment of pulmonary diseases [72]. Our research team has worked extensively with berberine-loaded LCNs, demonstrating their superior *in vitro* anticancer activity against A549 lung cancer cells [60-62], as well as a potent antioxidant, anti-inflammatory, and anti-senescence activity in cigarette smoke-induced BCi-NS1.1 human airway basal cells [58, 73] and antioxidant and anti-inflammatory activity in lipopolysaccharide (LPS)-induced mouse RAW264.7 macrophages [59].

In the present work, we tested a monoolein-based berberine LCN formulation (BM-LCNs) against a model of airway remodelling and EMT obtained by stimulating BEAS-2B human bronchial epithelial cells with TGF-β [32]. We show that BM-LCNs significantly attenuate the functional and molecular features associated with TGF-β-induced remodelling and EMT. This study highlights the enormous potential of nanoparticle-based drug delivery systems in enhancing the use of phytoceuticals for the treatment of lung diseases, providing proof of the applicability of BM-LCNs as a potential therapeutic agent tackling the aberrant airway remodelling process that drives the pathogenesis of CRDs.

2. Materials and Methods

2.1. Formulation and Physicochemical Characterisation of BM-LCNs

Berberine hydrochloride (Cat. #B3251), monoolein (Cat. #CRM44893), and poloxamer 407 (Cat. #16758) were purchased from Sigma-Aldrich, Australia, and were used for the preparation of BM-LCNs. BM-LCNs were formulated using the ultrasonication method, and characterised for physicochemical characteristics such as particle size, polydispersity index, zeta potential, entrapment efficiency, morphology, and *in vitro* release, as reported in a previous study [62]. Briefly, 200 mg monoolein were melted at 70 °C in a glass vial. Poloxamer 407 (20 mg) was dissolved in 4.8 mL deionized water and heated to 70 °C in a glass vial. Berberine powder (5 mg) was added to the melted monoolein and vortexed until completely dissolved. Then, the poloxamer 407 solution was added to the berberine-monoolein solution until formation of a coarse dispersion. The coarse dispersion was finally subjected to size reduction using a probe sonicator, using an amplitude of 80 for 5 minutes, with 5-seconds-on and 5-seconds-off cycles.

2.1 Cell Culture

The human bronchial epithelial cells (BEAS-2B, ATCC #CRL-9609) were a kind gift from Professor Alaina Ammit (Woolcock Institute of Medical Research, Sydney, NSW, Australia). These cells were used at passages between 15 and 25 throughout the study, and were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Australia, Cat. #D6046) supplemented with 5% fetal bovine serum (Sigma-Aldrich, Australia, Cat. #F9423), 100 unit/ml penicillin and 100 µg streptomycin (Pen-Strep solution, Sigma-Aldrich Australia, Cat. #P4333) in a humidified atmosphere containing 5% CO_2.

2.2 Cell viability assay - MTT

The MTT cell viability assay was performed as reported in a previous study [59]. The BEAS-2B cells were seeded at a density of 5,000 cells/well in a transparent, clear-bottom 96-well plate and left to attach overnight. The following day, the cells were incubated in the presence of 5 ng/mL human TGF-β1 (R&D Systems Biotechnology, Minnesota, USA, Cat. # 754BH005), at 37 °C for 24 h. The cells were then incubated for 24 more hours in the presence of BM-LCN concentrations ranging between 0 and 10 µM or with empty LCNs at dilutions representative to the BM-LCNs concentrations tested. Then, 250 µg/mL MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich, Australia, Cat. #M2003) was added to each well and incubated at 37°C for further 4 h. Upon incubation, the supernatant was removed, and the formed formazan crystals were dissolved using 100 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Australia, Cat. #D8418). The absorbance was read at 570 nm wavelength using a TECAN Infinite M1000 plate reader (Tecan Trading AG, Switzerland).

2.2 Cell viability assay – Trypan Blue staining

The impact of BM-LCNs on cell viability has also been assessed using Trypan Blue staining, similarly to how reported in a previous study [62]. Briefly, 10,000 cells/well were seeded in a 48-well plate and left to attach overnight. The following day, the cells were incubated in the presence of 5 ng/mL human TGF-β1, at 37 °C for 24 h. The cells were then incubated for 24 more hours in the presence of BM-LCN concentrations ranging between 0 and 10 µM. After incubation, the cells were treated with 300 µL 1X Trypsin-EDTA solution (Sigma-Aldrich, Australia, Cat. # T4299), incubating at 37 °C for 2 minutes to allow cell detachment. The

trypsin was then inactivated by adding 300 µL FBS-supplemented DMEM, and the cells were centrifuged at 500 x g, for 5 minutes, at room temperature. The cell pellet was then resuspended in 20 µL FBS-supplemented DMEM and mixed at a 1:1 ratio with 0.4% Trypan Blue solution (ThermoFisher Scientific, Australia, Cat. #15250061). The number of live cells/mL was counted using a Neubauer Improved haemocytometer, using a light microscope at 10X magnification.

2.3 Wound healing assay

To assess the anti-migratory activity of BM-LCNs on TGF-β-stimulated BEAS-2B cells, the wound healing assay was performed. Briefly, 100,000 BEAS-2B cells/well were seeded into 6-well plates and incubated at 37 °C overnight. The following day, the cell monolayer was scratched using the tip of a sterile 200 µL pipette tip, and the wells were washed five times with sterile PBS (Sigma-Aldrich, Australia, Cat. #P3813). The cells were then incubated in the presence of 5 ng/mL TGF-β1 alone or in the presence of 5 ng/mL TGF-β1 and 0.5µM BM-LCNs for up to 48 h. The distance between the edges of the scratch was measured under a light microscope, at 10X magnification, at 0, 24, and 48 h time points. The percentage of wound closure was normalized as a percentage compared to the control (untreated) group.

2.4 Human cytokine protein array

The effect of BM-LCNs on the expression of cytokines and other proteins in TGF-β-induced BEAS-2B cells was studied using the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA, Cat. #ARY022B), as described previously [58]. The cells were seeded in a 6-well plate at a density of 100,000 cells/well and left to attach overnight. The following day, the cells were incubated in the presence of 5 ng/mL TGF-β1 and incubated at 37 °C for 24 h. The cells were then incubated for another 24 h in the presence of 0.5 µM BM-LCNs. Following incubation, the cells were lysed with 500 µL RIPA buffer (ThermoFisher Scientific, Australia, Cat. #89900) supplemented with protease inhibitor tablets (Roche Diagnostics GmbH, Mannheim, Germany, Cat. #11697498001). An amount of 300 µg of proteins from each experimental group was loaded onto each array and incubated overnight at 4 °C. The further incubation steps with antibodies and chemiluminescent reagents were performed following the manufacturer's instructions. The arrays were photographed using a ChemiDoc MP (Bio-Rad, Hercules, CA, USA) and the pixel density for each spot was analysed with ImageJ (version 1.53c, Bethesda, MD, USA).

2.5 NO levels determination with Griess reagent

The relative levels of NO released by BEAS-2B cells in the culture supernatants were determined using the modified Griess reagent (Sigma-Aldrich, Australia, Cat. #G4410). The cells were seeded in a 6-well plate at a density of 100,000 cells/well and left to attach overnight. The following day, the cells were incubated in the presence of 5 ng/mL TGF-β1 and incubated at 37 °C for 24 h. The cells were then incubated for another 24 h in the presence of 0.5 µM BM-LCNs. Following incubation, the culture supernatants were collected, and 100 µL of the supernatants were added to a clear-bottom, transparent 96-well plate. The supernatants were mixed in a 1:1 ratio with the Griess reagent and the plates were incubated for 30 min at room temperature in dark. The relative NO levels were determined using a TECAN Infinite M1000 plate reader (Tecan Trading AG, Switzerland) by measuring the absorbance at 540 nm. Unconditioned cell culture media was used as a blank, and its absorbance was subtracted from the absorbance of each sample. Relative NO levels were reported as a percent change compared to the untreated group.

2.6. Statistical analysis

The data are presented as mean ± SEM. The data were analysed by ordinary one-way ANOVA, followed by Tukey multiple comparison test, using GraphPad Prism (v.9.4, GraphPad Software, San Diego, CA, USA). A two-tailed of p-value <0.05 was considered statistically significant for pairwise comparisons.

3. Results

3.1. Identification of an optimal concentration of BM-LCNs for treating TGF-β-stimulated BEAS-2B cells

To find a non-lethal BM-LCNs concentration to treat BEAS-2B cells, the MTT assay and the Trypan Blue assay were performed to assess the cell viability following treatment of TGF-β-induced BEAS-2B cells with increasing BM-LCNs concentrations. The results are shown in Figure 1a (MTT assay) and Figure 1b (Trypan Blue assay). Furthermore, the toxicity of empty LCNs on BEAS-2B cells was tested through MTT assay (Figure 1c). Treatment with BM-LCNs concentrations of 0.25 and 0.5 µM resulted in a slight, not statistically significant, reduction in cell viability of 4.5% and 12.8%, respectively (Figure 1a, $p>0.05$ against the TGF-β-treated group for both groups). At higher concentrations of 1, 2.5, 5, and 10 µM, treatment with BM-LCNs caused a significant reduction of cell viability of 28.0%, 29.4%, 26.5%, and 51.9%, respectively (Figure 1, $p<0.0001$ against the TGF-β-treated group for all groups). In the Trypan Blue assay, treatment with BM-LCNs concentrations of 0.25 and 0.5

μM resulted in a slight, not statistically significant, increase in cell viability of 0.8% and 1.5%, respectively (Figure 1b, $p>0.05$ against the TGF-β-treated group for both groups). At higher concentrations of 1, 2.5, 5, and 10 μM, treatment with BM-LCNs caused a significant reduction of cell viability of 18.1% ($p<0.01$ against the TGF-β-treated group), 30.2% ($p<0.001$ against the TGF-β-treated group), 54.7% ($p<0.0001$ against the TGF-β-treated group), and 89.4% ($p<0.0001$ against the TGF-β-treated group), respectively (Figure 1b). The highest non-toxic BM-LCNs concentration resulted to be 0.5 μM across both assays, and this concentration was used to treat cells in the following experiments. Furthermore, treatment of BEAS-2B cells with empty LCNs used at a concentration corresponding to 0.25, 0.5, and 1 μM BM-LCNs resulted in a slight, not significant, reduction in cell viability (of 1.4%, 1.6%, and 4.2% respectively ($p>0.05$ against the untreated group, Figure 1c). Treatment with higher concentrations of empty LCNs, corresponding to 2.5, 5, and 10 μM BM-LCNs, resulted in a significant reduction of cell viability of 15.8% ($p<0.001$ against the untreated group), 16.5% ($p<0.0001$ against the untreated group), and 27.9% 16.5% ($p<0.0001$ against the untreated group), respectively (Figure 1c). Treatment with either TGF-β alone or with TGF-β and 0.5 μM BM-LCNs did not result in significant changes in the cells' morphology (Figure 1d).

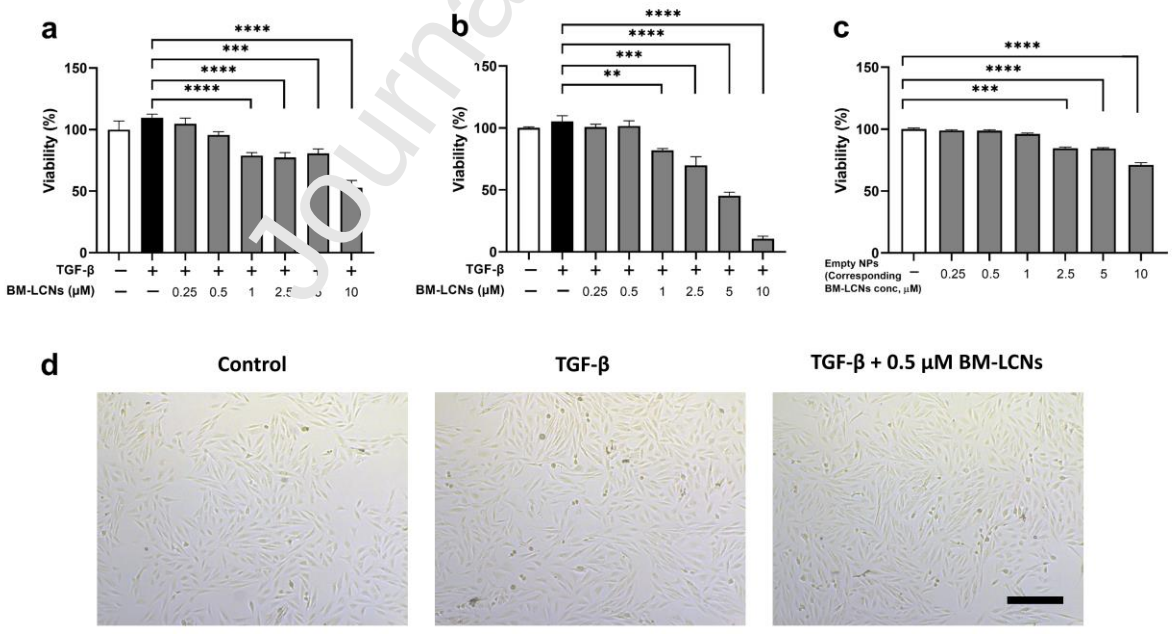

Figure 1. Impact of BM-LCNs treatment on the cell viability and morphology of TGF-β-induced BEAS-2B. BEAS-2B cells were incubated for 24 h in the presence of 5 ng/mL TGF-β and for a further 24 h with increasing concentrations of BM-LCNs (0.25, 0.5, 1, 2.5, 5, 10

μM, Figure 1a, b). Subsequently, the MTT assay (a) or the Trypan Blue assay (b) was performed to assess cell viability. BEAS-2B cells were incubated for 24 h in the presence of increasing empty LCN concentrations, corresponding to 0.25, 0.5, 1, 2.5, 5, 10 μM BM-LCNs, and the MTT assay was performed to assess cell viability (c). The BEAS-2B cell morphology upon treatment with TGF-β alone or with TGF-β and 0.5 μM BM-LCNs is shown in (d). Scale bar = 300 μm. The results in (a-c) were normalised as a percentage compared to untreated control and indicated as mean ± SEM (n = 3, **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$ with one-way ANOVA test).

3.2. Anti-migratory activity of BM-LCNs in TGF-β-induced BEAS-2B cells

The effect of treatment with 0.5 μM BM-LCNs on the migratory capacity of TGF-β-induced BEAS-2B cells was assessed through the wound healing assay after 24 and 48 h of treatment (Figure 2a and 2b, respectively). At the 24 h time, the percentage of wound closure of the untreated group was 36.3% (Figure 2a). Treatment with TGF-β resulted in a significant 42% increase in the percentage of wound closure compared to the untreated group (percentage wound closure of 51.5%, $p<0.0001$, Figure 2a). Simultaneous treatment with BM-LCNs reversed the effect of TGF-β, resulting in a significantly lower percent wound closure similar to the untreated group (34.8%, $p<0.0001$ vs TGF-β-induced group, Figure 2a). At the 48 h time point, the percentage of wound closure of the untreated group was 65.5% (Figure 2b), and treatment with TGF-β resulted in a significant 18% increase in the percentage of wound closure compared to the untreated group (percentage wound closure of 77.3%, $p<0.01$, Figure 2b). Simultaneous treatment with BM-LCNs reversed the effect of TGF-β, resulting in a significantly lower percent of wound closure which was similar to the untreated group (63.0%, $p<0.01$ vs TGF-β-induced group, Figure 2b). Representative figures of the cells at the indicated time points are shown in Figure 2c.

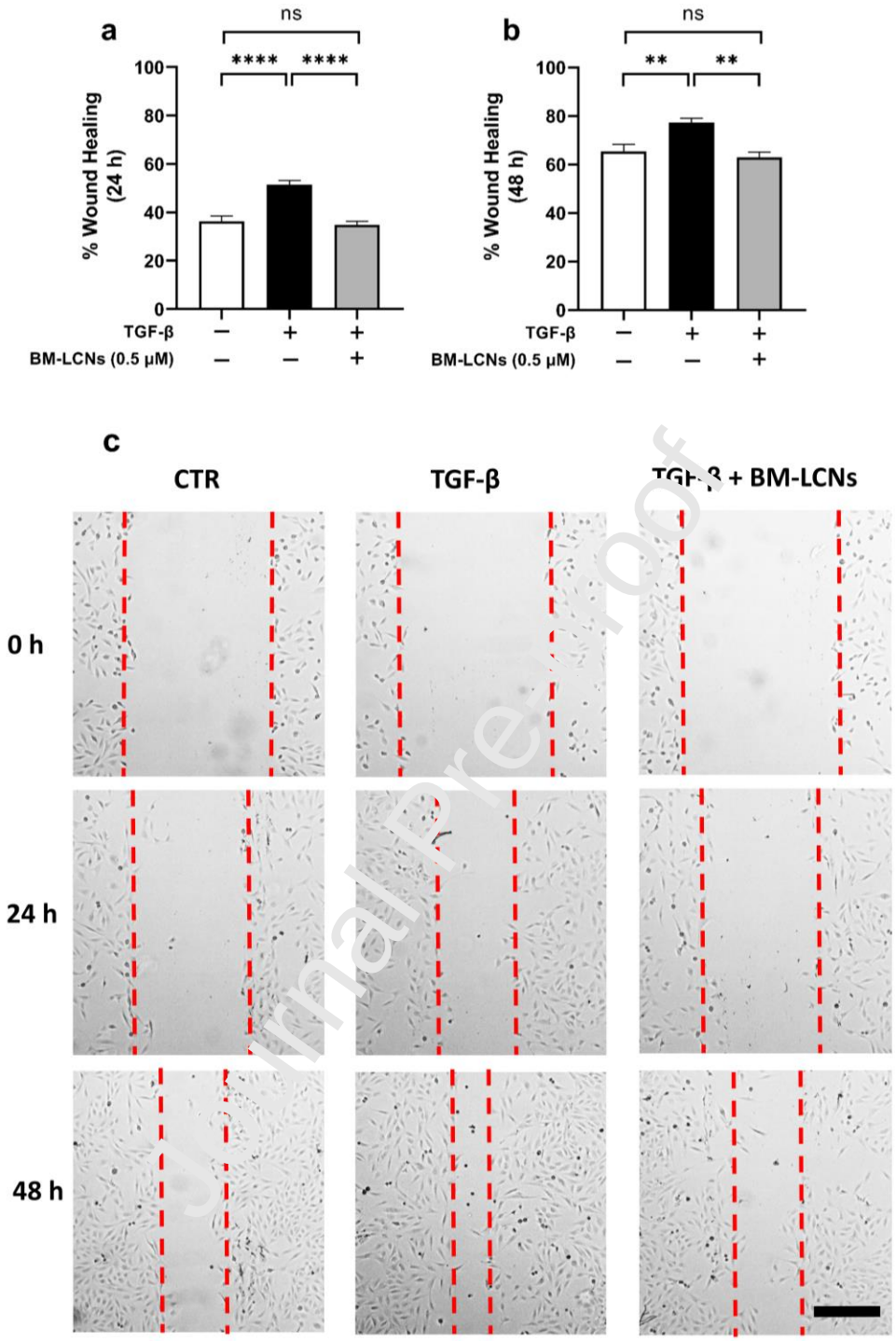

Figure 2. Anti-migratory activity of BM-LCNs in TGF-β-induced BEAS-2B cells. The wound was created by scratching, with a sterile pipette tip, a layer of BEAS-2B cells. Cells were then treated with 5 ng/mL TGF-β1 with or without the presence of 0.5 μM BM-LCNs. Photographs were acquired using a light microscope at 10x magnification. The distance between the edges of the wounds was measured before treatment (0 h) and after 24 h (a) and 48 h (b) incubation in order to calculate the percent wound closure. Representative pictures of

the cells at the indicated time points are shown in (c). Magnification: 10X; Scale bar = 300 µm. Values are expressed as mean ± SEM (n = 3; ns: $p>0.05$; **: $p<0.01$; ****: $p<0.0001$ with one-way ANOVA test).

3.3. BM-LCNs counteract the protein expression signature induced by TGF-β

The relative protein levels of endoglin, basic FGF, myeloperoxidase, thrombospondin-1, VEGF, myeloperoxidase, and cystatin C are shown in Figure 3, as detected using the Human XL Cytokine Protein Array. Treatment with 0.5 µM BM-LCNs counteracted the action of TGF-β by reducing the expression of endoglin, basic FGF, myeloperoxidase, thrombospondin-1, and VEGF (Figure 3a to 3e, respectively), which are induced by TGF-β, as well as by partially restoring the expression of cystatin C, which is suppressed by TGF-β (Figure 3f). A representative array for each experimental group is shown in Figure 3g. In particular, treatment of BEAS-2B cells with TGF-β resulted in a nearly statistically significant increase in the signal intensity correlated to the levels of endoglin (1.14-fold, $p = 0.07$, Figure 3a), as well as a statistically significant increase in the signal of basic FGF (1.37-fold, $p<0.01$, Figure 3b), and myeloperoxidase (1.35-fold, $p<0.05$, Figure 3c). Simultaneous treatment with BM-LCNs resulted in a significant reduction of the signal intensity for all these proteins. The signal corresponding to endoglin was reduced by 15.7% ($p<0.05$, Figure 3a), the signal corresponding to basic FGF was reduced by 21.6% ($p<0.05$, Figure 3b), and the signal corresponding to myeloperoxidase was reduced by 24.0% ($p<0.01$, Figure 3c). A similar trend, although not statistically significant, was observed for thrombospondin-1 (THBS1) and VEGF. Upon treatment with TGF-β, THBS1 signal was increased by 1.58-fold and VEGF signal was increased by 2-fold (Figures 3d and 3e, respectively, $p>0.05$). Treatment with BM-LCNs reduced the signal related to the expression of these proteins by 34.4% ($p>0.05$, Figure 3d) and 21.2% ($p>0.05$, Figure 3e), respectively. With regards to cystatin C, its expression was significantly reduced by 65% upon treatment with TGF-β ($p<0.0001$, Figure 3f). Treatment with BM-LCNs resulted in a 1.68-fold increase in the signal correlated to cystatin C ($p = 0.053$, Figure 3f).

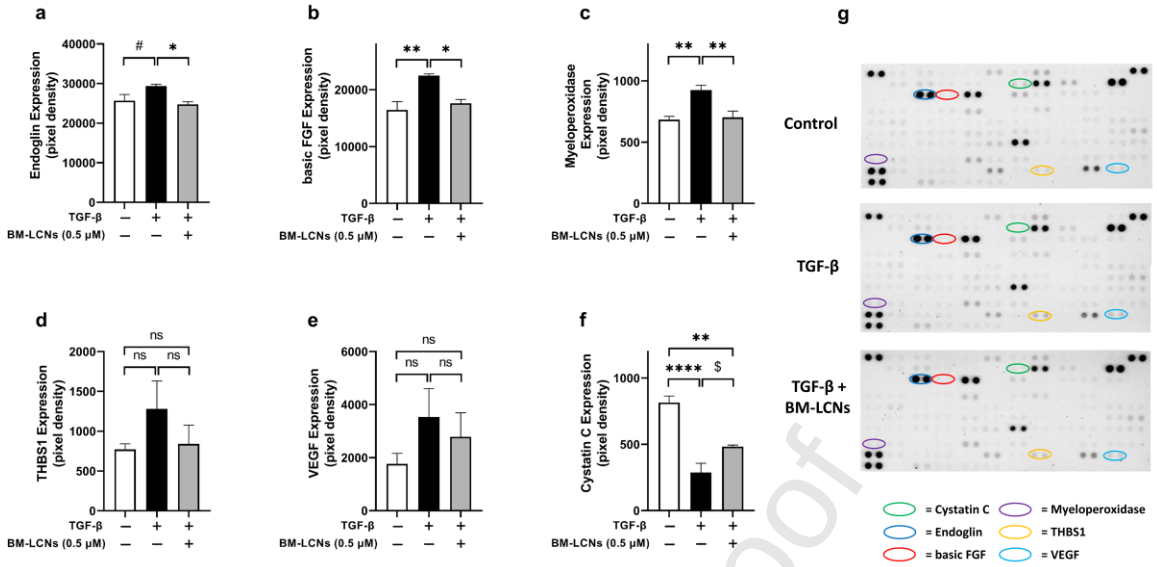

Figure 3. BM-LCNs counteract the expression patterns of proteins induced by TGF-β. BEAS-2B cells were treated with 5 ng/mL TGF-β for 24 h with or without a successive 24 h treatment with 0.5 µM BM-LCNs. The relative protein expression levels of endoglin (a), basic FGF (b), myeloperoxidase (c), thrombospondin-1 (THBS1) (d), VEGF (e), and cystatin C (f) were determined using the Human XL Cytokine Protein Array. A representative array for each group is shown in (g). Values in (a-f) are expressed as mean ± SEM (n=4, *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$; #: $p=0.07$; $: $p=0.053$ with one-way ANOVA test).

3.4. BM-LCNs restore baseline levels of NO

The effect of BM-LCNs on NO production in BEAS-2B cells was assessed by measuring NO levels in the cell culture supernatant using the Griess reagent. The results are shown in Figure 4. Treatment of BEAS-2B cells with TGF-β resulted in a significant 36.4% reduction of the NO levels compared to untreated group ($p<0.01$, Figure 4). The subsequent treatment with BM-LCNs restored the secretion of NO to the same levels as the untreated group ($p<0.01$ against the TGF-β-treated group, Figure 4). No statistically significant difference was observed between the untreated group and the TGF-β + BM-LCNs treated group (Figure 4).

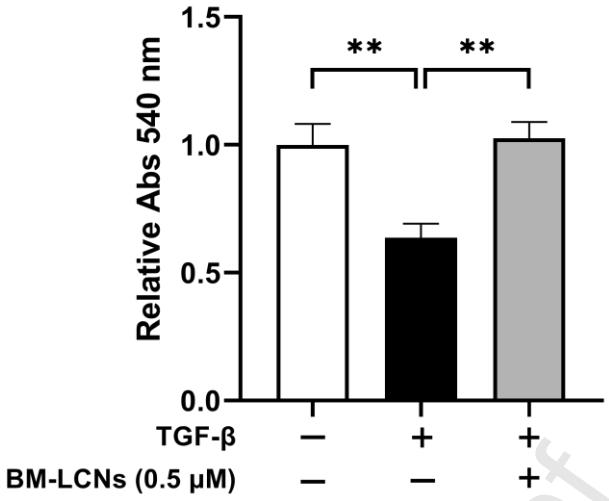

Figure 4. BM-LCNs restore baseline levels of NO. BEAS-2B cells were treated with 5 ng/mL TGF-β1 for 24 h with or without a successive 24 h treatment with 0.5 μM BM-LCNs. The NO levels in the cell culture supernatant were determined using the Griess reagent and measuring the absorbance at 540 nm. The values indicated are mean ± SEM (n=3; **: $p<0.01$ with one-way ANOVA test).

4. Discussion

CRDs are among the leading causes of morbidity and mortality worldwide, causing substantial medical and economic burdens [8]. Cigarette smoking is considered to be among the main causative factors for this heterogeneous group of diseases due to the fact that it results in the exposure of the respiratory system to thousands of different noxious chemicals, promoting chronic inflammation, oxidative stress, and consequentially severe tissue damage [11, 74, 75]. Traditional therapies for CRDs are mainly aimed at improving disease symptoms, and they are often ineffective at restoring the destroyed airways and lung parenchyma [76]. Furthermore, the currently available pharmacological strategies for these ailments are hampered by severe side effects [21]. This is particularly true in the case of LC due to the elevated toxicity of the currently used chemotherapeutic drugs [24].

A common feature shared between the CRDs is the process of airway remodelling, consisting of radical structural changes occurring in both the large and small airways and contributing to severe airway obstruction [30, 35]. The main structural alterations in airway remodelling include EMT of epithelial layers, excessive collagen and mucus secretion, and thickening of

the basement membrane. These processes are orchestrated by TGF-β, whose expression and signalling are aberrant in virtually all CRDs [36, 77]. In this context, a therapeutic agent tackling the TGF-β signalling would be advantageous. It would find widespread clinical application as a therapy for all diseases where tissue remodelling and fibrosis play a fundamental role [78].

The plant world represents an endless source of inspiration for novel compounds, collectively termed phytoceuticals or nutraceuticals, with the most disparate pharmacological activities [56]. Many phytoceuticals are known to downregulate TGF-β signalling and therefore show great promise in treating CRDs and fibrotic disorders [63]. One of these compounds is berberine, which has been shown to suppress TGF-β expression and signalling and subsequent cell motility, proliferation, and EMT *in vitro* in many types of cancer [79-84] and is embedded with potent multi-organ antifibrotic activity *in vivo* [63]. Despite its potent biological activity, the clinical application of berberine is limited by a poor pharmacokinetic profile, mainly deriving from its scarce permeability. This results in the necessity to administer large doses of berberine to achieve therapeutic efficacy, with an increased risk of adverse effects [66]. To overcome this important obstacle to the clinical application of berberine and other phytoceuticals, several types of advanced drug delivery systems are currently being developed [35, 56].

The present study shows that berberine encapsulated in a liquid crystalline nanoparticle formulation attenuates some of the TGF-β-induced remodelling features in BEAS-2B human bronchial epithelial cells. In particular, treatment of TGF-β-induced BEAS-2B cells with BM-LCNs significantly reduced the cells' increased motility up to 48 h of treatment, as assessed through wound healing assay, resulting in an extent of migration that was comparable to that observed in the untreated group. This is in accordance with previous reports showing, in different cell systems, that berberine is capable of inhibiting cell migration [62, 83].

The protein array experiment revealed fundamental mechanistic insights about the pathways impacted by berberine in counteracting TGF-β action. Endoglin is a known interactor of TGF-β receptors I and II. It is an auxiliary component of the TGF-β signalling machinery capable of modulating the downstream signalling [49]. Among its different functions, endoglin is known to regulate actin cytoskeletal organization in endothelial cells, and this could contribute to the increased angiogenesis observed in tissue remodelling [85]. The

present study is the first to report that berberine treatment results in the downregulation of endoglin levels, restoring them to levels compared to untreated cells. Other factors activated upon TGF-β signalling and contributing to various aspects of tissue remodelling, including angiogenesis, are thrombospondin-1, VEGF, and bFGF. In this study, treatment with BM-LCNs reduced the expression of these proteins to levels comparable to the untreated group. Besides being among the main activator factors of latent TGF-β, thrombospondin-1 is involved in physiological tissue repair and pathologic fibrosis in TGF-β-dependent and independent pathways [86]. The present study is the first to report that berberine inhibits the expression of thrombospondin-1.

With regards to VEGF and bFGF, these two cytokines are known to mediate TGF-β-induced remodelling and angiogenesis in various diseases, including asthma and COPD [45-47]. Furthermore, these two cytokines are considered among the most important inductors of angiogenesis in cancers such as NSCLC [87]. The finding that BM-LCNs counteract the TGF-β-induced upregulation of these proteins are in accordance with reports showing that berberine (i) suppresses the expression of bFGF in breast cancer cells, and (ii) downregulates the expression of VEGF in hepatocellular carcinoma cells, concomitantly inhibiting their angiogenic potential [88].

Myeloperoxidase is mainly produced by neutrophils and other body cells [89], and it plays a pivotal role in airway inflammation and tissue remodelling in diseases such as COPD [47]. In the present study, berberine significantly counteracted the TGF-β-induced increase in myeloperoxidase expression in BEAS-2B cells. This is in accordance with a study where berberine was shown to alleviate dextran sulfate sodium (DSS)-induced colitis in mice through the reduction of inflammation and oxidative stress, which was exerted via mechanisms including the downregulation of myeloperoxidase levels [90].

Treatment of BEAS-2B cells with TGF-β further resulted in the downregulation of Cystatin C protein expression. This is a negative regulator of TGF-β signalling [51]. Administration of Cystatin C, in particular, has been shown to have preclinical efficacy against the oncogenic activity of TGF-β in an in *vivo* model of breast cancer [92]. In the present study, BM-LCNs partially restored the TGF-β-induced downregulation of the expression of Cystatin C , further highlighting the potent activity of BM-LCNs in inhibiting TGF-β signalling.

Nitric oxide (NO) plays a fundamental role in preserving the epithelial phenotype of lung epithelial cells by preventing EMT [52]. In this context, one of the mechanisms by which

TGF-β induces EMT is the reduction of endogenous NO production via the downregulation and inhibition of eNOS [52], sGC, and PKGI [53]. In the present study, treatment of TGF-β-induced BEAS-2B cells with BM-LCNs significantly counteracted the effect of TGF-β, restoring the baseline NO production to levels comparable to the untreated group. An increased NO production induced by berberine was shown in a study by Wang et al (2009), in which treatment of high-fat-diet and streptozotocin-induced diabetic rats with berberine resulted in increased eNOS expression and, concomitantly, higher NO levels [93]. Interestingly, in a previous report, treatment of lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages with a similar berberine-LCN formulation resulted in a significant reduction of the elevated NO levels induced by LPS, which was exerted through the downregulation of the expression of the inducible NO synthase (iNOS) [59]. This shows proof of the multifaceted, context-dependent biological activity of berberine, that promotes physiological levels of NO and a generally healthy cell phenotype through its potent anti-inflammatory, antioxidant, and anti-fibrotic properties. The findings reported in this study are summarized in the Graphical Abstract of the present manuscript.

An important advantage of the present study is that BM-LCNs significantly counteracted TGF-β-induced remodelling features at an equivalent berberine concentration of 0.5 µM. This concentration is substantially lower (10- to 600-fold) compared to the average concentration range of free berberine powder that was shown to be active in counteracting TGF-β-induced features in previous reports (5-300 µM) [79-84]. Although these mentioned studies were performed on different cell lines and with different experimental setups, this strong discrepancy in active berberine concentration reflects the fact that nanoparticle-based drug delivery systems such as LCNs allow improved delivery of the therapeutic cargo, with resulting lower doses necessary to achieve a significant therapeutic effect. This is in agreement with previous reports from our research team showing that encapsulating phytoceuticals in LCNs or other nanoparticle-based delivery systems resulted in potent activity at lower concentrations than the free molecule [58, 59, 94-96].

Despite the promising activity of BM-LCNs in counteracting TGF-β-induced remodelling features, the present study is not exempt from limitations. The main limitation is that these study findings are reported only on TGF-β- induced BEAS-2B bronchial epithelial cells,

which represent a rather simplistic *in vitro* model of airway remodelling that does not account for the totality of the cellular and molecular mechanisms involved. Despite the fact that epithelial cell proliferation is one of the key factors driving airway remodelling [31], and that TGF-β signalling plays a central role in this process [97],the lung, like any other organ, is composed of different cell types that, together, orchestrate its function in health and disease. Airway remodelling itself is an extremely complex process, which involves several other cell types as well as other pro-fibrotic factors that work in synergism with TGF-β [98]. Similar studies performed on other cell types, such as alveolar epithelial cells, endothelial cells, and macrophages, would provide a more complete picture of the true potential of BM-LCNs in counteracting TGF-β-mediated aberrant airway remodelling. In this context, the use of *in vitro* models consisting of the co-culture of different cell lines [98, 99], especially in conjunction with microfluidic [100] and "airway-on-a-chip" devices [101], would provide a more accurate and detailed depiction of the airway remodelling process. Furthermore, to allow the translation of these *in vitro* results to the clinic, BM-LCNs should be tested on suitable animal models of asthma, COPD, and pulmonary fibrosis.

5. Conclusions

This study highlights the potent activity of BM-LCNs in counteracting TGF-β-induced remodelling features in human bronchial epithelial cells. This activity is exerted through the inhibition of TGF-β-induced cell migration, by regulating the expression of several cytokines and mediators dysregulated by treatment with TGF-β, and by restoring physiological baseline NO levels. The findings reported in this manuscript provide further proof of the multifaceted applicability of BM-LCNs as a potential therapy for CRDs where aberrant tissue remodelling plays a pivotal role. However, in order to achieve clinical translation, the results of this finding must be further confirmed and validated by investigating the effect of BM-LCNs on more complex *in vitro* systems, as well as on *in vivo* models of CRDs.

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7. Data Availability Statement

The data presented in this study are available on request from the corresponding authors.

8. Conflict of Interest

The authors have no conflict of interest to declare.

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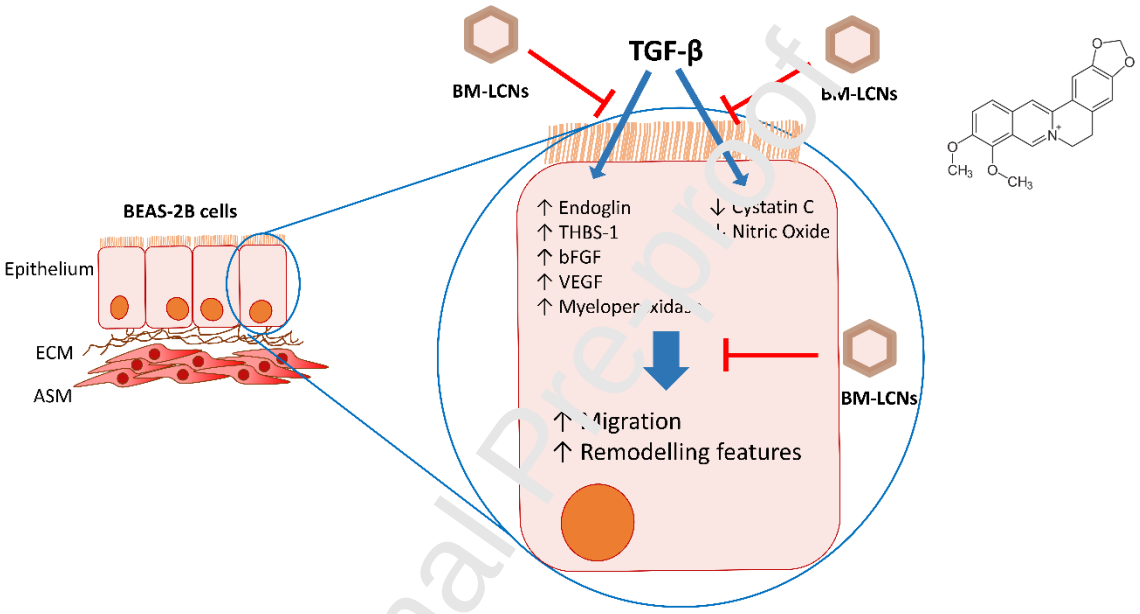
Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical Abstract



Highlights

- TGF-β is the main orchestrator of airway remodelling in chronic respiratory diseases
- We encapsulated berberine in liquid crystalline nanoparticles (BM-LCNs)
- BM-LCNs counteract TGF-β-induced remodelling features in BEAS-2B bronchial cells
- BM-LCNs reverse TGF-β effects on cell motility, protein expression, and NO secretion
- Encapsulation of berberine in LCNs grants potent activity at low concentrations