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RESEARCH PAPER

Immunomodulatory Effects of a Low-Dose Clarithromycin-Based Macrolide Solution Pressurised Metered Dose Inhaler

Mehra Haghi • Alessandro Saadat • Bing Zhu • Gaia Colombo • Gregory King • Paul M. Young • Daniela Traini

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ABSTRACT

Purpose The aim of this study was to assess the effects of low-dose clarithromycin, formulated as solution pressurized metered dose inhaler, following deposition on the Calu-3 respiratory epithelial cells.

Methods Clarithromycin was deposited on the air-interface culture of Calu-3 cells using a modified Andersen cascade impactor. Transport of fluorescein-Na, production of mucus and interleukin-8 release from Calu-3 cells following stimulation with transforming growth factor-β and treatment with clarithromycin was investigated.

Results The deposition of clarithromycin had significant effect on the permeability of fluorescein-Na, suggesting that the barrier integrity was improved following a short-term treatment with clarithromycin (apparent permeability values were reduced to $3.57\times10^{-9}\pm2.32\times10^{-9}$ cm.s^{-1}, compared to $1.14\times10^{-8}\pm4.30\times10^{-8}$ cm.s^{-1} for control). Furthermore, the amount of mucus produced was significantly reduced during the course of clarithromycin treatment. The concentration of interleukin-8 secreted from Calu-3 cells following stimulation with transforming growth factor-β resulted in significantly lower level of interleukin-8 released from the cells pre-treated with clarithromycin (5.2 ± 0.5 ng.ml^{-1} clarithromycin treated *vs.* 7.7 ± 0.8 ng.ml^{-1} control, respectively).

Conclusions Our data demonstrate that treatment with clarithromycin decreases the paracellular permeability of epithelial cells, mucus secretion and interleukin-8 release and therefore, inhaled clarithromycin holds potential as an anti-inflammatory therapy.

KEY WORDS Andersen cascade impactor · Calu-3 · epithelial transport · mucus

ABBREVIATIONS

ABS	Acrylonitrile butadiene styrene
ACI	Andersen cascade impactor
AIC	Air interface culture
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
ELISA	Enzyme-linked immunosorbent assay
FEV_1	Forced expiratory volume in 1 second
flu-Na	Fluorescein sodium
FVC	Forced vital capacity
HBSS	Hank's Balanced Salt Solution
HFA	Hydrofluoroalkane
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
JAMA	Junction adhesion molecule-A
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate buffer solution
pMDIs	Pressurised metered dose inhalers
RGB	Red-green-blue
RSV	Respiratory syncytial virus
TEER	Transepithelial electrical resistance

M. Haghi · B. Zhu · P. M. Young · D. Traini (✉)
Respiratory Technology Woolcock Institute of Medical Research and Discipline of Pharmacology, Sydney Medical School
The University of Sydney, Sydney, NSW, Australia
e-mail: Daniela.traini@sydney.edu.au

A. Saadat · G. Colombo
Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

G. King
Sydney Central Medical School, The University of Sydney, Sydney, NSW Australia

Present Address:
M. Haghi
School of Pharmacy, Graduate School of Health, The University of Technology, PO Box 123, Broadway NSW 2007, Sydney, Australia

TGF Transforming growth factor
TNF Tumor necrosis factor

INTRODUCTION

Macrolide antibiotics are widely used for the treatment of lung diseases, in which inflammation is a major component (1). It has been shown that macrolides, such as clarithromycin, affect the migration of neutrophils and inhibit neutrophil oxidative burst (2). Furthermore, macrolides impair the production of pro-inflammatory cytokines, such as interleukins (IL)-1b, -6, -8, and tumor necrosis factor (TNF)-α in cultured human bronchial epithelial cells (3,4).

The beneficial anti-inflammatory effects of macrolides on cytokine production have been investigated in *ex vivo* animal models, with both roxithromycin (5) and clarithromycin (6). Clinical evidence is also evident to support the use of macrolide antibiotics for cystic fibrosis (CF) treatment (7). It has been suggested that both erythromycin and clarithromycin suppress endothelin-1 synthesis and release by human bronchoepithelial cells (8). Furthermore, macrolides have been shown to have an effect on neutrophil chemotaxis (9) and/or inhibition of the extracellular matrix remodeling by targeting matrix-metalloproteases (10). The underlying mechanisms of macrolides in the body, however, are not yet fully understood, although one hypothesis is that they act *via* an immune-modulatory effect (11,12) and anti-inflammatory actions by suppression of NF-κB (13). For an excellent review of immunological actions of macrolides the authors refer the reader to an article by Kanoh and Rubin (14).

Implications for CF Treatment

Several clinical studies have reported conflicting results with respect to the anti-inflammatory properties of macrolide compounds in CF. For example a study by Pukhalsky *et al.* (2004) (15) reported significant positive results in 27 CF patients after 250 mg of clarithromycin administration on alternate days during 12–months long therapy. A marked reduction of cytokine level in both sputum and plasma, as well as a significant improvement in both FEV_1 and FVC were observed. In comparison, a study by Ordonez *et al.* (2001) (16) on 10 adult patients that received 3 weeks of placebo, followed by 6 weeks of twice daily clarithromycin 500 mg found no significant difference in pulmonary function or sputum neutrophil number, IL-8, free neutrophil elastase or myeloperoxidase. A more recent randomised controlled clinical trial of 63 CF patients by Robinson *et al.* (17), treated with either placebo or 500 mg of oral clarithromycin twice daily for 5 months with a 1-month wash out, also found no positive results and concluded that clarithromycin was not effective in treating CF lung disease.

Irrespective of this conflicting information from short-term trials, data from numerous but mainly small investigations, have suggested that long-term treatment with low dose macrolides in patients with non-CF bronchiectasis could have a positive influence on reducing the frequency of infections, sputum volume, and inflammation (18–20). Furthermore, in a study by Wong *et al.* no change from the baseline was observed in the lung function tests of the group treated with azithromycin, compared with the control (18).

Implications for Treatment of COPD

The use of low dose macrolides in chronic obstructive pulmonary disease (COPD) is still an area of debate. Two studies (i.e. Banerjee *et al.*, (21) and Seemungal *et al.* (22)), established a positive effect of prophylactic macrolide treatment in moderate-to-severe COPD. Both studies were relatively small and to assess the clinical potential harmful effects of these classes of drugs with the benefits will need further investigation.

Implications for Treatment of Asthma

Asthma is another disease marked by airways inflammation, which usually requires long-term treatment, and published reports have demonstrated that macrolides have an anti-inflammatory effect through different mechanisms (8,23,24). Even though some clinical data indicated a positive effect for long term, low dose use of macrolides in asthmatic patients, at present these data are insufficient to recommend routine use of macrolides for the control of asthma.

Other Respiratory Diseases

Other than CF, Asthma, COPD and bronchiectasis therapy, clarithromycin has been found to be effective in other lung diseases. In a very recent study, clarithromycin stopped lung function decline in airway-centered interstitial fibrosis, where classic treatment with corticosteroids did show little efficacy (25). In human respiratory syncytial virus (RSV) infections, clarithromycin has also been proven to have an immune-modulatory effect, significantly suppressing RSV-induced production of IL-6, IL-8, and regulated on activation, normal T-cell expressed and secreted, using pulmonary epithelial cell A549 (26). Furthermore, it was observed that clarithromycin may be beneficial in patients with acute lung injury from mechanical ventilation, since previous studies on ventilator-induced lung injury in mice showed decreased neutrophil recruitment into the alveolar spaces when clarithromycin was administered (27,28).

An Alternative to Oral Therapy - Approach

The immune modulatory effect of macrolides has been reported with oral low dose treatment, which is often too low to have a clinically significant antimicrobial effect (29,30). Therefore, the former is most probably the likely mechanism of low dose macrolides. To the authors' knowledge no inhalable formulation containing clarithromycin is commercially available as an anti-inflammatory respiratory drug. Indeed, therapy is usually *via* the intravenous or oral route and the drug used for its antibiotic and/or anti-inflammatory properties.

For a targeted delivery system, the administration of clarithromycin directly to the lung would be advantageous. Benefits of pulmonary clarithromycin therapy include but are not limited to: localized delivery (31) and reduced systemic side effects (32). Therefore, administering macrolides as inhaled therapy could be beneficial to achieve an immune modulatory effect locally. Solution-based pressurised metered dose inhalers (pMDIs) have been widely used as the effective treatment for different lung diseases for years (33). The main advantages over other respiratory drug delivery forms are: dose consistency over product lifetime, long-term chemical stability (34) and satisfactory aerosol performance (35). While the formulation and stability of low dose clarithromycin as a solution pMDI has been previously described by the authors (36), the aim of the present work was to assess the feasibility of localized clarithromycin administration to the lung *in vitro* by investigating the effectiveness of the formulated pMDI clarithromycin after deposition on a lung epithelial cell line. Furthermore, data collected on the toxicity, barrier integrity and inhibitory effects on inflammation and mucus secretion using Calu-3 epithelial cell monolayers grown at an air-liquid interface are also presented.

MATERIALS AND METHODS

Cell Culture

Passages 37–41 of Calu-3 cells (HTB-55) (American Type Cell Culture Collection (ATTC), Rockville, USA) were grown in complete Dulbecco's Modified Eagle's medium: F-12 (Sigma-Aldrich, Sydney, Australia) containing 10% (*v*/*v*) fetal calf serum (Gibco, Invitrogen, Sydney, Australia), 1% (*v*/*v*) non-essential amino acid solution (×100) (Sigma-Aldrich, Sydney, Australia), and 1% (*v*/*v*) L-glutamine solution (200 mM) (Gibco, Invitrogen, Sydney, Australia) and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. In order to establish the air-liquid interface (ALI) model, cells were seeded onto Snapwell® polyester inserts (0.4 μm pore size, 1.12 cm^2 surface area) (Corning Costar, Lowell, MA, USA) at a density of 5×10^5 cells/cm^2 and the monolayers were allowed to differentiate under air-interface for 15–19 days. Fiegel *et al.* (37) has previously used the ALI to develop physiologically relevant models of the pulmonary epithelial barrier that would allow for quantitative characterization of therapeutic aerosols *in vitro*. For this study this period of time in culture was selected based on the study by Haghi *et al.* (38) that determined the optimum time in culture for Calu-3 cells to differentiate, form tight junctions and produce mucus in culture.

Cell Viability Assay

The toxicity of clarithromycin (School of Pharmacy, Shahid Beheshti Medical University, Tehran, Iran) was assessed by measuring the viability of Calu-3 cells in a liquid covered culture, following 3 days drug exposure to increasing concentrations of clarithromycin, as previously described (39). Briefly, Calu-3 cells were seeded in a 96 well plate. After 24 h, Calu-3 cell cultures were exposed to a range of clarithromycin concentrations (from a minimum of 0.61 nM to a maximum of 80,000 nM). Plates were incubated for 72 h after which the cells were analysed for cell viability using CellTiter 96® Aqueous assay (MTS reagent) (Promega, Madison, USA). The CellTiter 96® Aqueous assay contains a tetrazolium compound (MTS) and an electron coupling reagent (PES) that has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution of the formazan product. Absorbance was measured at 490 nm using a spectrophotometer and associated software (Spectramax M2 and Soft Max pro 4.8, Molecular Devices, Sunnyvale, CA, USA) and the value was directly proportional to cell viability (%). The half maximal inhibitory concentration (IC_{50}) values were defined as the clarithromycin concentration that produced a decrease of 50% in cell viability compared to the untreated control. The experiment was performed in triplicate. Data was fitted to the Hill equation using the General Fit function of KaleidaGraph 4.1 software.

Preparation and Physico-Chemical Characterization of Clarithromycin pMDIs Solution Formulation

Pressurized metered dose solution formulations containing 2% (*w*/*w*) clarithromycin and 10% (*w*/*w*) ethanol (Biolab, Clayton, Victoria, Australia) as co-solvent in HFA134a, delivering 100 μg per actuation, were prepared and analyzed for their physicochemical characteristics and aerosol performances according to the method described by Saadat *et al.* (36). The formulation was found to be physically stable for up to 30 days between 4 and 37°C and had aerodynamic characteristics that made it suitable for inhalation drug delivery.

Use of the Cell Integrated Modified ACI for Assessing pMDI Clarithromycin Drug Deposition

The novel integrated modified Andersen cascade impactor (ACI) described by Haghi *et al.* (38) was used to assess clarithromycin drug deposition on Calu-3 epithelial cells. Briefly, custom-made flat plates for accommodating Snapwells were designed using CAD (ANSYS Design Modeler release 13, ANSYS Inc, PA, USA), built in acrylonitrile butadiene styrene (ABS) using a 3D printer (Dimension Elite, MN, USA), and were used as substitute for 'standard' Andersen cascade impactor plates. Each plate can accommodate up to eight Snapwells at the same time.

Fourteen days post seeding, the Snapwells, containing Calu-3 cells at air-interface configuration, were placed on stage 4 of the ACI (aerodynamic cut-off diameter 2.1–3.3 μm). The ACI was sterilized by soaking all components in 80% ethanol to minimize the possibility of contamination over the 5 days period of the study. Two and five actuations of clarithromycin into the modified ACI resulted in the deposition of 0.51± 0.10 μg or ~0.5 μg and 1.10±0.16 μg or ~1.1 μg on each Snapwell. After particle deposition, the inserts were transferred to a 6 well plate containing the culture medium and returned to incubator for 1, 3 or 5 days prior to analysis.

Transepithelial Electrical Resistance Measurements (TEER)

The transepithelial electrical measurement (TEER) was performed using an EVOM Voltohmmeter (World Precision Instruments, FL, USA) with STX-2 chopstick electrodes to study the barrier integrity Calu-3 cells.

Fluorescein Sodium Transport Experiments

Fluorescein sodium (flu-Na) (Sigma-Aldrich, Sydney, Australia) (MW 367 Da) was used to assess paracellular permeability following drug deposition. To ensure that the integrity of the monolayer was maintained during the course of the experiment, on days 1, 3 and 5 after clarithromycin deposition (days 15, 17 and 19 post seeding) the culture medium was replaced with 600 μl of pre-warmed HBSS. Two hundred fifteen microliter of a 2.5 mg.ml−1 flu-Na solution was added to the apical compartment. Samples from the basal compartment of each well were drawn over 4 h. The fluorescence was measured using a fluorescence plate reader (Spectramax M2 and Soft Max pro 4.8, Molecular Devices, Sunnyvale, CA, USA), using excitation and emission wavelengths of 485 and 520 nm, respectively.

Histological Cell Surface Staining for Mucus Detection

Twenty-four hours prior to clarithromycin deposition, the mucus covering the Calu-3 cells was partially dissolved by incubating the cells with 1 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) (Gibco, Invitrogen, Sydney, Australia) for 90 min according to the method described by Kereda *et al.* (40). The following day, clarithromycin was deposited and cells were incubated for 1, 3 and 5 days. Alcian blue 1% (pH 2.5) in 3% acetic acid (Fronine laboratory, Sydney, Australia) was used to stain the mucus on the surface of Calu-3 monolayers according to the method by Haghi *et al.* (41). Briefly, the Snapwells were washed with phosphate buffer solution (PBS) and fixed using paraformaldehyde (4%w/v) (Sigma-Aldrich, Sydney, Australia). The cells were then stained using Alcian blue and rinsed multiple times with PBS. The filter membranes were mounted on glass slides using Entellan new mounting medium (ProSciTech, Thuringowa, Australia) and sealed. Images were then taken using an attached DP71 camera (Olympus, Japan). The images were post processed using Apple Automater (v 2.0.4 Apple Inc., Cupertino, California, USA) to center crop 400×300 pixel JPEG images. Each image was analyzed using ImageJ (v1.42q, NIH) with Color Profile (Dimiter Prodanov; Leiden University Medical Center, Leiden, Netherlands) and Colour Inspector 3D v2.0 (Kai Uwe Barthel; Internationale, Medieninformatik, Berlin, Germany) plug-ins. A three-dimensional color-space was produced representing the 8-bit red-green-blue (RGB) value of each image. The ratio of blue (RGB_B ratio) was calculated by dividing the mean RGB_B by the sum of the RGB values for each image ($RGB_R + RGB_G + RGB_B$). The mean ratio of five images was used as an indication of the degree of mucus production at days 15, 17 and 19, for each treatment and control.

Enzyme-Linked Immune-Sorbent Assay for IL-8 Measurements

Transforming growth factor (TGF)-β (R&D Systems, Minneapolis, MN, USA) was used at concentration of 1 $ng.ml^{-1}$ to induce IL-8 production in the Calu-3 cells grown on Snapwells 14 days post seeding. Clarithromycin (0.5 and 1.1 μg) was deposited onto the cells at day 15. For control samples, at day 14, the pMDIs with no drug (only propellant and solvent) were actuated onto the Calu-3 cells grown on Snapwells placed in the ACI and after 24 h TGF-β was added to the culture plate. The culture medium was sampled after 24 and 48 h to measure the concentration of IL-8 in the culture media. ELISA technique was used to measure the secreted IL-8 was using a commercial kit, according to the manufacturer's protocol. The Human IL-8 ELISA Kit II (BD OptEIA™, catalogue number 550999). The kit was purchased from BD Biosciences (San Diego, California, USA). The quantities of

secreted IL-8 in the test samples were determined using a standard curve generated with purified recombinant human IL-8 provided with the kit. The range of the IL-8 assay was 2–1000 pg.ml^{-1}.

High Performance Liquid Chromatography

The amount of drug deposited on Calu-3 cells in Snapwells was quantified using a Shimadzu HPLC system consisting of a LC20AT pump, a SIL20AHT autosampler and an SPD-20A UV–VIS detector (Shimadzu, Sydney, Australia). Sample analysis was conducted using a Triart C18 column (4.6× 250 mm) (YMC, Kyoto, Japan). The mobile phase was prepared as described by Saadat *et al.* (36). The flow rate was set at 1 mL.min^{-1} with an injection volume of 100 µl and UV detection wavelength of 207 nm. The retention time of clarithromycin was approximately around 4.45 min and linearity was confirmed between 0.1 and 200 µg.ml^{-1} (R^2= 0.999).

Statistical Analysis

Analysis of variance was calculated using ANOVA, followed by post-hoc multiple comparisons analysis. Differences were deemed significant for $p \leq 0.05$. Unless otherwise stated, data are represented in terms of mean and standard deviation.

RESULTS

The current study aimed to evaluate the anti-inflammatory and muco-inhibitory properties of clarithromycin formulated at low dose solution pMDI after deposition directly on Calu-3 epithelial cells *in vitro*.

Cell Viability Assay

To better understand the range of concentrations suitable for pulmonary delivery, the dose response cytotoxicity profile of clarithromycin on Calu-3 cells was established and is shown in Fig. 1. Cells viability was calculated with reference to the untreated cells, where average absorbance was normalized to 100% viability.

The viability assay demonstrated that Calu-3 cells could tolerate a wide range of clarithromycin concentrations. The IC_{50} of Calu-3 cell monolayers treated with clarithromycin was 38.73±3.92 µM.

TEER Measurements Following Clarithromycin Deposition

Clarithromycin, 0.5 and 1.1 µg respectively, were deposited onto Calu-3 cells at day 14 post seeding and TEER values measured at day 15 (1 day) and 19 (5 days). There was no statistical difference in TEER after 24 h deposition for either dose ($P>0.05$). After 5 days in culture, the increase in the TEER was found to be not statistically significant for both doses, when compared to control cells 580±97 Ω.cm^2, with values being 650±61 Ω.cm^2 and 683±47 Ω.cm^2 5 days following the deposition for 0.5 and 1.1 µg clarithromycin, respectively.

Apparent Permeability to flu-Na Following Clarithromycin Deposition

0.5 and 1.1 µg of clarithromycin were deposited onto Calu-3 cells at day 14 post seeding and the permeability values of flu-Na were measured at day 15 (1 day), 17 (3 days) and 19 (5 days). There was no statistical difference in permeability after 24 h deposition for either dose ($P>0.05$). There was no

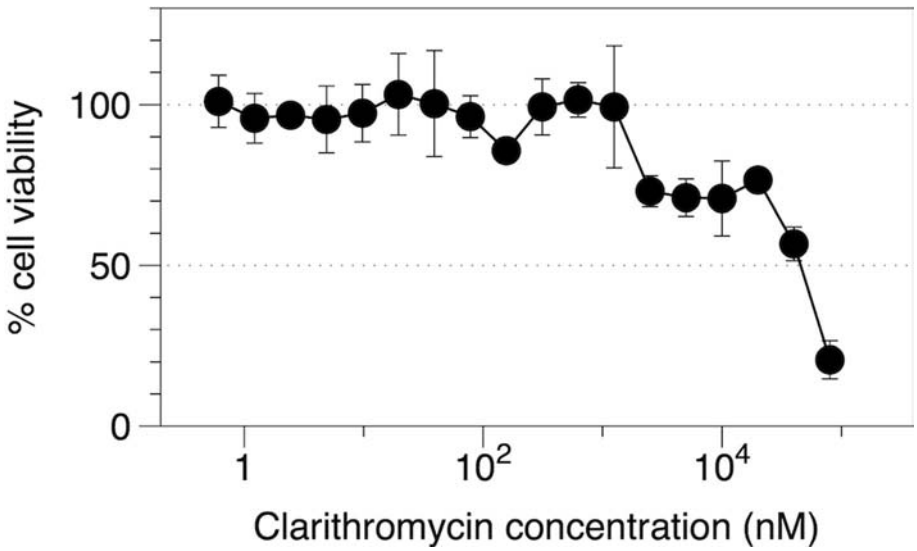

Fig. 1 The effect of clarithromycin on Calu-3 cell viability after 72 h drug treatment. ($n=3$, ± StDev).

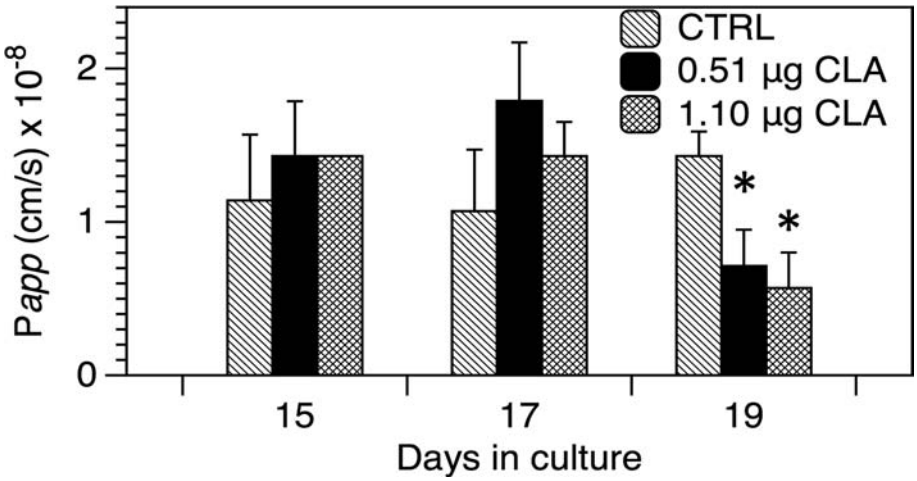

Fig. 2 Apparent permeability of flu-Na following approximately 0.51 and 1.10 μg clarithromycin deposition on Calu-3 cells. ($n=3$, ± StDev).

significant difference also at day 17, 3 days after drug deposition between the permeability values of the clarithromycin. However, after 5 days in culture, the permeability of flu-Na was found to be significantly lower for both doses, when compared to control cells, with permeability values being $7.14\pm2.35\times10^{-9}$ and $3.57\pm2.32\times10^{-9}$ cm.s^{-1} 5 days following the deposition for 0.5 and 1.1 µg clarithromycin, respectively (Fig. 2).

Muco-Inhibitory Effect of Clarithromycin on Calu-3 Cell Monolayers

The effect of clarithromycin on mucus production was investigated 1, 3 and 5 days following administration of the drug onto air interface Calu-3 epithelial cell model. Alcian blue was used to stain cellular acidic mucus secreted by the Calu-3 cells cultured under AIC conditions. It has been previously demonstrated that mucus accumulation is time dependent (41) and that long-term treatment with clarithromycin reduces the amount of sputum production, probably by inhibiting airway secretions, and increases sputum elasticity (11). Furthermore other authors have demonstrated that the presence of mucus considerably obstructs transmucosal movement of modestly sized macromolecules (42) and its presence obstructs drug transport (43). Untreated controls showed a mucus secretion profile consistent with earlier findings by Haghi *et al.* (41). When 0.5 and 1.1 µg of clarithromycin was deposited on the Calu-3 cells, a significant time dependent inhibition of mucus production was observed, compared to the untreated controls (Fig. 3). The extent of mucus inhibition was quantified using RGB_B ratio values, which were calculated, based on microscopic images taken of the stained mucus using ImageJ (Fig. 4). The RGB_B ratio of clarithromycin treated Calu-3 on Snapwells, was significantly lower compared to the control. At the end of the experiment on day 19, RGB_B ratio of 0.541 ±0.010 and 0.534 ± 0.015 was observed for the 0.5 and 1.1 µg clarithromycin treated Calu-3 cells, respectively, in comparison to the control samples that showed a value of 0.600±

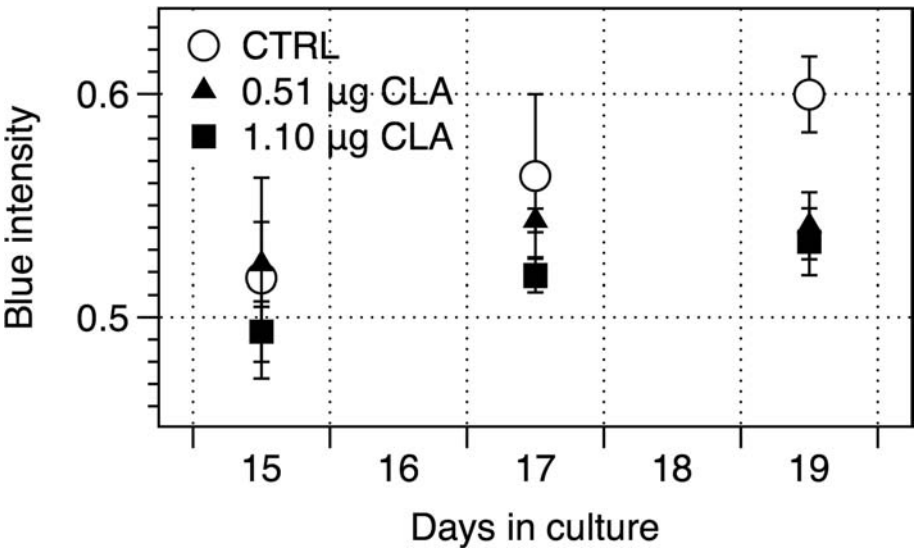

Fig. 3 Time-dependent muco-inhibitory effect of clarithromycin on Calu-3 cells ($n=3$, ± StDev)

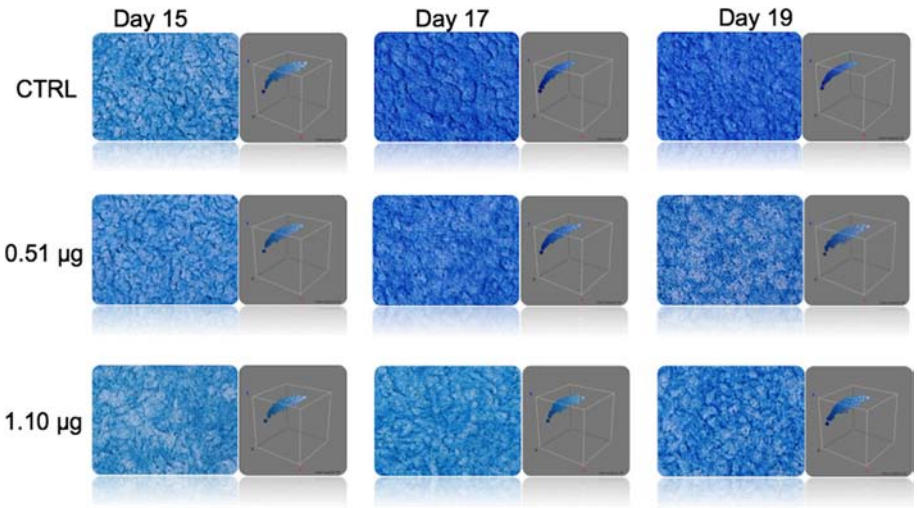

Fig. 4 Mucus staining of Calu-3 cells following deposition of ~0.50 and 1.10 μg clarithromycin and the RGB colour space analysis of the images.

0.017. Therefore, when clarithromycin was deposited on the Calu-3 monolayers grown under AIC conditions, a significant inhibition in mucus production was observed over the 5 days period post treatment, suggesting a potential beneficial effect of the clarithromycin on mucus inhibition.

Effect of Clarithromycin on IL-8 Production in Calu-3 Cells

IL-8 levels were measured at 24 and 48 h after stimulation of the Calu-3 cells with TGF-β. No statistically significant difference was observed between samples from the culture media for each treatment with clarithromycin ($P > 0.05$). A significant difference was observed in the level of IL-8 between the control and the cells previously treated with clarithromycin (Fig. 5). IL-8 levels were measured to be 8.4 ± 0.9 ng.ml^{-1} and 7.7 ± 0.8 ng.ml^{-1} after 24 and 48 h of stimulation with TGF-β, respectively, while the level of IL-8 after 24 and 48 h of exposure to TFG-β for the cells pre-treated with clarithromycin was 5.2 ± 0.9 ng.ml^{-1} (for pre-treatment with 0.5 µg) and 5.2 ± 0.5 ng.ml^{-1} and 5.3 ± 0.4 ng.ml^{-1} (for pre-treatment with 1.1 µg), respectively.

DISCUSSION

This is the first study to investigate the effects of low dose inhalable clarithromycin deposition directly on Calu-3 bronchial epithelial cells. It is shown that clarithromycin, once deposited on the epithelial cells, resulted in increased epithelial barrier integrity, short-term IL-8 reduction and a decrease in mucus production.

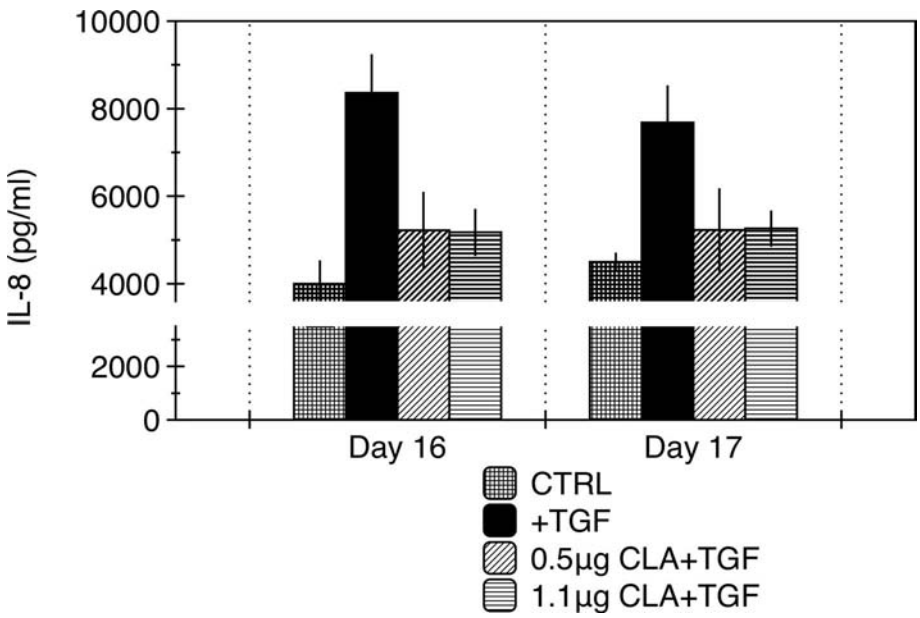

Fig. 5 Effect of clarithromycin (deposition at day 14) on the levels of IL-8 in the culture of Calu-3 cells after treatment with TGF-β (day 15), ($n = 3$, ± StDev).

Epithelial cells modulate the immune response through the release of cytokines and chemokines and regulate the production of mucus, antimicrobial substances and surfactants. Therefore, by forming a physical and chemical barrier, epithelial cells are the first obstacles for pathogens. Epithelial cell remodeling and damage is a typical characteristic of inflammatory respiratory diseases such as asthma and COPD. The restoration of a normal epithelial barrier by pharmacotherapy has been associated with improvement in clinical outcomes (44,45).

Modulation of tight junction proteins has been observed by adding azithromycin in the culture of primary human bronchial epithelial cells (11,46). The study showed that azithromycin mode of action involved the transition of claudin-1 and -4, Occludin and junction adhesion molecule-A (JAMA) to an intracellular location, hence regulating the tight junctions. A similar effect was observed when the Calu-3 cells were treated with clarithromycin in the present study. A dose dependent reduction in paracellular transport and increase in TEER was measured 1, 3 and 5 days post treatment. It is therefore envisaged that clarithromycin contributes to the integrity of epithelial barrier by stabilization of cellular tight junctions.

Altered mucus secretion and impaired mucus clearance are observed in chronic airways diseases such as asthma, COPD and bronchiectasis (47). Intranasal administration of clarithromycin is shown to inhibit goblet cell hyperplasia by inhibition of the MUC5AC (a major gel-forming mucin in the airways) through the activation of the NF-κB, while *in vitro* studies have shown the inhibition of TNF-α induced mucus secretion in human nasal epithelial cells (48). One proposed mechanism of action for beneficial effects of clarithromycin in diffuse panbronchiolitis is based on a direct effect on the function of the airway epithelia that overproduce mucin. The study showed that following oral treatment with clarithromycin, production of Muc5AC was reduced in a murine model (49). In the present study, a significant decrease in the mucus production was observed following deposition of clarithromycin on Calu-3 cells. Interestingly, the muco-inhibitory effect was only observed after the partial removal of the mucus from the Calu-3 surface. During the course of a previous experiment, where the cell surface was completely covered with mucus, treatment with clarithromycin did not result in any significant difference in the amount of mucus produced (data not shown). This finding could be due to the fact that the Calu-3 cells grown in the air interface culture model lack the mucociliary clearance system mechanism; therefore, for the muco-inhibitory effects of clarithromycin to be significant, a clearance mechanism or a pre removal of the mucus is required. This finding might indicate that treatment with a mucus-clearing agent in patients with impaired mucociliary clearance could be required for improving clinical outcomes.

Macrolides are reported to show anti-inflammatory and immune modulatory properties both *in vitro* and *in vivo* (12). Modulation of the systemic inflammatory response by clarithromycin has been proposed as the most probable explanation for the observed clinical benefits (50). Studies have shown that the immune-modulatory effects of macrolides occur with very low doses (well below the antimicrobial doses) (51). The current study highlights the muco-inhibitory and IL-8 reducing effects of clarithromycin following deposition of clarithromycin on the surface of Calu-3 sub-bronchial epithelial cells. The doses of clarithromycin used in the present study resulted in a decrease in IL-8 secretion in Calu-3 cells. Calu-3 untreated cells (stimulated with TGF-β) showed approximately ~1.5 times more IL-8 release compared to the cells pretreated with clarithromycin. Similar results were observed in the study by Cigana *et al.* (52), where the treatment of cystic fibrosis airway epithelial cells with azithromycin reduced the production of IL-8 mRNA and protein by 40%. This study suggested azithromycin's role in the signaling pathway by regulating the NF-κB activation. In a study by Takizawa *et al.* erythromycin and clarithromycin were shown to suppress mRNA levels and decrease IL-8 expression (~25%) in both normal and inflamed human bronchial epithelial cells by ELISA, northern blot analysis, quantitative reverse transcription and polymerase chain reaction (RT-PCR) techniques (53). In the current study the deposition of 0.5-1.1 μg clarithromycin resulted in ~35–40% reduction in IL-8 secretion from Calu-3 cells (measured by ELISA technique), comparable with the previous findings. Taken together, the findings of our study are in good agreement with immune-modulatory effects reported in different epithelial cell culture models. Therefore, the observed inhibitory effect of clarithromycin on mucus secretion in the present study could be explained by the immune-modulatory activity of clarithromycin on the NF-κB signalling pathway.

CONCLUSIONS

In vitro studies on Calu-3 in the air interface model showed clarithromycin to be non-toxic at the concentration range studied and have mucus inhibitory properties.

The findings of this study on the effect of clarithromycin on Calu-3 epithelial cells provide further evidence in explaining the clinical benefits in management of chronic lung diseases. The novel clarithromycin pMDI formulation had beneficial effects in barrier integrity, regulation of anti-inflammatory cytokine (IL-8) and inhibition of mucus production. The transport of clarithromycin across the epithelium, after deposition on the epithelial surface still requires further investigation.

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