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Targeting MMP-13 in bronchial epithelial repair

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Abstract

Background: Viral infection of the bronchial epithelium disrupts the barrier properties of the epithelium in healthy individuals and those with lung disease. Repair of the bronchial epithelium is dependent of the formation of a provisional fibrin matrix and migration of epithelial cells to cover denuded areas, followed by proliferation and differentiation.

Objective: The objective was to test the hypothesis that poly I:C, a model of viral infection, limits epithelial repair through the stimulated release of matrix metalloproteinase-13 (MMP-13).

Methods: Confluent layers of cultured normal human primary bronchial epithelial cells (NHBE) and SV-40 virus transformed 16HBE14o- bronchial epithelial cells were mechanically wounded, and video microscopy used to measure the rate of wound closure over 2 hours, in the absence and presence of poly I:C (1-20 $\mu\text{g}/\text{ml}$). MMP-13, tissue factor and endothelin release were measured by ELISA. The effect of inhibitors of MMP-13 activity and expression and a non-specific endothelin receptor antagonist, bosentan, on the rate of epithelial repair was investigated

Results: Poly I:C limited the rate of epithelial repair, and NHBE were significantly more sensitive to poly I:C effects than 16HBE14o- cells. NHBE, but not 16HBE14o-, released MMP-13 in response to poly I:C. Inhibitors of MMP-13 activity (WAY 170523) and expression (dimethyl fumarate) significantly enhanced the rate of repair. Bosentan enhanced the rate of bronchial epithelial repair by a mechanism that was independent of MMP-13.

Conclusions and clinical relevance: Bronchial epithelial repair is limited by endothelin and by MMP-13, a protease that degrades coagulation factors, such as fibrinogen, and matrix proteins essential for epithelial repair. Further studies with primary cells from patients are needed to confirm whether repurposing bosentan and inhibitors of MMP-13 expression or activity, for inhalation may be a useful therapeutic strategy in diseases where repeated cycles of epithelial injury and repair occur, such as asthma and COPD.

Introduction:

The bronchial epithelium plays a key role in immune responses to the environment, and its integrity is paramount in defence against infection, allergens and pollution. Inherited or acquired defects in innate defences in asthma and COPD render the bronchial epithelium susceptible to environmental factors, with cycles of epithelial damage and repair playing a key role in both the initiation and progression of these chronic airways diseases [1, 2]. Respiratory viral infections, particularly with rhinovirus (RV), the most prevalent respiratory virus, cause exacerbations of asthma and COPD, which in turn are associated with increased morbidity and mortality [3, 4]. People with asthma and healthy individuals are equally at risk

of upper respiratory tract infection with RV however those with atopic asthma have more frequent and more severe lower respiratory tract symptoms [5]. Respiratory viral infections cause epithelial barrier disruption that predisposes to antigen sensitisation in asthma [1] and bacterial infection in COPD [3, 4]. Therapeutics that improve bronchial epithelial repair increase the resistance of the airways to these sequelae, limiting the consequences of viral infection as well as other aggravating factors that disrupt the barrier function, such as proteolytic allergens, oxidants in cigarette smoke, air pollutants and pro-inflammatory cytokines [6].

Viral infection of the airways activates epithelial cells and disrupts epithelial tight junctions and adherens junctions, reducing the barrier function of the bronchial epithelium [7, 8, 9]. RNA viruses such as RV and respiratory syncytial virus (RSV) replicating within host cells produce double stranded RNA (dsRNA), which is recognised by Toll-like receptor 3 (TLR3) on endosomes. TLR3 binding of dsRNA, or a synthetic analogue, polyinosinic:polycytidylic acid (poly I:C), induces expression of pro-inflammatory cytokines and disrupts the epithelial barrier function in both primary normal human bronchial epithelial cells (NHBE) and the 16HBE14o- transformed bronchial epithelial cell line [7,8]. Disruption of the epithelial barrier is independent of autocrine or paracrine effects of released cytokines [8,9], but reflects direct effects of TLR3 signalling through the TIR-domain-containing adapter-inducing interferon- β (TRIF) pathway leading to decreased expression of members of the tight junction claudin protein family [7], disruption of the actin cytoskeleton and redistribution of tight junction (occludin and ZO-1) and adherens junction (E-cadherin and β -catenin) proteins from intercellular contacts to the cytoplasmic compartment without change in expression levels [8, 9].

It follows that repair of the epithelium is essential for recovery of barrier function. The restitution of normal airway epithelium involves the formation of a provisional fibrin matrix [10] and migration of neighbouring epithelial cells over the first 12-24 hours to cover denuded surfaces as the primary repair mechanism, followed by proliferation of progenitor cells to restore cell numbers and differentiation to restore function [11]. Within the airway, injury to the epithelium is followed by an inflammatory response involving neighbouring epithelial cells, fibroblasts, macrophages, endothelial cells and plasma exudation [11]. The released soluble factors supporting epithelial repair include growth factors, cytokines, matrikines and MMPs [11]. MMP-1, MMP-7 and MMP-9 are proposed to facilitate epithelial repair by promoting cell migration [11].

We previously showed that bronchial epithelial repair following mechanical disruption was dependent on the local expression of coagulation factors including tissue factor (TF), fibrinogen and Factor XIIIa in NHBE and 16HBE14o- cell monolayers and the formation of a provisional fibrin matrix [10]. It has been shown that MMP-13 (collagenase-3) impairs fibrin formation via degradation of fibrinogen [12] and other coagulation factors [13] as well as matrix proteins. Further, poly I:C as a model of most replicating virus [14] strongly

stimulated expression of its receptor (TLR-3), release of pro-inflammatory cytokines, and a large 25-fold increase in MMP-13 expression, without change in TIMP-1 or TIMP-2, in small airway epithelial cells [15]. We hypothesised a role for MMP-13 in limiting bronchial epithelial repair following virus infection, which has not previously been demonstrated.

We therefore investigated expression of MMP-13 in cultures of the 16HBE14o- bronchial epithelial cell line (16HBE) and normal primary bronchial epithelial cells (NHBE) exposed to poly I:C as a model of viral infection, the effect of increased expression of MMP-13 on the rate of repair following mechanical wounding of confluent bronchial epithelial cell layers, and the potential for inhibitors of MMP-13 activity and/or expression to enhance the rate of bronchial epithelial repair.

Materials and Methods:

Cell Culture

Cells of the SV-40 virus transformed human bronchial epithelial cell line, 16HBE14o-, were cultured in minimal essential media (MEM; GibcoBRL, Paisley, UK) containing 10 % heat inactivated-foetal calf serum (FCS; Sigma F9665), 2 mM L-glutamine (GibcoBRL) and 1% antibiotic/antimycotic (GibcoBRL, 100 units penicillin G, 100 µg streptomycin, 250 ng amphotericin B/ml) in 75 cm² tissue culture grade flasks at 37°C in 5 % CO₂. At 80-90 % confluence the cells were passaged using 0.05 % trypsin-0.02% EDTA (Sigma 59418C). After re-suspending in 1 ml full media the cells were counted and seeded at 100,000 cells per well in a 24-well plate, and cultured for 2 days to confluence in 250 µl full medium.

Normal human bronchial epithelial cells (NHBE) in bronchial epithelial growth medium (BEGM) were purchased from Lonza (Basel, CH), and are derived from the bifurcation of the trachea, and are therefore a mixture of bronchial and tracheal epithelial cells. Cells were grown in 75 cm² tissue culture grade flasks and sub-cultured at 100,000 cells per well into 24-well plates, flasks and plates pre-coated with collagen IV at 10 µg/cm², and cultured in fully supplemented BEGM (BulletKit[®]: Lonza, CH) at 37°C in a 5% CO₂ incubator until confluent. NHBE cells were used with a maximum of 3 passages and experiments were carried out with cells from 3 different donors.

Mechanical wounding

At confluence, scrape damage was applied using a P200 yellow pipette tip to produce a single wound 0.8 mm wide. Cell layers were washed and covered with 250 µl fresh warm medium containing 1-20 µg/ml poly I:C (Sigma) [14,15], in the absence and presence of 1 µM WAY 170523 (Tocris Bioscience) [16,17], 1-50 µM dimethyl fumarate (Sigma) [18] or 0.01-1 µM bosentan (Sigma) [19], at the concentrations indicated in the legends and on the

figures. Control cells were untreated. Each condition was replicated in triplicate within each independent experiment. Damaged cultures were incubated for 24 hours for real-time video microscopy (see below). After 24 hours, supernatants were removed, cell debris cleared and supernatants stored at -80°C prior to analysis.

Video microscopy

24-well plates were incubated for 24 hours at 37°C with 5% CO₂ on the stage of a Zeiss Axiovert 200M inverted microscope for live cell imaging. A 5X objective was used and bright field images were acquired every 15 minutes for 24 hours. Volocity software (Version 6.1; Quorum Technologies, Guelph, Ontario) was used to obtain movies for each well and also to measure gap distances (µm) across each wound at 5 locations at each time point. Time to closure and initial rate of closure over the first two hours as µm/hour were calculated (mean±SEM) using MS Excel.

Measurement of total MMP-13, TF and endothelin (ET) in cell culture supernatants;

Total MMP-13, TF and ET were measured by ELISA using Duo-Sets from R&D Systems according to the manufacturer's instructions.

MMP-13 standards were added to ELISA plates in the concentration range 62.5-4000 pg/ml, and NHBE samples diluted 1:2 for analysis in duplicate. We determined the lower limit of detection for the assay to be 21.91 pg/ml and the intra-assay coefficient of variance to be 4.9% (n=40).

TF standards were added to ELISA plates in the concentration range 7.8-500 pg/ml and samples diluted 1:2 for analysis in duplicate. We determined the lower limit of detection for the assay to be 4.81 pg/ml and the intra-assay coefficient of variance to be 4.8% (n=28).

ET-1 standards were added to ELISA plates in the concentration range 3.9-250 pg/ml and samples diluted 1:2 for analysis in duplicate. We determined the lower limit of detection for the assay to be 0.09 pg/ml and the intra-assay coefficient of variance to be 6.4% (n=39).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (Version 7, La Jolla, CA, USA). Data for TF were analysed using one-way ANOVA. Data for the effect of WAY 170523, DMF and bosentan on the rate of epithelial repair were analysed by two-way ANOVA with Bonferroni's correction for multiple comparisons.

Results

Poly I:C significantly inhibited the initial rate of repair of both the bronchial epithelial cell line and primary NHBE cells (Figure 1). Poly I:C had a significantly greater effect and was at least an order of magnitude more potent at inhibiting wound repair in NHBE cultures over 2 hours (Figure 1B) and 24 hours (Figure 2) compared to 16HBE cultures (Figure 1A). Over 24 hours the wounded 16HBE cell layers had completely repaired in the absence or presence of poly I:C. However, although wounded NHBE layers cultured in media alone for 24 h were largely restored to confluence (Figure 2D), in the presence of 20 µg/ml poly I:C cells were scattered and the wound failed to heal (Figure 2E).

We investigated the effect of a specific inhibitor of MMP-13 activity, WAY 170523, on the epithelial repair response in the presence of poly I:C. Results (Figure 1A and B) show that in the presence of WAY 170523 (1 µM) there was no effect of poly I:C on the rate of epithelial repair, and that WAY 170523 significantly inhibited the effect of poly I:C (20 µg/ml and 1 µg/ml) in 16HBE and NHBE epithelial cell models, respectively.

We subsequently investigated whether the increased sensitivity of NHBE to poly I:C was due to higher levels of MMP-13 in NHBE compared to 16HBE cell cultures. Figure 3 shows that NHBE constitutively express and release MMP-13 into cell culture supernatants (1203±222.3 pg/ml at baseline). MMP-13 levels were stimulated by poly I:C in a concentration-dependent manner and were significantly increased above baseline on incubation with 10-20 µg/ml poly I:C (4133±159 pg/ml with 20 µg/ml poly I:C). Conversely, concentrations of MMP-13 in 16HBE14o- cell supernatants were below the lower limit of detection.

In view of the evidence that MMP-13 activity (Figure 1) limits the rate of bronchial epithelial repair in the cell cultures, we next investigated the effect of dimethyl fumarate (DMF), an inhibitor of MMP-13 expression in chondrocytes in the range 1-50 µM [18]. In the same concentration range DMF inhibited poly I:C-induced MMP-13 expression, with a significant 50% inhibition of MMP-13 in the presence of 20 µg/ml poly I:C, from 4133±159 to 2068±743 pg/ml (Figure 3).

In the same concentration range DMF alone had no effect on the rate of repair of 16HBE cell layers, but at 10-50 µM, DMF significantly enhanced the rate of epithelial repair in the presence of 20 µg/ml poly I:C (Figure 4A). Further, the repair of NHBE monolayers strongly inhibited by poly I:C in the concentration range 1-20 µg/ml, was restored by DMF (50 µM) at all concentrations of poly I:C (Figure 4B).

In view of our previous findings demonstrating an important role for coagulation in bronchial epithelial repair, initial experiments investigated the effect of poly I:C on TF protein expression over 24 hours (Figure 5). Wounding alone had no significant effect on TF antigen levels in supernatants, as we had previously observed [10], but poly I:C at 10 and 20 µg/ml stimulated a significant increase in TF in both intact and wounded 16HBE cell layers

(Figure 5A). However, poly I:C had no effect on TF protein expression in wounded NHBE cell layers at any concentration (Figure 5B).

The non-specific endothelin receptor antagonist, bosentan (1-10 μ M), was previously shown to inhibit MMP-13 release from primary bronchial epithelial cells stimulated with TNF α [19]. We therefore investigated the effect of poly I:C on endothelin release from NHBE (Figure 6A) and the effect of bosentan of the rate of NHBE repair (Figure 6B).

Poly I:C stimulated a significant increase in the release of endothelin (Figure 6A). Bosentan in the concentration range 10-1000 nM potently and effectively reversed the inhibitory effect of poly I:C (20 μ g/ml) on the rate of NHBE repair (Figure 6B). However, bosentan in the concentration range 10-1000 nM had no effect on MMP-13 release from NHBE (n=3, not shown) and, conversely, exogenously added endothelin-1 (1 – 100 nM) did not stimulate MMP-13 release (not shown).

Discussion

This is the first report that epithelial-derived MMP-13 limits epithelial repair, that poly I:C significantly increases MMP-13 release from mechanically wounded NHBE and that inhibitors of MMP-13 activity and expression enhance bronchial epithelial repair. Our study also shows that factors supporting fibrin formation, namely significantly increased TF and limited MMP-13 release, render 16HBE cells relatively resistant to the effects of poly I:C on the rate of bronchial epithelial repair compared to NHBE.

The difference in the effect of poly I:C on the repair response in 16HBE and NHBE cell-based models was striking. Although poly I:C inhibited the rate of cell migration and wound repair in both cell types, NHBE were significantly more sensitive to the effect of poly I:C (Figures 1 and 2). This may partly reflect the significantly increased expression of TF, a coagulation factor essential for epithelial repair [10], in the 16HBE cells, but not in the NHBE cells, in response to poly I:C. The difference in the rate of repair may also reflect significant poly I:C induced MMP-13 expression in NHBE cells (Figure 3), but not in 16HBE cells in which MMP-13 expression remained low. MMP-13 expression is positively regulated by BCYRN1, a c-MYC-activated long non-coding RNA, in non-small-cell lung cancer cells [20]. Expression of BCYRN1 is extremely low in the 16HBE cell line, which may account for the low levels of MMP-13 released from these cells [20]. BCYRN1 is generally not detected in normal tissue [20] and physiological expression of MMP-13 is limited to tissues undergoing rapid extracellular matrix remodelling [13]. Overall, the data indicate that the 16HBE cell line,

although widely used, may not be a suitable model to measure the rate of normal bronchial epithelial repair.

Targets for MMP-13 include type I, II, III, IV, X, XIV collagens, tenascin C, aggrecan core protein, perlecan, fibronectin, fibrinogen, factor XII, fibrillin and fibrillin-rich microfibrils, as well as the serpins PAI-2 and α 2-antichymotrypsin [13]. We previously showed that normal bronchial epithelial repair following mechanical wounding is dependent on coagulation factor expression and fibrin formation [10]. In our model of normal bronchial epithelial repair following viral infection, increased MMP-13 activity is proposed to degrade coagulation factors and matrix proteins essential for successful epithelial repair. The MMP-13 inhibitor WAY 170523 significantly enhanced the rate of repair of NHBE cell layers, suggesting a potent inhibitory effect of MMP-13 activity on normal bronchial epithelial repair.

Secretion of MMP-13 was previously shown to be strongly induced by poly I:C acting on small airway epithelial cells [15], an effect mediated via release of IFN- β and interferon receptor pathways [21]. Increased expression of MMP-13 was also reported in primary tracheobronchial airway epithelial cells infected with both respiratory syncytial virus and influenza A [22]. Together with our findings in NHBE, increased expression and release of MMP-13 may be common to viral infection of epithelial cells throughout the respiratory tract. Increased MMP-13 expression in alveolar macrophages and type II pneumocytes in COPD [23] and the recently reported role of MMP-13 in lung destruction in a viral exacerbation model of COPD [24], together with increased MMP-13 gene expression in the upper and lower respiratory tract epithelium in response to viral infection in asthma [25, 26] which is not different to the normal response, suggest that targeting MMP-13 may be a viable treatment option in both of these diseases.

Inhibitors of MMP-13 activity, including selective inhibitors [27, 28] and specific neutralising antibodies [29] that avoid the side effects of broad spectrum MMP inhibitors are currently in development for the treatment of arthritis, cancer and cardiac disease, with the potential for repurposing for respiratory disease. Other approaches include inhibition of MMP-13 expression. DMF was previously shown to attenuate MMP-13 expression in isolated chondrocytes by inhibiting the JAK/STAT3 pathway [18]. Our results provide evidence that DMF similarly attenuates MMP-13 expression in bronchial epithelial cells and has a positive effect on epithelial repair. DMF was originally developed to treat inflammatory skin disease such as psoriasis and was recently repurposed to treat multiple sclerosis [30]. Others recently proposed repurposing DMF as an anti-inflammatory therapy for asthma [31] and we now suggest that DMF may also limit inflammation indirectly through positive effects on bronchial epithelial repair.

Of particular further interest are the recent reports that the leukotriene receptor antagonist montelukast not only suppresses inflammation in a poly I:C-induced asthma exacerbation model in mice [32], but also suppresses LPS-induced MMP-13 expression in mouse osteoblasts [33]. However, the effects of montelukast on bronchial epithelial repair in our model remain to be investigated.

Bosentan was previously shown to inhibit MMP-13 release from primary bronchial epithelial cells stimulated with TNF α [19]. However, although we demonstrated that poly I:C (20 μ g/ml) induced endothelin release from NHBE (Figure 6A), the positive effect of bosentan on the rate of epithelial repair in the presence of poly I:C (20 μ g/ml) was not related to inhibition of MMP-13 release. Alternatively, endothelin-1 was previously shown to result in slower migration of primary bronchial epithelial cells across uncoated filters [34]. In our experiments, the strong positive effect of bosentan on epithelial repair in the presence of poly I:C may reflect inhibition of endogenously produced endothelin-1 acting in an autocrine fashion on endothelin receptors A and/or B expressed on primary bronchial epithelial cells [35] to limit the rate of cell migration. Figure 7 illustrates our proposed mechanisms for defective bronchial epithelial repair following virus infection, identifying targets and drugs to improve repair.

Endothelin-1 is a potent bronchoconstrictor and pro-inflammatory mediator that plays a role in the pathogenesis of lung diseases, including asthma, and in the co-morbidities associated with COPD [36]. A role for endothelin receptor antagonists and the repurposing of bosentan, approved for pulmonary arterial hypertension, for asthma was proposed [37]. Although treatment with oral bosentan for 4 weeks did not improve lung function or symptom score in difficult to control asthma [38], it was suggested that local delivery via inhalation may enhance the potency and efficacy of repurposed drugs for allergy and asthma, while avoiding the systemic adverse side effects [37]. Animal models have indicated the feasibility of this approach for bosentan [39, 40].

The limitations of the study that influence the final conclusions are that analysis was done only in normal NHBE and the results have yet to be confirmed in diseased cells. Additionally, the results were not confirmed in differentiated epithelial cells, using the air-liquid interface (ALI) model. However, others [41] have shown that NHBE in ALI close by only 20% even at 48 hours, which was suggested to be due to technical issues with using ALI for wound repair assays.

Overall, the mechanisms by which respiratory viruses limit the repair response are not well understood and this is an important research area for identifying therapeutic targets aimed at enhancing epithelial repair. Chronic airways diseases such as asthma and COPD are associated with repeated cycles of injury and repair. Derangements in bronchial epithelial function may contribute to epithelial fragility and airway remodelling [42] and the epithelium is therefore a particular target for focused therapeutic interventions in asthma [43, 44] and COPD [2]. Overall, our results lead us to propose a novel MMP-13-dependent

mechanism for defective bronchial epithelial repair following virus infection. However, further studies using primary cells from patients are needed to confirm whether MMP-13 inhibitors and endothelin receptor antagonists repurposed for inhalation may be useful therapeutic approaches in asthma and COPD.

Conflict of interest statement;

The authors declare no conflict of interest.

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Figure legends

Figure 1. The effect of poly I:C and the MMP-13 inhibitor, WAY 170523, on bronchial epithelial repair. Poly I:C (black bars) limits the rate of epithelial wound repair, and WAY 170523 (1 μ M) restores the rate of repair (grey bars) in (A) 16HBE cells (n=8) and (B) NHBE (n=3 or 4). * p<0.05, ** p<0.01, *** p<0.001 compared with the no poly I:C control. p<0.05 compared to poly I:C alone.

Figure 2. Confluent NHBE layers wounded mechanically with a pipette tip (A) were analysed using video microscopy over 24 h, with recordings made every 15 minutes. Wounds starting to repair in media alone at 2 h (B) failed to start repair in the presence of 20 μ g/ml poly I:C at 2 h (C). Cell layers cultured in media alone for 24 h were largely restored to confluence (D) but in the presence of 20 μ g/ml poly I:C cells were scattered and the wound failed to heal (E). Image width = 1707 μ m.

Figure 3. MMP-13 expression in NHBE. A. Poly I:C (black bars) increases expression and release of MMP-13 from NHBE, while 50 μ M DMF (grey bars) inhibits expression and release of MMP-13 from NHBE stimulated with 20 μ g/ml poly I:C. * p<0.05, ** p<0.01 compared with the no poly I:C control. p<0.05 compared to poly I:C alone (n=3).

Figure 4. DMF enhances bronchial epithelial repair. A. DMF alone (black bars) had no effect on the rate of epithelial repair in 16HBE cells, but in the presence of 20 μ g/ml poly I:C (grey bars) DMF at 10 μ M and 50 μ M significantly enhances the rate of repair compared to the effect of poly I:C alone. * p<0.05, ** p<0.01 compared with DMF alone. ★p<0.05 compared to poly I:C alone, (n=3). B. Poly I:C (1-20 μ g/ml) alone (black bars) inhibits the rate of NHBE repair and addition of 50 μ M DMF (grey bars) significantly reversed the effect of poly I:C at all concentrations tested. **p<0.01 compared to the no poly I:C control. ★ p<0.05, ★★ p<0.01 compared to the poly I:C alone control (n=3 or 4).

Figure 5. The effect of poly I:C on TF release by bronchial epithelial cell layers. Poly I:C strongly stimulated TF release from intact and wounded 16HBE bronchial epithelial cell layers, ** p<0.01, *** p<0.001 compared with the no poly I:C control (n=4). Poly I:C had no effect on TF release from wounded NHBE cell layers (n=4).

Figure 6. The effect of endothelin-1 on NHBE repair. A. Poly I:C (20 μ g/ml) induces endothelin-1 release in NHBE. B. Poly I:C (grey bars) alone (20 μ g/ml) completely inhibits

NHBE repair. The dual endothelin receptor antagonist bosentan, at 10-1000 nM, reverses the inhibitory effect of 20 µg/ml poly I:C on NHBE repair responses. ** p<0.01, *** p<0.001 compared to the no poly I:C control, ** p<0.01, *** p<0.001 compared to the poly I:C alone control (n=3).

Figure 7. Proposed mechanisms for defective bronchial epithelial repair following virus infection, identifying targets and drugs to improve repair. TLR-3 activation with poly I:C increases endothelin-1 (ET-1) and MMP-13 release from NHBE. ET-1 is proposed to limit the repair response through direct effects on cell migration, and these effects can be blocked by the dual ET-1 receptor antagonist bosentan. MMP-13 is proposed to limit repair through degradation of coagulation factors and matrix proteins, including fibrinogen, which are essential for epithelial repair. Inhibitors of MMP-13 activity (WAY170523) and expression (DMF) therefore improve the repair response in the presence of poly I:C.

Targeting MMP-13 in bronchial epithelial repair – Figures

Figure 1.

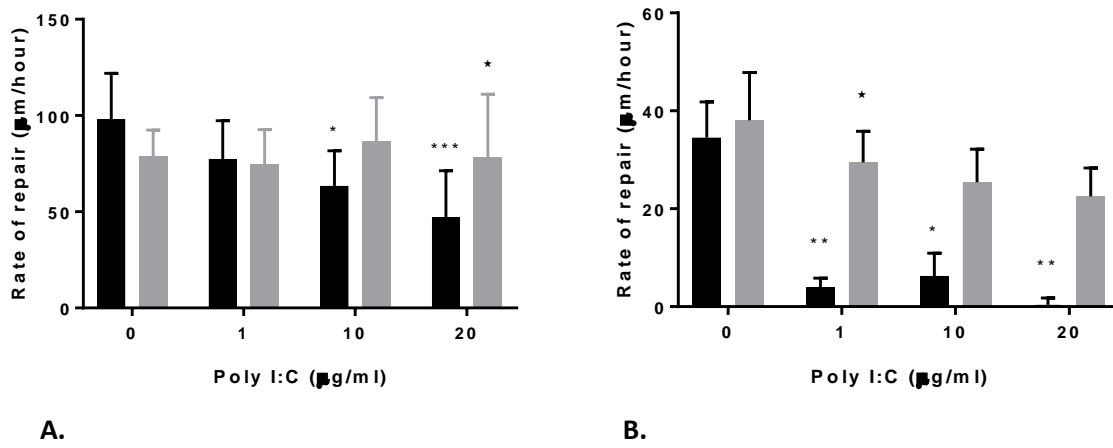


Figure 2.

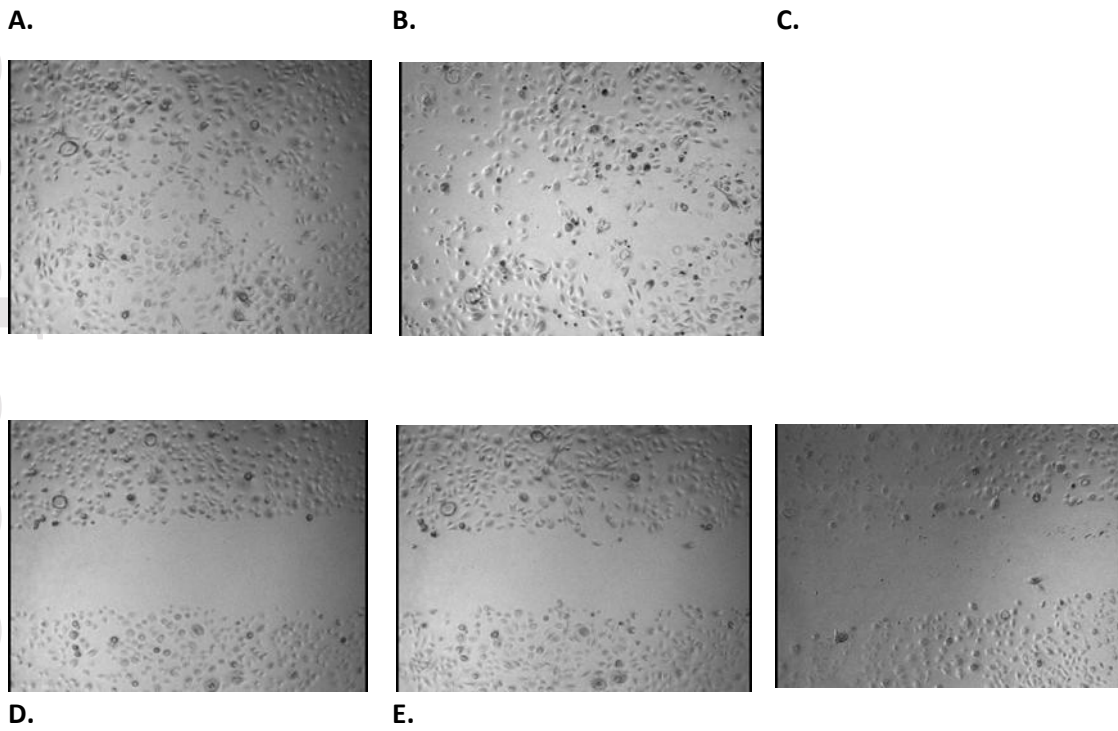


Figure 3.

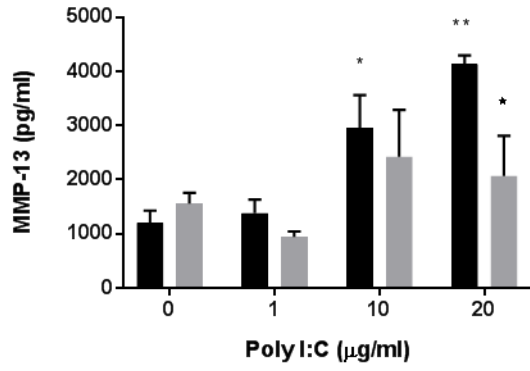


Figure 4.

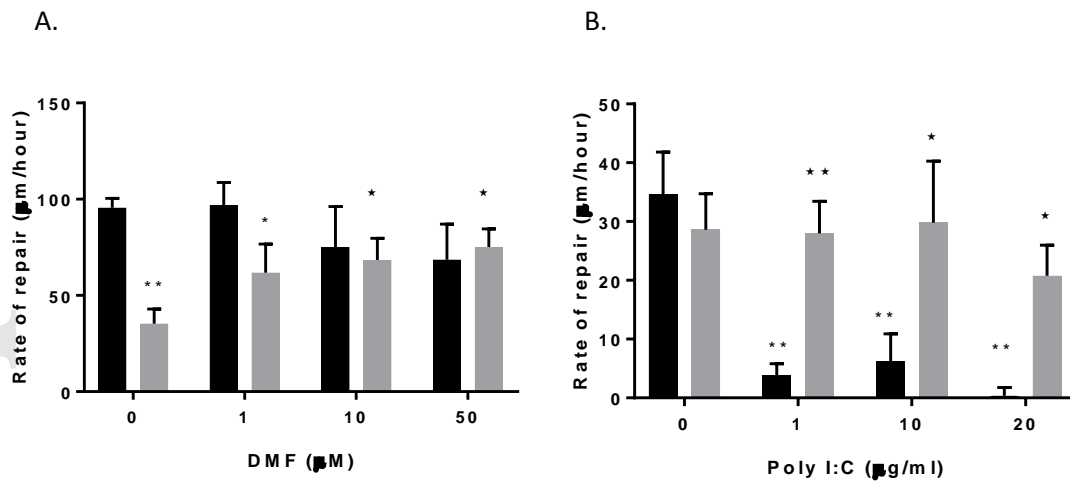
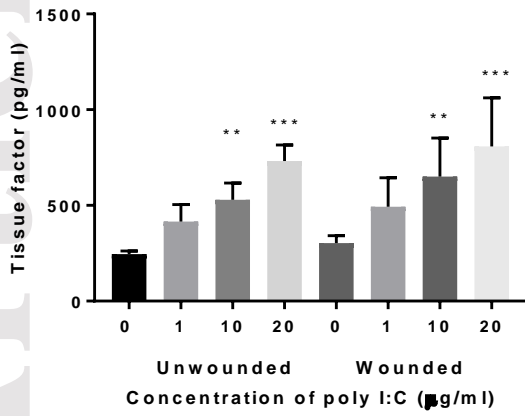
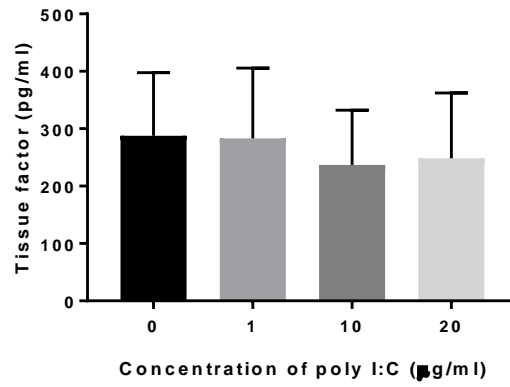


Figure 5.

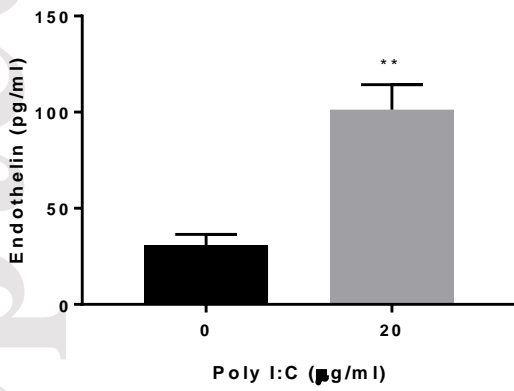


A.

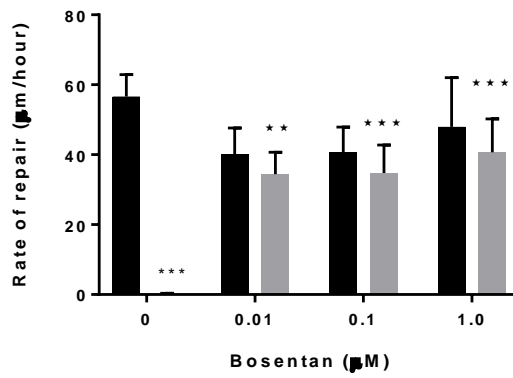


B.

Figure 6.



A.



B.

Figure 7.

