Anti-inflammatory effects of DX-890, a human neutrophil elastase inhibitor☆

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Abstract

Background: Neutrophil elastase (NE)-mediated inflammation contributes to lung damage in cystic fibrosis (CF). We investigated if DX-890, a small-protein NE inhibitor, could reduce neutrophil trans-epithelial migration and reduce activity released from neutrophils and NE-induced cytokine expression in airway epithelial cells.

Methods: Activated blood neutrophils (CF and healthy) treated ±DX-890 were assayed for NE activity. Transmigration of calcein-labeled neutrophils was studied using a 16HBE14o− epithelial monolayer. IL-8 release from primary nasal epithelial monolayers (CF and healthy) was measured after treatment ±DX-890 and NE or CF sputum.

Results: DX-890 reduced NE activity from neutrophils (CF and healthy) and reduced neutrophil transmigration. DX-890 pre-treatment reduced IL-8 release from epithelial cells of healthy or CF subjects after stimulation with NE and CF sputum sol. All improvements with DX-890 were statistically significant (p<0.05).

Conclusions: DX-890 reduces NE-mediated transmigration and inflammation. NE inhibition could be useful in managing neutrophilic airway inflammation in CF.

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1. Introduction

Persistent neutrophil influx to the airway is a feature of cystic fibrosis. Failure of the macrophage clearing system for apoptotic cells leads to neutrophil death by secondary necrosis and the release of high levels of pro-inflammatory mediators including the proteolytic enzyme neutrophil elastase (NE) [1] and oxidants which inactivate the anti-proteases α1-antitrypsin and secretory leukoprotease inhibitor which normally inactivates extracellular NE, thus protecting the airway [2].

Extracellular NE degrades many components of the extracellular matrix including elastin, collagen types I-IV, proteoglycan and fibronectin [3] as well as complement factors and their receptors [4], immunoglobulins [5] and surfactant proteins [6], further compromising the immune defense. NE perpetuates inflammation by stimulating airway epithelial cells to release pro-inflammatory mediators such as the chemokine IL-8 [7–10]. NE also induces goblet cells and submucosal glands to secrete mucin proteins [11] and impairs the ability of macrophages to recognize and phagocytose apoptotic neutrophils [12].

Inhibition of NE may reduce CF airway inflammation but, to date, no NE inhibitors have been licensed for use in treating airway inflammation in CF. A comprehensive review of
synthetic, recombinant and natural inhibitors of NE can be found elsewhere [13].

DX-890 (Dyax Corp., USA) is a potent and selective small-protein inhibitor of NE which was discovered by phage display [14]. This study investigated if DX-890 could reduce NE activity from activated neutrophils and IL-8 secretion from airway epithelial cells, and if DX-890 could reduce neutrophil trans-epithelial migration.

2. Methods

All materials were from Sigma Aldrich unless stated otherwise. This study was approved by the Office for Research Ethics Committees Northern Ireland (ORECNI) and all participants gave written informed consent.

2.1. Neutrophil isolation

Neutrophils were isolated from heparinized venous blood of volunteers by a dual density centrifugation method using Histopaque-1077 and Histopaque-1119 according to the manufacturer’s instructions (Sigma Aldrich). In all experiments neutrophil samples were >98% viable as determined by Trypan blue staining. Blood was either from clinically stable F508del homozygous CF patients attending outpatient clinics in Belfast City Hospital, or from healthy volunteers.

2.2. Neutrophil elastase activity from activated neutrophils

Neutrophils (1.25 × 10⁴ cells) were primed by pre-incubation with cytochalasin b (5 μg/ml) ± DX-890 (10 nm–1 μM) for 10 minutes at 37 °C. Neutrophils were then stimulated with fMLP (100 nM) for a further 10 minutes before addition of the colorimetric substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-pNA (100 ng/ml; Elastin Products). Cleavage of substrate was immediately monitored colorimetrically at 405 nm every 45 seconds for 10 minutes. Each reaction (200 μl) was performed in triplicate in a 96-well plate. Initial reaction rates are reported as the change in absorbance per minute.

2.3. Neutrophil transmigration

2.3.1. Production of the epithelial monolayer

16HBE14o⁻ cells (1 × 10⁶ cells/ml) were seeded in the apical chambers of collagen coated Transwells (Corning Costar). Cells were fed every second day with minimal essential media (MEM; Invitrogen) with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (250 μg/ml apical, 600 μl basolateral) until transepithelial electrical resistance (TEER), measured every second day, stopped rising linearly, indicating a confluent, polarized monolayer.

2.3.2. Calcein labeling of neutrophils

Blood neutrophils from healthy volunteers were fluorescently labeled by incubation with 1.5 μM calcein-AM for 20 minutes in the dark at 37 °C, followed by two PBS washes and centrifugation (200g, 10 minutes, room temperature). Cells were kept in darkness during and after the labeling procedure to avoid photocytotoxicity associated with calcein-AM.

2.3.3. Neutrophil transmigration

Culture media was washed from the epithelial monolayer with HBSS (Gibco) and 600 μl fMLP (100 nM in HBSS) was added to the basolateral compartment. Aliquots of calcein-labeled neutrophils (1 × 10⁶ cells/ml; 200 μl) were pre-incubated ± DX-890 (500 nM) at 37 °C for 10 minutes, then added to the apical chamber of Transwells and the plate was incubated at 37 °C for 2 hours. Following incubation, liquid in the apical chamber was aspirated, and the upper compartments were washed with HBSS to remove any neutrophils that had not transmigrated through the monolayer to the basolateral side of the membrane. Fluorescence was measured using a bottom-reading probe and an area scan of the entire well. Excitation and emission wavelengths were 495 nm and 520 nm respectively.

2.4. Preparation of the soluble fraction of CF sputum

Spontaneously expectorated sputum samples were collected from CF inpatients in the adult CF ward in Belfast City Hospital. Samples were processed using PBS as described by Nixon et al. [15]. Sputum sol from seven patients was pooled before being used to stimulate airway epithelial cells.

2.5. Nasal epithelial cell culture

Nasal epithelial cells (NEC) were obtained by nasal brushing from CF (F508del homozygous) and healthy volunteers as previously described [16]. Participant characteristics are described in Table 1. Cells (passage 2-4; 1.5 × 10⁵ cells/ml) were seeded in a collagen coated 24-well plate. The next day, cells were approximately 80% confluent and were pre-treated with DX-890 (200–500 nM) for 30 minutes, then stimulated with NE 50 nM or CF sputum sol 1:100 for 4 hours. Culture media was replaced and cells were incubated for 24 hours. Cell-free supernatant was stored at −20 °C for future IL-8 measurement.

Table 1

<table>
<thead>
<tr>
<th>Characteristics of volunteers in the nasal epithelial cell study.</th>
<th>Cystic fibrosis (n=10)</th>
<th>Healthy (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>50.0</td>
<td>57.1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>22 (22.25)</td>
<td>28 (23.5, 31.5)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.5 (18.9, 22)</td>
<td>20.3 (20.1, 21)</td>
</tr>
<tr>
<td>FEV₁ (%) (% predicted)</td>
<td>65 (55, 74)</td>
<td>–</td>
</tr>
<tr>
<td>FVC* (L)</td>
<td>3.04 (2.51, 4.03)</td>
<td>–</td>
</tr>
</tbody>
</table>

All data were median and interquartile range apart from the percentage of males (median only). Lung function testing was not performed for healthy participants. BMI: body mass index, FEV1: forced expiratory flow in one second, FVC: forced vital capacity.
2.6. Statistical analysis

Data are non-parametric and are therefore presented as median ± interquartile range. Comparisons within groups were made using Wilcoxon signed rank test or the Kruskal–Wallis test. Comparisons between groups (i.e. between CF and non-CF groups) were made using the Mann–Whitney U test. A p value ≤ 0.05 was considered significant.

3. Results

3.1. Inhibition of NE activity in neutrophils

NE activity in non-stimulated neutrophils was similar between healthy and CF neutrophils. fMLP-induced NE release was higher from healthy neutrophils than from CF neutrophils (p = 0.025). Pre-treatment of neutrophils with DX-890 reduced activity of NE released after fMLP stimulation. DX-890 10 nM partially reduced NE activity but DX-890 100 nM and 1 μM reduced NE activity to below the level of spontaneous release in both healthy and CF neutrophils (Fig. 1).

3.2. Neutrophil transmigration

TEER values of 16HBE14o− monolayers increased over time and reached a maximum of 0.6 ohms/cm² after 7–15 days, indicating confluence (data not shown).

Median (range) spontaneous neutrophil transmigration was 25.2% (22.2, 38.3). Transmigration was significantly increased in response to the chemoattractant fMLP to 47.6% (39.7, 87.1) (p = 0.001). DX-890 pre-treatment of neutrophils significantly reduced transmigration in response to fMLP to 36.1% (20.9, 53.75) (p = 0.02; Fig. 2).

3.3. Nasal epithelial cells

A DX-890 concentration of 500 nM was necessary to inhibit NE activity in CF sputum sol 1:100. DX-890 did not induce IL-8 secretion and did not adversely affect cell viability at doses up to 500 nM (data not shown). Therefore DX-890 was used at doses up to 500 nM in the epithelial cell study.

Baseline IL-8 secretion was similar between healthy and CF epithelial cells (median 1.5 μg/ml and 1.3 μg/ml respectively). NE 50 nM upregulated IL-8 secretion in healthy (2.5 μg/ml) and CF epithelial cells (2.4 μg/ml). CF sputum sol 1:100 also upregulated IL-8 secretion in healthy (2.6 μg/ml) and CF epithelial cells (2.0 μg/ml) (Fig. 3).

Pre-incubation with DX-890 (200 nM) reduced NE-induced IL-8 secretion in healthy (median 2.2 μM/g/ml) and CF epithelial cells (median 1.7 μg/ml). Similarly, pre-incubation with DX-890 (500 nM) was associated with reduced CF sputum sol-induced median IL-8 secretion in healthy epithelial cells (1.8 μg/ml) and CF cells (1.7 μg/ml) (Fig. 4).

4. Discussion

Neutrophilic inflammation of the lung is a hallmark of cystic fibrosis and greatly contributes to the pathogenesis of lung disease. DX-890 reduces the release of active NE from fMLP-stimulated neutrophils and reduces migration of neutrophils through an epithelial monolayer in vitro. DX-890 also reduced IL-8 release from CF and healthy NEC following stimulation with NE and CF sol.

NE is essential for the antimicrobial activity of neutrophils, both in degradation of phagocytosed microbes and those caught in neutrophil extracellular traps (NETs) [17]. The necessity of NE in the immune defense against Gram negative bacteria is demonstrated by the mortality of NE deficient mice [18].
paradox of NE is that, while it is necessary for normal function of the immune system, over-abundance has degradative and pro-inflammatory effects in diseases such as cystic fibrosis. Neutrophilia occurs in the CF lung from infancy [19] and much of the subsequent lung damage can be attributed to neutrophil necrotic products, NE in particular. Reduction of airway NE levels could break the destructive cycle of airway inflammation and reduce lung damage in CF.

DX-890 is a potent small peptide inhibitor of NE that has previously been shown to reduce lung injury and neutrophil accumulation in the lungs of rats [20]. We investigated if DX-890 could modify neutrophil function, specifically NE activity following fMLP stimulation of the cell. No difference in NE release was observed between CF and normal neutrophils at baseline, but following stimulation with fMLP, more NE was released by normal neutrophils. Brockbank et al. found that CF blood neutrophils release more spontaneous NE than normal blood neutrophils, but after stimulation with fMLP more NE was released from normal neutrophils [21]. Another study showed that CF and normal neutrophils release similar amounts of spontaneous NE but CF neutrophils release more NE following stimulation with TNF-α and IL-8 [22]. The disparity in the behavior of neutrophils across these studies may be due to differences in isolation and treatment as well as the stimulus used to provoke degranulation. We demonstrated that DX-890 reduced activity of NE released by both healthy and CF blood neutrophils in response to fMLP.

Another strategy for dampening neutrophilic inflammation in the airway is to reduce migration of neutrophils from the bloodstream to the airway. The role, if any, played by neutrophilic proteases in transmigration is controversial. Several rodent studies have demonstrated that NE inhibitors decrease neutrophil migration to the airway following stimulation such as lung injury [20, 23–25], although other studies do not support these findings [26–28]. Rees et al. found that a NE inhibitor protected against rodent lung injury induced by the instillation of NE or CF sputum sol [29]. Our results demonstrate that pre-incubation of neutrophils with DX-890 reduces transmigration through an epithelial cell monolayer in response to fMLP. Delacourt et al. demonstrated that DX-890 reduced neutrophil migration through a Matrigel basement membrane [20]. Following the observation that following mechanical ventilation, NE −/− mice had significantly less neutrophil infiltration into the lung than wild type, Kaynar and colleagues demonstrated the necessity of NE for neutrophil migration across endothelium but not Matrigel [24]. Results from our model build upon observations from the Delacourt study and suggests that NE activity is involved in at least two portions (interstitium and epithelium) of the neutrophils’ transmigration from blood vessel to airway lumen.

Extracellular NE drives inflammation by stimulating nasal epithelial cells (NEC) to secrete pro-inflammatory mediators such as IL-8. This study demonstrates that DX-890 reduced IL-8 release induced by NE and CF sol from CF and non-CF NEC. In these experiments, the dose of DX-890 used greatly exceeded the stimulatory dose of NE and was sufficient to negate NE activity in samples (data not shown). CF sputum sol contains many factors capable of stimulating IL-8 secretion such as other proteases and bacterial components and it is not therefore surprising that DX-890 only partially reduces IL-8 secretion. It is more difficult to speculate as to why DX-890 did not completely prevent NE-induced IL-8 secretion. In other studies that carried out similar experiments using different NE inhibitors [30–32], NE and the inhibitor were co-incubated before application to the cells. In this study DX-890 was added to cells prior to stimulation with NE. This pre-incubation of DX-890 may have caused inactivation of DX-890 via interaction with cellular secretions, cell membranes or culture media components, reducing the concentration of DX-890 available for

**Fig. 3.** IL-8 release from epithelial cells in response to NE and CF sputum sol. IL-8 release from healthy and CF nasal epithelial cells in response to NE 50 nM and CF sol 1:100. Data are median and interquartile range and are duplicate values for n = 6 experiments. *Wilcoxon signed rank test p ≤ 0.05 compared to non-treated (normal cells). †Wilcoxon signed rank test p ≤ 0.05 compared to non-treated (CF cells).

**Fig. 4.** Effect of DX-890 on IL-8 secretion induced by NE and CF sol. (A) DX-890 (200 nM) reduces IL-8 release induced by NE 50 nM from healthy and CF nasal epithelial cells. (B) DX-890 (500 nM) reduces IL-8 release induced by CF sol 1:100 in healthy and CF nasal epithelial cells. Data are presented as median and interquartile range and are duplicate values for n = 6 experiments. *Wilcoxon signed rank test p ≤ 0.05.
subsequent inhibition of NE activity. It is possible that if NE and DX-890 were pre-incubated prior to stimulation of cells, the prevention of IL-8 secretion would be greater.

DX-890 has already demonstrated tolerability in rodents [23] and in a Phase I clinical trial in humans [33]. In conclusion, this study showed that DX-890 inhibits NE activity in neutrophils and reduces neutrophil transmigration through an epithelial cell monolayer. DX-890 also dampens secretion of IL-8 from primary nasal epithelial cells in response to stimulation with NE and CF sputum sol. These data support the further investigation of DX-890 for the treatment of airway diseases involving neutrophilic inflammation, such as cystic fibrosis.

Acknowledgements

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References