REVIEW

Inhibition of airway proteases in cystic fibrosis lung disease

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ABSTRACT: Progressive lung disease determines the morbidity and mortality of cystic fibrosis (CF) patients.

CF lung disease is characterised by endobronchial inflammation sustained by bacterial infections and an ongoing accumulation of airway neutrophils. Activated or necrotic neutrophils liberate proteases that cause damage to structural, cellular and soluble components of the pulmonary microenvironment.

Among various proteases released by airway cells, elastase is considered to play the major role in CF lung disease. Based on this concept, several therapeutic approaches have been developed to inhibit free elastolytic activity, including small synthetic chemical compounds, semi-synthetic inhibitors and natural inhibitors of free elastase.

The present review summarises and discusses the pathophysiological rationales, methodological requirements and clinical implications of inhibition of airway proteases in cystic fibrosis lung disease.

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STATEMENT OF INTEREST
None declared.

espite various therapeutic advances, >90% of cystic fibrosis (CF) patients still die due to respiratory failure. The airways of the majority of patients with CF are chronically infected with bacterial pathogens, especially *Pseudomonas aeruginosa* [1]. As a consequence, large numbers of neutrophils accumulate in CF airways, resulting in sustained endobronchial inflammation [2]. The duration and intensity of the airway inflammation, however, appear to be much more pronounced than required for the removal of the offending pathogens. This excessive inflammatory response is probably related to the disease-causing mutations in the CF transmembrane conductance regulator (CFTR) gene. However, the exact pathogenic mechanisms involved have not been completely elucidated. Increased mucus viscosity, impaired local host defence, and innate and adaptive immune mechanisms seem to be involved. Other mechanisms, such as gain of function resulting from mutated CFTR causing an enhanced endoplasmic reticulum stress response and pro-inflammatory activity may also play a role [3]. As a result, CF airways exhibit sustained inflammation that causes harm to the lungs, leading to irreversible collateral damage caused by chronic airway destruction and remodelling.

Despite several lines of pathophysiological evidence to support the concept of an overwhelming pro-inflammatory response in CF airways [2], only a few clinical studies have successfully established anti-inflammatory strategies in patients with CF lung disease, such as the application of ibuprofen [4–6] and recombinant human DNase [7]. Other studies have demonstrated the ineffectiveness or the occurrence of adverse side-effects of approaches involving inhaled steroids [8] and a leukotriene (LT)B$_4$ antagonist [9]. A major difficulty in evaluating anti-inflammatory treatments in clinical trials remains selection of appropriate outcome measures (table 1). Possible outcome parameters include lung function (spirometry), bronchoalveolar lavage (BAL) fluid (BALF) analysis, sputum analysis, sputum microbiology, chest computed tomography (CT), infant and toddler lung function such as measured *via* lung clearance index, frequency of pulmonary exacerbations, quality-of-life assessments, aerosol deposition and clinical symptom scores. The various outcome measures should be considered according to the phase of the clinical trial, as depicted in table 1. The references given in table 1 indicate studies in which the respective outcome measures have been used.

Traditionally used pulmonary outcomes, such as forced expiratory volume in one second (FEV1), reflect major changes in pulmonary function but are considered less appropriate for monitoring more subtle changes. Therefore, novel outcome parameters, such as CT scan results, may become increasingly important but still require more extensive validation. Other outcome measures, such as exacerbation rate or quality of life, might additionally help in the monitoring of CF patients' lung disease, and should be considered when performing clinical studies. Inflammatory outcome markers in blood, sputum or urine have been shown to be especially helpful in the assessment of antibiotic treatment [20–22].

Therefore, it remains an ongoing challenge for clinical researchers to define and carefully evaluate outcome measures that permit the monitoring of airway inflammation and investigation of the effect of anti-inflammatory interventions in patients with CF lung disease. For a more extensive discussion concerning outcome measures in clinical trials of CF, see a previous review [23].

PROTEASE–ANTIPROTEASE IMBALANCE IN CF AIRWAYS

Previous studies have demonstrated that CF airway fluids contain large amounts of pro-inflammatory mediators, especially interleukin (IL)-8, which attract large numbers of neutrophils into the airways [24]. This should provide an initial line of immune defence against bacterial pathogens. However, the recruited airway neutrophils seem unable to clear the local pathogens, but instead accumulate, undergo secondary necrosis and liberate their intracellular components, such as serine proteases. Serine proteases are physiologically important in both intracellular [25, 26] and peri-/extracellular

| TABLE 1 | Outcome measures of clinical trials in cystic fibrosis lung disease | | | |

	Phase[#]			[Ref.][¶]
	I	II	III	
Quality of life		++	+++	[10]
Frequency of pulmonary exacerbations		++	+++	[11]
Infant and toddler lung function, including LCI		+++		[12]
Chest CT		+++	+++	[13]
Aerosol deposition	+++	++		[14]
Bronchoalveolar lavage fluid analysis	+++	++		[15]
Sputum analysis	++	++		[16]
Symptom score	+++	+++	+++	[17]
Lung function (spirometry)	+++	+++	+++	[18]
Sputum microbiology	+++	+++	+++	[19]

LCI: lung clearance index; CT: computed tomography. ++: moderate; +++: optimal. [#]: scale (++ or +++) indicates degree to which outcome measure is suited to clinical trial phase; [¶]: study in which the outcome measure has been implemented.

killing, as recently demonstrated by the identification of neutrophil extracellular traps that capture bacteria and facilitate a close interaction of the pathogen with released proteases, finally resulting in bacterial killing [27–30].

In chronic disease conditions, the amount of proteases exceeds the neutralising capacity of antiproteases. The protease–antiprotease imbalance hypothesis of chronic lung disease suggests that protease levels in the lower respiratory tract are a major determinant of pulmonary damage and emphysema [31]. This hypothesis is corroborated by the finding that disease severity in patients with chronic bronchitis and pulmonary emphysema is positively related to pulmonary protease load and inversely related to the antiprotease shield [32].

In CF patients, several studies have found a correlation between proteases on the one hand and tissue damage and disease severity on the other [33–36]. The local protease–antiprotease imbalance in CF airway fluids has been demonstrated to be established by the age of 1 yr, and is probably sustained chronically thereafter [33]. The protease–antiprotease imbalance in CF airways is probably not due to an intrinsic CFTR-associated lack of antiproteases since CF patients exhibit normal serum and bronchoalveolar levels of antiproteases [33].

Among the variety of proteases released by disrupted neutrophils, human leukocyte elastase (HLE) is considered the major factor causing harm to the surrounding pulmonary microenvironment [37]. The scanty data available demonstrate that the vast majority of the elastinolytic activity in CF sputum can be attributed to the neutrophil-derived HLE, when assessed in terms of micrograms of elastin degraded per millilitre of solution per minute. Accordingly, HLE was responsible for 90% of the activity, protease 3 for 7% and soluble mediators released from macrophages, along with *P. aeruginosa*-derived proteases, made up 3% [38]. Nevertheless, it is very difficult to compare the various proteolytic activities of different neutrophil-derived enzymes directly, since the relevance of the target structures and different microcompartments within the airspaces are unknown. Free HLE degrades several extracellular matrix components, such as elastin, collagen, proteoglycans, fibronectin and other proteins, thereby causing pulmonary tissue destruction [31, 39–43]. In addition, HLE is capable of inactivating the antiprotease tissue inhibitor of metalloproteases (TIMP) and elafin [44], which, in turn, amplify overall proteolytic activity *in situ*. For a more extensive discussion of HLE functions, reference should be made to previously published reviews [45–48].

With ongoing CF lung disease, a prolonged endobronchial protease activity (PEPA) is established. This term has been chosen since, in contrast to acute lung disease, in which a temporarily increased extracellular protease activity can also be observed, the protease–antiprotease imbalance in CF is sustained for an ongoing period of time, and thus is prolonged. PEPA has been documented very early in CF airways by means of BAL in children with CF diagnosed by neonatal screening and regular follow-up [15, 49]. These studies suggested a close relationship between inflammation and infection. However, the causative sequence between pulmonary infection and airway inflammation is still incompletely

understood. In particular, the longitudinal course of PEPA, as well as the effect of antibiotic or nonantibiotic treatments on PEPA, are yet to be characterised.

PEPA INACTIVATES SOLUBLE AND CELLULAR COMPONENTS OF THE PULMONARY IMMUNE SYSTEM

Apart from the issues that remain unclear, there is a broad consensus that PEPA causes harm to various structural, cellular and soluble components of the lung [50]. In the following sections, attention is drawn to the mechanisms by which PEPA inactivates the pulmonary host defence and thereby permits pathogens to colonise CF airways. In order to provide an overview of the proteins targeted by HLE activity, they have been divided into the soluble and cellular targets of the pulmonary immune system, and are summarised and discussed below.

Soluble immune targets

HLE has been found to affect a variety of soluble components of the pulmonary immune response (fig. 1a–d). In particular, the complement opsonic C3 fragment iC3b is cleaved on the surface of bacteria by HLE [51]. HLE has the capacity to cleave immunoglobulins, resulting in Fc and Fab fragments and leading to a decrease in both phagocytosis and killing of *P. aeruginosa* [52]. HLE has also been shown to degrade surfactant protein (SP)-A and -D, which impairs the innate host defence of the bronchoalveolar space [53–56]. HLE causes further changes in the host defence response seen in CF by cleaving and inactivating bacterial flagellin [57], and also by degradation and inactivation of annexin I, a calcium-dependent phospholipid-binding protein thought to have anti-inflammatory properties [58]. In addition, HLE seems to be a potent proenzyme activator and inducer of gene expression of matrix metalloprotease (MMP)-2 and cathepsins [59, 60]. The upregulation of MMP-2 activity may then lead to cleavage and inactivation of further key immune proteins, such as β-defensins and lactoferrin. HLE was found to be an activator of MMP-9 [61] and an inactivator of the MMP inhibitor TIMP-1 [44], resulting in an imbalance in MMP and TIMP. Recently, these findings have been confirmed at concentrations of HLE demonstrated in CF lung disease [62, 63]. The increased activation of MMP-9 in CF lung disease has many possible downstream inflammatory effects, including activation of latent transforming growth factor (TGF)-β [64] and the possible liberation of collagen fragments chemotactic for neutrophils [65]. HLE may also contribute to the perpetuation of lung inflammation by degrading complement and releasing C5a, a potent chemoattractant for neutrophils, and by increasing airway serous cell mucus production [66, 67].

Cellular targets

The main cellular targets of PEPA are shown in figure 1e–h. Phagocytosis of apoptotic neutrophils results in increased production of TGF-β, which, in turn, reduces the production of pro-inflammatory cytokines by neutrophils. Alveolar macrophages remove apoptotic neutrophils from the respiratory tract *via* phosphatidylserine receptor (PSR/CD36), thereby facilitating the resolution of inflammatory processes [68, 69]. Free HLE has been found to cleave PSRs from alveolar macrophages, which impairs the clearance of apoptotic cells [70]. As a result, the HLE-mediated impairment of phagocytosis results in

decreased production of TGF-β and amplification of pro-inflammatory responses [71]. Furthermore, HLE was found to induce IL-8 production by bronchial epithelial cells and LTB_4 expression in macrophages [72, 73]. The induction of IL-8 expression was found to be mediated *via* an IL-1 receptor-associated kinase 1/myeloid differentiation factor (MyD)88/nuclear factor-γ-dependent pathway, suggesting the involvement of Toll-like receptors (TLRs) [72]. Moreover, HLE cleaves complement receptor (CR)1 [51, 74], tumour necrosis factor receptor (TNFR)II [75], CD43 [76], CD16 (low-affinity immunoglobulin G receptor (FcγRIII)) [76] and, as demonstrated recently, the high-affinity IL-8 receptor CXC chemokine receptor (CXCR) 1 [77] on the neutrophil surface. By means of these mechanisms, HLE disables bacterial killing (CXCR1), phagocytosis (CR1 and CD16), adhesion (TNFRII) and spreading/aggregation (CD43) of neutrophils. With respect to adaptive immunity, HLE cleaves CD4 and CD8 on T-cells, which reduces the cytotoxicity and cytokine production by T-cells [78]. HLE was also found to cleave CD14 on fibroblasts and monocytes, resulting in decreased responsiveness to lipopolysaccharide [79, 80].

Taken together, unopposed HLE is able to undermine several key components of the innate and adaptive pulmonary immune response, which, in combination, results in impairment of pulmonary host defence in CF airways. In particular, impairment of the complement system [51, 74] and IL-8/CXCR1-mediated neutrophil functions [77] may be of major relevance since both mechanisms were found to be operative *in vitro*, as well as in humans with CF lung disease *in vivo*. BERGER *et al.* [74] showed that neutrophils upregulate both CR1 and CR3 upon activation with *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP). BALF neutrophils isolated from CF patients showed increased CR3 expression, similar to f-MLP-stimulated blood cells, but CR1 was decreased on BALF neutrophils compared to the f-MLP-stimulated blood cells. The loss of CR1 on CF BALF cells could be reproduced by treating isolated neutrophils with HLE, whereas CR3 was resistant to HLE. In a subsequent study, the same group demonstrated that the complement fragment iC3b on the surface of opsonised bacteria is cleaved by proteolytic activity and that this mechanism is operative *ex vivo* [51]. Taken together, the loss of opsonins on bacteria, as well as cleavage of CR1 on airway neutrophils, results in a local pulmonary opsonin–receptor mismatch that impairs phagocytosis of bacterial pathogens in CF lung disease [81].

It was recently found that, besides the elastase-mediated impairment of the complement system, IL-8-mediated neutrophil functions are also disabled in CF lung disease [77]. IL-8 mediates its functions *via* two receptors, CXCR1 and CXCR2 [82]. It was demonstrated that CXCR1 but not CXCR2 conveys oxidative and nonoxidative antibacterial neutrophil functions [77]. However, CXCR1 is cleaved in CF airways, which impairs the stimulating effect of IL-8 on bacterial killing by airway neutrophils. Soluble cleaved CXCR1 fragments were further found to stimulate IL-8 production in bronchial epithelial cells, mediated *via* a TLR2/MyD88 pathway. Inhibition of proteases by inhalation of α_1-antitrypsin (α_1-AT) *in vivo* largely prevented CXCR1 and CR1 cleavage and improved bacterial clearance in CF patients *in vivo*.
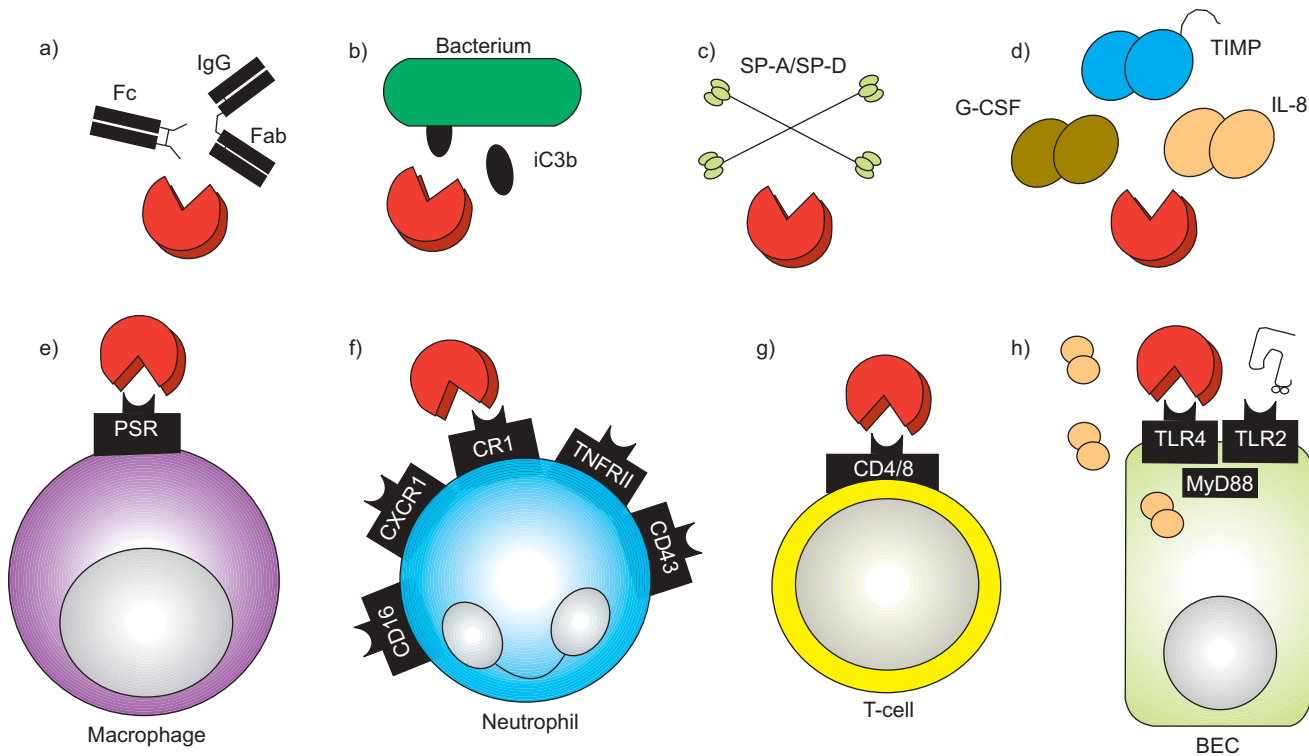
FIGURE 1. Effects of free neutrophil elastase (red) on: a–d) soluble; and e–h) cellular components of the pulmonary immune system. a) Elastase cleaves immunoglobulin (Ig)G, resulting in shed Fc and Fab fragments. It: b) cleaves iC3b on opsonised bacteria; and c) degrades surfactant proteins (SP)-A and -D, thereby impairing the innate host defence in the bronchoalveolar space. d) Elastase processes granulocyte colony-stimulating factor (G-CSF), tissue inhibitor of metalloproteinase (TIMP) and interleukin (IL)-8. These events lead to an inhibition of growth processes (G-CSF), increased activity of matrix metallproteases and a more active form of IL-8 as chemoattractant on neutrophils. e) Elastase cleaves phosphatidylserine receptors (PSRs) from alveolar macrophages, thereby impairing the clearance of apoptotic cells from the bronchoalveolar space. f) It cleaves complement receptor (CR)1, CD16, tumour necrosis factor receptor (TNFR)II, CXC chemokine receptor (CXCR)1 and CD43 on the neutrophil surface, thereby disabling the bacterial phagocytosis (CR1, CD16), killing capacity (CXCR1), adhesion (TNFRII) and aggregation (CD43) of neutrophils. g) Elastase further cleaves CD4 and CD8 on T-cells, which reduces cytotoxicity and cytokine production. h) In bronchial epithelial cells (BECs), elastase, as well as cleaved soluble CXCR1 fragments, induce IL-8 production mediated *via* Toll-like receptor (TLR) 2/4 and the common adapter protein, myeloid differentiation factor 88 (MyD88), thereby amplifying the pro-inflammatory neutrophil influx into cystic fibrosis airways.

When viewed in combination, the cleavage of CR1 and iC3b, as well as CXCR1, impairs both complement-mediated phagocytosis and the direct antibacterial functions of neutrophils, and may act synergistically to disarm the innate immune response in CF airways *in vivo*.

ASSESSMENT OF PEPA

Since the protease–antiprotease system is complex and involves several interdependencies with nonprotease factors, the present review focuses primarily on HLE and its natural inhibitor α_1-AT. Most commonly, HLE is measured using a chromogenic activity assay that is standardised and sensitive [83]. The amount of α_1-AT present is determined by immunodetection, either by ELISA or *via* immunoprecipitation. α_1-AT can be inactivated by oxidation and binding to HLE. Therefore, in addition to the amount of α_1-AT that is bound to HLE in complexes, its functional capacity to neutralise HLE can also be assessed. Since preanalytical issues, such as sample type, sample preparation and further processing, are critical to the accurate assessment of PEPA, they are addressed in the following sections. In particular, the use of BAL and sputum are compared, and the shortcomings and pitfalls of each method are discussed below.

Bronchoalveolar lavage

Bronchoscopy with BAL can be performed in patients of all age groups (infants and children requiring general anaesthesia or deep sedation), and localised areas within the lungs can be precisely targeted [84]. Depending upon the volume of lavage fluid instilled, either more proximal (*e.g.* 1 mL·kg body weight^{-1}) or more distal areas (*e.g.* 2–4 mL·kg body weight^{-1}) are reached. However, it is an invasive procedure. The rate of adverse events, mostly fever or localised infiltration, might increase with the volumes instilled and the severity of the underlying lung disease. A further consideration when assessing the overall proteolytic condition of the airways of CF patients is that CF lung disease is typically more pronounced in the bronchi and bronchioles, whereas BAL samples both bronchial and alveolar compartments.

The BALF recovered should be freed of mucus by filtration, followed by the separation of cells from a cell-free supernatant by centrifugation. The supernatant is routinely used for assay of HLE activity. The normal range of HLE activity expected in human volunteers (but not in children) has been determined (discussed further later). None or only minor free elastolytic activity is normally detectable in healthy individuals, whereas anti-elastolytic reserve (α_1-AT) is physiologically present.

Induced or spontaneous sputum

By inhalation of hypertonic agents, such as 6% sodium chloride, sputum can be induced in almost every subject. The technique of sputum induction is safe and now widely used as a therapeutic and diagnostic option [16, 85, 86]; potential broncho-obstruction induced by the procedure may be alleviated by pretreatment with a bronchodilator. The recovery of the sputum, however, depends heavily upon the cooperation of the individual. The majority of young children aged <6 yrs do not reliably spit out sputum that is induced in their bronchial tree, but instead frequently swallow it. Therefore, this technique was considered to be reliably used only in those aged ⩾6 yrs. However, a recent study reported the successful application of sputum induction in CF children with a median age of 3 yrs [87]. Despite further studies being required for the assessment of practicability and safety in this young age group, the latter study suggested that sputum induction can also be applied in CF infants aged <6 yrs.

Nevertheless, several limitations should be considered when using sputum. First, induced sputum is thought to be more variable than BALF, especially when using serial sampling, since serial specimens may not sample the same region within the lung. This is of particular relevance in CF lung disease since CF inflammation seems to inhomogeneously affect different pulmonary sites [88]. Secondly, sputum is a complex network of material expelled from the lungs, and, as such, is very difficult to separate into its original components without influencing them. Usually sputum plugs are selected and dissolved either with the help of chemicals (dithiothreitol, DNase and other enzymes) or mechanically. When applying this technique, it is possible that intracellular granule contents from rupturing cells or cells trapped in the nets of plugs are liberated. Furthermore, compounds may be bound to mucus or other surfaces exposed during processing of the sample and thus lost to analysis. Thus, preanalytical issues are critical to the processing of sputum and should be evaluated and optimised prior to clinical studies.

Normal values for induced sputum cellular counts and composition have been established in both adults and children [89, 90]. In CF patients, a limited number of studies have been performed involving a broad range of CF subjects (discussed further later). In particular, the assessment of induced sputum has proven helpful in studying the effect of antibiotic treatment [20–22, 86]. The value of induced sputum in studying more subtle effects of anti-inflammatory therapies remains unclear as no study of anti-inflammatory drugs published to date using outcome measures obtained from induced sputum has convincingly demonstrated an effect. Recently, MAYER-HAMBLETT *et al.* [91] reported the findings of a meta-analysis involving 269 CF patients from four US multicentric studies on the association between expectorated sputum biomarkers and lung function (FEV1). Among several biomarkers studied, HLE levels correlated with FEV1 both cross-sectionally and longitudinally, whereas IL-8 and other putative biomarkers showed no association with lung function in these patients, suggesting HLE may be useful as a biomarker in CF lung disease. In conclusion, both BALF and sputum are useful for the study of proteolytic activity/PEPA in CF lung disease, but the differences between the two techniques, especially with

regard to the patient's age, sampling region and preanalytical issues, should be considered.

CLINICAL SITUATIONS WITH DIFFERING PEPA

CF lung disease is variable and compartmentalised in space and time, ranging from early lung disease without clinical symptoms to progressive lung disease with destruction of lung tissue and loss of pulmonary function. Given the broad range of CF lung disease, appropriate patient stratification is a prerequisite for clinical studies [23]. This section describes different stages of CF lung disease, with their typical scenarios and relevant implications of inhibition of HLE.

Early lung disease with no established chronic infection and inflammation (clinically stable)

This group comprises young patients, who usually exhibit a lung function within the normal range and show no clinical symptoms outside their exacerbations. BALF studies revealed that even CF infants aged 1 yr exhibit increased neutrophil counts and HLE activity, depending upon the bacterial pathogens in their BALF [92, 93]. Owing to the technical difficulties of inducing sputum in this population [86], BAL is required to study these patients over time. No long-term data are available regarding these patients with respect to their HLE status. Given the potential anti-inflammatory benefit of inhaled HLE inhibitors [94], the examination of such a population is of pivotal interest in examining whether or not HLE-directed therapeutics are able to prevent the establishment of persistent airway inflammation, improve clearance of pathogens within the airways, and, finally, decrease progression to chronic obstructive airways disease.

Later lung disease with localised destruction and significant PEPA (clinically stable)

The lung function of these subjects may well be within the normal range, although some compromise is likely, and chest CT scans can clearly depict the extent and sites of localised pulmonary destruction [95, 96]. With inappropriate resolution of acute exacerbation, localised destructive and chronic lung disease is established in certain areas of the lung. Induced sputum obtained from subjects with this condition is likely to represent a mixture of healthy areas within the lung and areas of significant proteolytic activity and destruction. The later sputum fractions more precisely represent BALF composition [97]. The central question is whether treatment with inhaled protease inhibitors is able to overcome the excessive proteolysis in the area of localised destruction without adversely affecting other pulmonary areas in which antiproteolytic conditions pre-exist. In this context, the effect of inhaled α_1-AT in conserving the integrity of the lungs over time might be of major importance in the microenvironment of localised destruction. The use of mixed induced sputum (with collection of material from the whole lung) is useful in the determination of whether or not overall control of elastolytic activity can be achieved. The analysis of sequential sputum fractions, conversely, may be helpful for resolution of the spatial distribution within the lungs [97]. However, this approach has not yet been used in studies of inhaled protease inhibitors and remains to be established in future investigations.

Late lung disease with general destruction and inflammatory activity (clinically stable)

Patients with multilocalised lesions usually show a surplus of HLE in their sputum. Such patients typically exhibit chronic bacterial infection and reduced lung function. The degree of free HLE activity depends upon the overall severity of the lung disease. Previous studies of inhaled α_1-AT in CF patients with progressive lung disease [95, 98] demonstrated that the HLE can be neutralised totally [98] or partially [95] depending upon the dose inhaled, the deposition mode and the method of obtaining airway fluids (BAL or sputum). Antiprotease use may be reasonable in this patient population in order to prevent further progression of pulmonary destruction.

Acute exacerbation

With acute disease exacerbation, protease activity is thought to be increased in CF airways. However, owing to difficulties in defining exacerbations precisely in CF, as well as study logistics, well-designed clinical studies addressing the role of proteases in CF exacerbations are still lacking [11, 99]. Thus the following critical questions remain unanswered. 1) Does anti-HLE treatment reduce the duration of exacerbations, extent of damage, post-exacerbation sputum production and airway hyperreactivity? 2) Has co-treatment with antibiotics an impact upon the course of exacerbations? 3) Do antibiotics have an additional effect on protease activity or soluble/cellular targets of PEPA in CF airways, as demonstrated for colistin [100]? Potential harmful effects, such as the expansion of bacterial growth or enhancement of symptoms, should be observed very closely in this context.

INHIBITION OF AIRWAY NEUTROPHIL ELASTASE

In order to inhibit excess HLE activity, there are currently three classes of inhibitor available: 1) small synthetic chemical compounds; 2) semi-synthetic inhibitors obtained by modification of their natural counterparts; and 3) natural inhibitors of HLE (table 2).

Synthetic inhibitors of neutrophil elastase

Since there is increasing evidence that HLE-mediated tissue damage often occurs at the tight interface between neutrophil and substrate, α_1-AT might have difficulty in accessing this

area due to its large size (52 kDa) and negative charge [101]. This and other pharmacological and economic issues suggested the development of synthetic and semi-synthetic serine protease inhibitors (serpins), as summarised later. A large number of synthetic chemical compounds have been synthesised and partially tested for inhibition of HLE [102]. In theory, these low-molecular weight inhibitors might be superior to α_1-AT on the basis of their size, resulting in better accessibility to narrow proteolytic sites within the CF lung [103–105]. However, due to unexpected toxicity or further relevant issues, none of the synthetic inhibitors have yet been introduced into clinical practice. Both peptide (such as ICI 200355; ICI Americas, Wilmington, DE, USA) and nonpeptide HLE inhibitors (ONO-5046; Ono Pharmaceutical Co. Ltd, Osaka, Japan) have been developed. Sivelestat, for instance, (ONO-5046/Elaspol) is a reversible selective inhibitor of HLE, which was initially launched in Japan. A phase 2 clinical study in patients with acute lung injury was suspended due to unfavourable effects of the compound. Midesteine (MR-889; Medea Research, Milan, Italy) represents another reversible low-affinity inhibitor of HLE, which was found to reduce mucus viscosity due to its interaction with thiol groups. It appears safe and is undergoing clinical evaluation for chronic obstructive lung disease. Specifically for CF, DMP-777 has been tested in phase 2 studies; however, its development was discontinued by the company. All other developments are in much earlier phases [106], and no results have yet been published.

Semi-synthetic inhibitors

Semi-synthetic inhibitors are usually obtained by chemical modification of natural human protease inhibitors. For example, engineered protein inhibitor of human neutrophil elastase (EPI-hNE-4) is derived from the Kunitz-type domain of inter-α-inhibitor [107, 108], which is a natural inhibitor of HLE normally present in plasma. EPI-hNE-4 is a 56-amino-acid-long fragment with a very low equilibrium dissociation constant of 4 pM for HLE, and has the advantage that it is derived from humans and is resistant against proteolysis and oxidation [107]. Studies in animals as well as using sputum from CF children, revealed successful inhibition of HLE and a decrease in neutrophil migration [109]. EPI-hNE-4 is currently being tested in phase 2 studies in patients with CF. SUPG-032 represents a nonglycosylated recombinant modified human α_1-antichymotrypsin in which six reactive-site amino acids have been replaced by those of α_1-AT, and is active against HLE, cathepsin G and protease 3. The hybrid compound also inhibits superoxide generation by neutrophils. Unfortunately, its further development was suspended. Finally, mention should be made of a fusion protein containing both α_1-AT and secretory leukocyte protease inhibitor (SLPI) activity, the current development status of which is unknown.

Natural inhibitors

Natural inhibitors of HLE comprise α_1-AT, monocyte/neutrophil elastase inhibitor (MNEI) and serpins, such as SLPI and elafin/pre-elafin. Studies with α_1-AT are addressed separately in the section below. SLPI, a 12-kDa highly-cationic hydrophobic nonglycosylated single-chain protein, is produced in the central airways by serous glandular cells and in the lower respiratory tract by Clara cells and goblet cells, and appears to

TABLE 2	Classes of inhibitor of human neutrophil elastase (hNE)	
Synthetic	**Semi-synthetic#**	**Natural**
Sivelestat	EPI-hNE-4 (inter-α-inhibitor)	α_1-antitrypsin
Midesteine	SUPG-032 (α_1-antichymotrypsin)	SLPI
	SLAPI (α_1-antitrypsin and SLPI)	Pre-elafin
		Monocyte/neutrophil elastase inhibitor

EPI: engineered protein inhibitor; SLAPI: α_1-antitrypsin–secretory leukocyte protease inhibitor (SLPI) fusion protein. #: obtained by modification of the natural mother compounds shown in parentheses.

be an efficient inhibitor of HLE activity in the airways. SLPI and α_1-AT are regarded as the major defence proteins against excessive HLE, providing 10–50% of the total HLE-inhibitory capacity [110, 111]. Since the reactivity of SLPI differs from that of α_1-AT, both antiproteases may act complementarily *in vivo*. MCELVANEY *et al.* [112] studied the effect of 1 week's inhalation of recombinant human SLPI (100 mg twice daily) in 16 adult patients with CF. As assessed by BAL, the number of neutrophils and levels of IL-8 were reduced and HLE activity was decreased for >12 h. However, when only 50 mg were used for 2 weeks, no such effects could be observed, clearly showing dose dependency. Elafin, a 6-kDa peptide similar to SLPI, is a specific elastase inhibitor, which is suggested to contribute up to 20% of the anti-HLE capacity of the lung *in vivo* [113, 114]. Both SLPI and elafin are upregulated by pro-inflammatory cytokines in airway epithelial cells [115, 116], but may act anti-inflammatorily since both were found to inhibit nuclear factor-κB activity and IL-8 and monocyte chemoat-tractant protein-1 production [117, 118]. To the best of the present authors' knowledge, pre-elafin and MNEI have not as yet been studied in patients with CF. However, animal data show a substantial reduction in *P. aeruginosa* counts in a mouse model of chronic infection after treatment with MNEI for up to 7 days [119]. Further clinical studies using natural inhibitors of HLE need to be conducted in CF patients in order to precisely evaluate their potency, beneficial and harmful effects, and usefulness in clinical settings.

Besides antiproteases, antibiotics were found to differentially affect proteases. Macrolide antibiotics were found to show HLE-inhibitory activity, whereas colistin increased HLE activity *in vitro* [100, 120].

INHIBITION OF FREE ELASTASE BY INHALED α_1-AT IN PATIENTS WITH CF

α_1-AT is a 52-kDa glycoprotein that is secreted mainly by hepatocytes, alveolar macrophages and neutrophils and acts as a potent serine protease inhibitor [121]. The interaction of α_1-AT with HLE results in a rapid and irreversible inactivation of the protease. Serum concentrations in healthy individuals range 20–60 μM. α_1-AT, released by the liver, diffuses into the lung. Although levels in the lung are lower (~2–7 μM), α_1-AT is considered to contribute >90% of the antiproteolytic capacity present in the pulmonary compartment [122]. When considering α_1-AT as a therapeutic agent, the source may be human serum or recombinant protein expressed in other systems, such as micro-organisms or transgenic animals [123]. A commercial α_1-AT preparation is available from pooled human plasma (Prolastin; Talecris Biotherapeutics, Research Triangle Park, NC, USA). Prolastin is well tolerated and effective *in vivo*, but the purity of the preparation is only ~60% [124]. Novel α_1-AT replacement preparations, such as Zemaira or Aralast, have shown equivalency with Prolastin in augmenting α_1-AT serum and alveolar epithelial levels and provide greater purity than conventional preparations (Zemaira>Aralast>Prolastin) [124–126]. However, clinical data on the use of these preparations in CF patients are unavailable to date.

Intravenous α_1-AT application was initially used in individuals with α_1-AT deficiency, has been reported to be safe and well tolerated [127], and is currently recommended for individuals with severe hereditary (PiZZ/null) α_1-AT deficiency [128, 129]. However, in CF airways, the amount of free proteases present is very high (mostly >10 μM and sometimes even up to 100 μM) and the affected sites in CF lungs may be barely accessible to α_1-AT. α_1-AT administered *via i.v.* was considered for treating CF patients. However, preliminary studies showed that large doses and repetitive administration would be required to achieve beneficial long-term effects [130]. Accordingly, aerosolised α_1-AT has gained increasing attention since: 1) the direct site of disease is targeted, with higher pulmonary concentrations achieved; 2) systemic side-effects are less probable; 3) smaller amounts of α_1-AT are required; and 4) no *i.v.* access is required.

Transgenic technology enables the high-volume production of active recombinant proteins, which is cost-efficient compared with purified human serum α_1-AT. PPL Therapeutics (Roslin, UK) have developed transgenic sheep that produce in their milk human α_1-AT that is almost identical to human-derived α_1-AT, with the exception of some of the side-chain sugars [131]. Since recombinant α_1-AT has a short half-life, *i.v.* administration is precluded but aerosol administration is feasible [132]. A pilot study in CF patients using recombinant α_1-AT derived from transgenic sheep showed no allergic effects up to doses of 500 mg recombinant α_1-AT·day^{-1} [133]. Nevertheless, use of recombinant α_1-AT may have the problem of antibody responses to traces of inhaled nonhuman proteins and may lead to anaphylactic reactions [134].

Tables 3 and 4 summarise published studies using BAL (table 3) and sputum (table 4) to assess the effect of inhaled α_1-AT in patients with CF lung disease.

A phase 1 study evaluated the biochemical effect of α_1-AT inhalation in CF patients and has provided important insight into the potential effects of aerosolised α_1-AT in CF lung disease [98]. Twelve adult subjects with CF inhaled 100–200 mg (1.5–3.0 mg·kg body weight^{-1}) α_1-AT twice daily for 1 week. The estimated efficacy of the nebuliser to deliver α_1-AT into the lungs was ~10–20%, resulting in pulmonary deposition of ~10–40 mg. Before inhalation, all subjects had their BALF HLE activity measured. If the epithelial lining fluid concentration of α_1-AT was increased to a level of >8 μM, all free HLE was blocked. Interestingly, post-treatment BALF did not inhibit neutrophil killing of *P. aeruginosa in vitro*, as was found using pretreatment BALF, suggesting that HLE effi-ciently impairs antibacterial neutrophil functions *in vivo*. This study in CF patients with an FEV1 of >60% of the predicted value clearly demonstrated free HLE in the alveolar compart-ment, which was neutralised dose-dependently and reached free anti-HLE capacity in some cases. In another smaller study, treatment with α_1-AT in the lower dose range led to reduced levels of total protein, numbers and amounts of proteins with a molecular weight of <20 kDa, and degradation products of SP-A [137]. In the latter study, no effect of α_1-AT on surfactant convertase, an alveolar enzyme hypothesised to be inhibited by serine protease inhibitors, was noted [138]. In healthy adults, the α_1-AT half-life is 69.2 h and the half-life of anti-elastolytic capacity is 53.2 h, suggesting that once daily dosing may be appropriate. However, the functional half-life under specific conditions, such as CF, may be different and remains to be determined.

In the only randomised placebo-controlled CF trial using induced sputum to assess the effect of different doses of inhaled α_1-AT (125, 250 and 500 mg), levels of myeloperoxidase, a surrogate for neutrophil activity, were reduced after α_1-AT treatment, but only in the group receiving the highest dosage of α_1-AT (500 mg) [133]. Similarly, levels of taurine, also a surrogate for neutrophils, were marginally reduced [139]. In line with this finding, it was found that the number of neutrophils, as well as pro-inflammatory cytokine levels, was reduced after α_1-AT inhalation [94]. Interestingly, in these studies, the HLE activity of sputum was not [133, 139], or was only partially [94], reduced. Another study involving 22 adult CF patients was published in abstract form only by BERGER et al. [136], showing a decrease in HLE levels but no changes in IL-8 levels and numbers of neutrophils upon aerosolised α_1-AT treatment. What are the reasons for this discrepancy between sputum and BAL α_1-AT studies? First, sputum and BAL sample different areas of the lungs. The more peripheral region sampled by BAL is expected to exhibit lower inflammatory activity and bacterial load [84]. CF lung disease is almost completely excluded from the alveolar space, and, even in severely damaged explanted lungs obtained after lung transplantation, only few inflammatory cells are observed in the alveoli [140]. Secondly, during preparation of BALF supernatant, the mucus recovered by lavage, containing trapped leukocytes and other elements attached to it, is often removed during filtration. In contrast, during preparation of sputum, cellular debris and apoptotic leukocytes may be included in the analysis. Finally, the different methods used to obtain sputum supernatant should be considered. When a sol phase is prepared, undetermined intra- and extracellular components can be released into the supernatant. This cannot be readily compared to a cell-free supernatant prepared by mechanical, enzymatic or biochemical means.

In an attempt to define the optimal regions within the lungs of CF patients for delivery of α_1-AT, preferential peripheral (alveolar) deposition was compared to more central (bronchial) deposition [94]. No differences in any outcome parameter were found between the two deposition methods. In combining both of these groups, the previously mentioned study showed that inhalation of α_1-AT increased α_1-AT levels and decreased levels of HLE activity, percentages of neutrophils, levels of pro-inflammatory cytokines and colony counts of P. aeruginosa. Lung function was not affected by α_1-AT inhalation, which may be due to the relatively short treatment period. Drawbacks of antiprotease therapy are that aerosolised antiproteases, as with most other drugs, can barely be deposited in the poorly ventilated areas of the lung that need treatment most. In addition, the pharmacokinetic and dynamic characteristics of a complex environment such as the CF lung are hard to establish. Thus, the challenge remains to efficiently deposit α_1-AT at the site of CF lung disease. It is, therefore, critical that the delivery system is optimised for maximal drug delivery over a brief period of time with minimal aerosol loss. This can be achieved by combining an efficient nebuliser, which generates the aerosol, with a device that guarantees an optimised breathing pattern. The AKITA (electronically regulated device for the controlled inhalation of therapeutic aerosols) device, programmed for the individual breathing pattern using a smart card, represents such a system. Using

TABLE 3 Bronchoalveolar lavage (BAL) studies of inhaled aerolised α_1-antitrypsin[#] (α_1-AT) in patients with cystic fibrosis (CF) and healthy volunteers

	Subjects n	Nebuliser	α_1-AT aerosol		Sample		Elastase µM		α_1-AT µM		[Ref.]
			Dose mg	DD to lungs[¶] mg (% dose)	Type	Time after last inhalation h	Before	After	Before	After	
Healthy	29	Pari LL/LC Plus	200	40 (20)	BALF, ELF	Kinetics	3.1±0.9	8.4±2.9	3.4±2.0	10.4±7.5 after 0.5 h	[135]
CF	12	Ultravent 10	1.5–3 mg·kg body weight⁻¹ (~90–180 mg) b.i.d. for 1 week	18–36 (10–20)	BALF, ELF	4	8.2±5.9	0 if >8 µM α_1-AT in ELF	4.4±0.5	8.8±2.9[+], 12.4±8.5[§]	[98]
CF	22	Pari LL	100, 200, 350 b.i.d. for 4 weeks	20, 40, 70 (20)	BALF, ELF	12	5.1±1.6	0 in all but one patient; inhibitory capacity 2.1±2.3	3.8±0.7	13.3±1.8	[136]
CF	8	Pari LC Plus	100 b.i.d. for 8 weeks	20 (20)	BALF, ELF	12	0.22±0.10[f]	0.06±0.03[##]	3.6±1.2%[¶+]	9.8±2.8%[¶+]	[137, 138]

Pari LL and Pari LC Plus are manufactured by Pari, Starnberg, Germany. Ultravent 10 is manufactured by Mallinckrodt Medical, St Louis, MO, USA. DD: delivered dose; BALF: BAL fluid; ELF: epithelial lining fluid. [#]: prolastin; [¶]: per inhalation; [+]: with lower dose; [§]: with higher dose; [f]: conversion from 1.6±0.7 U·mL⁻¹ given in original; [##]: conversion from 5.8±2.7 U·mL⁻¹ given in original; [¶+]: percentage of α_1-AT spot volume assessed from area and density of spot. 1 µM=52 µg·mL⁻¹ and 1 mg=0.019 µmol α_1-AT; 1 µM=30 µg·mL⁻¹ and 1 mg=0.033 µmol human leukocyte elastase (assuming all enzyme is active).

TABLE 4	Sputum studies of inhaled aerolised α_1-antitrypsin[#] (α_1-AT) in patients with cystic fibrosis (CF) and healthy volunteers									
	Subjects n	Nebuliser	α_1-AT aerosol		Sample		Elastase $\mu g \cdot mL^{-1}$		α_1-AT $\mu g \cdot mL^{-1}$		[Ref.]
			Dose in nebuliser mg	DD to lungs[¶] mg (% dose)	Type	Time after last inhalation h	Before	After	Before	After	
CF	52	AKITA with Pari LC Plus or LC Star	25 q.d.	25 (100)[+]	IS	16	30.2 ± 3.7	28.6 ± 4.8	17.1 ± 2.9	30.3 ± 5.5	[94]
CF	39	Pari LC Star	125–500 q.d. for 4 weeks	Placebo	S, SP	24	2.6 ± 3.8		NG	NG	[133]
				25 (20)			0.8 ± 1.7	p=0.21			
				50 (20)			2.8 ± 4.9	p=0.09			
				100 (20)			0.7 ± 1.9	p=0.31			
CF	17	Pari LC Plus	250 b.i.d. for 10 days	50 (20)	S, SP	12	$156 \pm 27^{§}$	150 ± 36^{f}	$124.8 \pm 20.8^{##}$	$421.2 \pm 98.8^{¶¶}$	[139]

AKITA is manufactured by Inamed, Gemünden, Germany. Pari LC Plus and Pari LC Star are manufactured by Pari, Starnberg, Germany. DD: delivered dose; IS: induced sputum; S: sputum; SP: sol phase; NG: not given; AKITA: electronically regulated device for the controlled inhalation of therapeutic aerosols. [#]: prolastin; [¶]: per inhalation; [+]: AKITA used so as to guarantee deposition of 25 mg α_1-AT in the lungs; [§]: conversion from 5.2 ± 0.9 µM given in original; [f]: conversion from 5.0 ± 1.2 µM given in original; [##]: conversion from 2.4 ± 0.4 µM given in original; [¶¶]: conversion from 8.1 ± 1.9 µM given in original. 1 µM=52 µg·mL^{-1} and 1 mg=0.019 µmol α_1-AT; 1 µM=30 µg·mL^{-1} and 1 mg=0.033 µmol human leukocyte elastase (assuming all enzyme is active).

this approach, ~85% of the aerosol can be delivered into the lungs [94]. This device is superior for the targeting and delivery of even relatively large amounts of aerosols into the lungs compared to previous systems. Automatic monitoring of compliance helps to define adherence to treatment.

IMPLICATIONS FOR CLINICAL TRIALS
Given the numerous targets of PEPA in CF lung disease, many potential outcome variables for clinical phase 1 and 2a studies of antiproteolytic treatment can be envisioned. These approaches will permit greater insight into the pathophysiology of CF lung disease. For future clinical studies of HLE inhibition in CF patients, however, it is important that the relevant target populations are clearly defined and stratified. Success in these trials will only be achieved by: 1) selection of representative study populations, 2) well-designed primary and secondary outcome parameters, 3) thoroughly characterised protocols for sample preparation, and 4) on-site training for compliance with these procedures. Finally, batch analysis of all variables for which storage is possible in an experienced central laboratory is essential. For phase 2b and 3 studies, outcome variables such as FEV1 and rate and time to exacerbation are appropriate; additional analysis of surrogate markers, e.g. cytokine analyses, in a study subcentre can be helpful in the interpretation of the results. Large studies of inhaled α_1-AT or other protease inhibitors are essential to the systemic evaluation of the effect of HLE inhibition as a therapeutic approach in CF lung disease.

SUMMARY
Inhibition of airway neutrophil elastase represents a causative approach to limiting the self-destructive process of neutrophil-derived protease activity in cystic fibrosis lung disease. Despite several attempts designed to achieve this goal, clinically useful concepts have only just started to evolve. α_1-Antitrypsin, the natural inhibitor of free elastase, has been employed in a few clinical studies and has yielded promising, but not yet convincing, results. Representative study populations, elaborated protocols for sample preparation, methodological accuracy and relevant end-points are warranted in order to further validate the usefulness of elastase inhibition in cystic fibrosis lung disease.

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