JPET Fast Forward. Published on April 20, 2006 as DOI: 10.1124/jpet.106.103440

JPET#103440

EPI-hNE4, a proteolysis-resistant inhibitor of human neutrophil elastase and potential anti-inflammatory drug for treating cystic fibrosis

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a/ Running title: EPI-hNE4, a neutrophil elastase inhibitor

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c/ pages: 29 + figs

tables: 0

figures: 5

references: 40

abstract: 233 words

introduction: 640 words

discussion: 1017 words

d/ Abbreviations

Abz, ortho–aminobenzoic acid ; α1-PI, α1-protease inhibitor ; cat G, cathepsin G ; EDDnp, N-(2,4-dinitrophenyl) ethylenediamine ; FRET, fluorescence resonance enegy transfer; HNE, human neutrophil elastase ; IL, interleukin ; MMP, matrix metalloprotease ; Pr3, protease 3 ; SLPI, secretory leucocyte protease inhibitor.

e/ Section

Inflammation and immunopharmacology

Abstract

EPI-hNE4 is a potent inhibitor of human neutrophil elastase derived from human inter-α-trypsin inhibitor and designed to control the excess proteolytic activity in the sputum of cystic fibrosis patients. We analyzed its resistance to the proteolysis it is likely to encounter at inflammatory sites *in vivo*. EPI-hNE4 resisted hydrolysis by neutrophil matrix metalloproteases and serine proteases that are released from activated neutrophils in inflammatory lung secretions, including MMP-8 and MMP-9, and the elastase-related protease 3 and cathepsin G. It also resisted degradation by epithelial lung cell MMP-7, but was broken down by the *Pseudomonas aeruginosa* metalloelastase pseudolysin, when used in a purified system, but not when this protease competed with equimolar amounts of neutrophil elastase. We also investigated the inhibitory properties of EPI-hNE4 at the surface of purified blood neutrophils and in the sputum of cystic fibrosis patients where neutrophil elastase is in both a soluble and a gel phase. The elastase at the neutrophil surface was fully inhibited by EPI-hNE4 and formed soluble complexes. The elastase in cystic fibrosis sputum supernatants was inhibited by stoichiometric amounts of EPI-hNE4, allowing titration of the protease. But the percentage of inhibition in whole sputum homogenates varied from 50 to 100% depending on the sample tested. EPI-hNE4 was rapidly cleaved by the digestive protease pepsin *in vitro.* EPI-hNE4 therefore appears to be an elastase inhibitor suitable for use in aerosols to treat patients with cystic fibrosis.

Introduction

The recurrent exacerbations of inflammation and infection that occur in cystic fibrosis (CF) lead to a decline in lung function, respiratory failure and ultimately to death. Many activated neutrophils enter the CF airway fluids where they participate in pathogen host defense, and contribute to chronic inflammation and lung destruction (Shapiro, 2002). Neutrophils recruited to inflammatory sites release reactive oxygen species and active serine proteases, one of which, neutrophil elastase (HNE), has focused the largest interest until now. Neutrophils also release the related serine proteases, protease 3 (Pr3) and cathepsin G (cat G), so that the overall proteolytic activity overwhelms the extracellular inhibitors present in the CF lung. The uninhibited HNE and related proteases degrade extracellular matrix molecules (Owen and Campbell, 1995). HNE is also involved in a variety of functions that prolong inflammatory responses, making it an attractive target for anti-inflammatory compounds. The HNE burden stimulates IL-8 production and lung permeability by degrading inter-endothelial and inter-epithelial junction proteins, thus fostering the continued recruitment of neutrophils and their transmigration (reviewed in (Moraes et al., 2003)). HNE is also a potent secretagogue, causing airways to become plugged in CF (Fahy et al., 1992). Lastly, it impairs the function of mucocilia (O'Riordan et al., 1997) and reduces the phagocytosis of apoptotic neutrophils by cleaving the phosphatidyl serine receptor on macrophages (Vandivier et al., 2002), so exacerbating the defective clearance of cells and mucus.

Several attempts have been made to use natural and recombinant protein inhibitors as antiproteases to treat the inflammation of CF lungs (McElvaney et al., 1991; McElvaney et al., 1992; Griese et al., 2001). Synthetic anti-elastase molecules such as acyl-enzyme inhibitors and transition-state inhibitors have also been tested (Ohbayashi, 2002). But no satisfactory results have yet been obtained, because of the toxicity and side effects of synthetic inhibitors or the unsuitability of the protein molecules for *in vivo* use (Konstan and

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Davis, 2002; Chughtai and O'Riordan, 2004). Nevertheless most of the inhibitors tested have beneficial effects that have encouraged further studies (Chughtai and O'Riordan, 2004).

EPI-hNE4 (DX-890) is a low molecular mass (6237 Da) inhibitor of HNE which was discovered and patented by Dyax Corp (Cambridge, Ma, USA). It was engineered using phage display (Roberts et al., 1992) and is expressed in *Pichia pastoris*. It is derived from the second Künitz domain of the light chain (bikunin) of the naturally occuring human protease inhibitor, inter-α-trypsin inhibitor (residues 285-340 ; Swiss-Prot accession number : PO2760), from which it differs at 5 positions (Becher et al., 2006). Replacement of the critical P1 Arg_{297} residue by an Ile residue confers a specificity for neutrophil elastase and makes it a potent HNE inhibitor with a Ki value of 5.45×10^{-12} M and a k_{on} value of 8×10^{6} $M^{-1}.s^{-1}$ (Delacourt et al., 2002). It protects the lungs of rats against HNE and CF sputum soluble fraction instilled into the trachea when given intra-tracheally or intravenously (Delacourt et al., 2002); it also protects the lungs of rats against repeated injuries (Honore et al., 2004).

We showed previously that EPI-hNE4 retains its inhibitory capacity upon nebulization (Grimbert et al., 2003). We have now assayed its resistance to those of proteases that it will encounter *in vivo* when given as an aerosol. These tests used serine proteases related to HNE (Pr3, and cat G) and matrix metalloproteases (MMP-8 and MMP-9) secreted by activated neutrophils or released from dead cells at inflammatory sites, MMP-7, the matrix metalloprotease with collagenolytic activity released from epithelial lung cells, and the bacterial metalloprotease pseudolysin secreted by *Pseudomonas aeruginosa*. We also tested the ability of EPI-hNE4 to inhibit membrane-bound HNE at the surface of purified neutrophils and the HNE in whole CF sputum because sputum elastase is present in both soluble and heterogenous gel phases. Finally, we determined the effect of pepsin on EPI-hNE4, since aerosolized drugs rapidly reach the digestive tract.

Methods

Materials

EPI-hNE4 was obtained from Debiopharm SA (Lausanne, Switzerland). Human neutrophil elastase (EC 3.4.21.37), protease 3 (EC 3.4.21.76), α1-PI and antichymotrypsin were obtained from Athens Research and Technology (Athens, Georgia). Cathepsin G (EC 3.4.21.20) was from ICN Pharmaceuticals (Orsay, France). MMP-7 (EC 3.4.24.33), proMMP-8 (EC 3.4.24.34), proMMP-9 (EC 3.4.24.35), and their respective fluorogenic substrates dinitrophenyl-RPLALWRS-OH, dinitrophenyl-PLAYWAR-OH and dinitrophenyl-PLGMWSR-OH were from Calbiochem (VWR, Strasbourg, France). Pseudolysin (E.C 3.4.24.26) was kindly provided by Prof. Jean Wallach (Université Claude Bernard, Lyon, France) or was purchased from Calbiochem (VWR, Strasbourg, France). Porcine pepsin (EC 3.4.23.1), bovine trypsin (EC 3.4.21.4), N-chlorosuccinimide and peroxidase-coupled goat anti-rabbit-IgG were from Sigma (St Quentin Fallavier, France). Lymphoprep™ and PBS without calcium and magnesium were from Invitrogen (Paisley, UK).

Patients

Sputum samples were collected after informed consent from 12 CF adult patients chronically infected with *Pseudomonas aeruginosa*. Approval was obtained from the Ethical Committee of our Institution.

Processing of sputum samples

Samples were homogenized in 10 mM PBS, pH 7.4 (3 mL/g sputum), and centrifuged at 10000 g for 10 min at 4°C. The pellets were suspended in PBS and supernatants centrifuged for another 10 min at 20000 g for further analysis.

Isolation of blood neutrophils

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Human neutrophils from healthy volunteers, who gage their informed consent, were purified essentially as previously described (Korkmaz et al., 2005). The neutrophils recovered at the bottom of the Lymphoprep^{TM} gradient were suspended in 200 µL PBS. Erythrocytes were lysed by placing them in 5 mL sterile distilled water for 20 sec; osmolarity was restored by adding 5 mL 2x concentrated PBS containing 8 mM EGTA. The suspension was then centrifuged at 500 g for 10 min at 25°C. The neutrophil pellet was washed twice in PBS containing 4 mM EGTA and neutrophils were suspended in this buffer at approximately 3 x 10^3 cells/µL and kept at room temperature with gentle stirring. The final pellet suspension was assessed by direct microscopic observation to consist of > 99 % neutrophils. It was used within a few hours of purification.

HNE assays

Free HNE activity was measured at 37 °C in 50 mM Hepes buffer, pH 7.4, 0.75 M NaCl, 0.05% Igepal CA-630 (v/v) using a fluorescence resonance energy transfer (FRET) peptide substrate : Abz-APEEIMRRQ-EDDnp (Korkmaz et al., 2004). HNE was titrated with α1-PI, the titer of which had been determined using bovine trypsin titrated with p-nitrophenyl-p–guanidinobenzoate. All HNE concentations reported throughout the text refer to that of the active protease in the reactional mixture. The hydrolysis of the Abz-peptidyl-EDDnp substrate was followed by measuring the fluorescence at λ_{ex} = 320 nm and λ_{em} = 420 nm in a Hitachi F-2000 spectrofluorometer. The system was standardized using Abz-FR-OH prepared from the total tryptic hydrolysis of an Abz-FR-paranitroanilide solution; its concentration was determined from the absorbance at 410 nm assuming ε_{410nm} = 8,800 M^{-1} cm^{-1} for p-nitroanilide. The concentrations of Abz-peptidyl-EDDnp substrate were determined by measuring the absorbance at 365 nm using ε_{365nm} = 17,300 M^{-1} cm^{-1} for EDDnp.

Membrane-bound HNE activity was quantified by comparing the rate of hydrolysis of Abz-APEEIMRRQ-EDDnp to that of titrated HNE in a detergent-free buffer (10 mM PBS, pH 7.4) at 28°C.

Titrated HNE was used to titrate a stock solution (10 mg/mL in 10 mM acetate buffer, pH 4.0) of EPI-hNE4. 1 to 30 µL of a 100-fold diluted EPI-hNE4 stock solution in 50 mM Hepes buffer, pH7.4, 150 mM NaCl, were incubated with a constant amount (10^{-8} M final) of HNE in 300 µL reaction buffer for 10 min at 37°C. The residual HNE activity was measured by adding 5 µL of substrate (15 µM final). EPI-HNE4 molar concentration was calculated from the value at the equivalence point (I/E molar ratio = 1).

Inhibition of HNE-related neutrophil serine proteases by EPI-hNE4

Free Pr3 and cat G were titrated with α1-PI and α1-antichymotrypsin respectively (Attucci et al., 2002; Korkmaz et al., 2004). Activities were measured in 50 mM Hepes buffer, pH 7.4, 0.75 M NaCl, 0.05 % (v/v) Igepal CA-630 for Pr3 and in 50 mM Hepes buffer, pH 7.4, 0.1 M NaCl, 0.01 % (v/v) Igepal CA-630 for cat G, using Abz-VADCADQ-EDDnp and Abz-TPFSGQ-EDDnp that are specifically cleaved by Pr3 and cat G respectively (Attucci et al., 2002; Korkmaz et al., 2004).

Pr3 or cat G (10^{-8} M final) were incubated for 10 min at 37°C with a range of EPI-hNE4 concentrations up to an inhibitor/protease molar ratio of 100. The residual activity of each protease was then measured.

The effects of Pr3 and cat G on the inhibition of HNE by EPI-hNE4 were tested at a sub-saturating concentration of EPI-hNE4 ([EPI-hNE4] / [HNE] = 0.75) using equimolar concentrations of the proteases (4.7×10^{-8} M final).

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Resistance of EPI-hNE4 to host and bacterial metalloproteases

ProMMP-8 and proMMP-9 were activated (Korkmaz et al., 2004) and the activities of MMP-8 and MMP-9 (10^{-8} M final) were measured with 20 μM final dinitrophenyl-PLAYWAR-OH and dinitrophenyl-PLGMWSR-OH fluorogenic substrates respectively, in the MMP buffer (50 mM Tricine, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 50 μM $ZnCl_2$), at 37°C. MMP-7 activity was measured with 20 μM (final) dinitrophenyl-RPLALWRS-OH in the HNE-Pr3 buffer, at 37°C. Pseudolysin activity was measured at a 10^{-8} M final concentration with 15 μM final fluorogenic substrate Abz-VADCAPQ-EDDnp in the HNE-Pr3 buffer, at 37°C.

MMP-7, MMP-8, MMP-9 and pseudolysin (10^{-10} M to 10^{-8} M final) were incubated with 2 x 10^{-6} M EPI-hNE4, in their respective activity buffers, for 30 min at 37°C. The mixtures were adjusted so that the final concentration of EPI-hNE4 was 2 x 10^{-8} M, and assayed against an equimolar amount of HNE. The residual activity of HNE was then measured.

The possible influence of metalloproteases on EPI-hNE4 binding to HNE was checked by mixing equimolar amounts (10^{-8} M) of each metalloprotease with HNE and incubating the mix with 10^{-8} M EPI-hNE4. The residual HNE activity was measured and compared to that of mixtures without metalloprotease.

The sensitivity of the HNE-EPI-hNE4 complex to degradation by each metalloprotease was assayed as follows. Equimolar amounts (2 x 10^{-6} M) of HNE and EPI-hNE4 were incubated for 10 min at 37°C and then with 10^{-7} M (final) metalloprotease for 30 min at 37°C. The residual HNE activity was measured.

EPI-hNE4 treatment with N-chlorosuccinimide

EPI-hNE4 and α1-PI (control) were treated with N-chlorosuccinimide (Korkmaz et al., 2005). The inhibitory capacities of the treated and native forms of the inhibitors were assayed after incubation with equimolar amounts (10^{-8} M) of HNE for 10 min at 37°C.

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Inhibition of HNE at the surface of purified blood neutrophils by EPI-hNE4

The inhibition of membrane-bound HNE (mHNE) by EPI-hNE4 was measured by incubating purified blood neutrophils whose number was adjusted so that the concentration of active HNE was 2 x 10^{-9} M (1 x 10^{5} to 5 x 10^{5} cells), with an equimolar amount of EPI-hNE4. Purified neutrophils or purified proteases (controls) were incubated with 15 µM Abz-APEEIMRRQ-EDDnp in polypropylene microplate wells selected for their low binding properties (Hard-Shell™ Thin-Wall Microplates, MJ Research, Waltham, MA, USA). Fluorescence was recorded as before using a microplate fluorescence reader (Spectra Max Gemini, Molecular Devices) under continuous stirring. Inhibition time curves were recorded using equimolar amounts of EPI-hNE4 and membrane-bound HNE (2 x 10^{-9} M final). Inhibitor and substrate were added simultaneously to the neutrophil suspension.

The fate of mHNE-EPI-hNE4 complexes was analyzed by incubating a 50-fold excess of neutrophils (10^{-7} M mHNE) with a stoichiometric amount of EPI-hNE4 (Korkmaz et al., 2005) for 5 min at room temperature. The suspension was centrifuged and the supernatant analyzed by SDS-PAGE under non-reducing conditions with 0.02 % (w/v) SDS in the sample buffer. Proteins were then blotted onto nitrocellulose and detected with rabbit polyclonal anti-EPI-hNE4 antibodies (1:1000) and peroxidase-coupled goat anti-rabbit-IgG (1:15000). Detection was performed with an ECL kit (Amersham Pharmacia Biotech, Orsay, France).

Inhibition of HNE in sputum samples by EPI-hNE4

The volumes of whole sputum homogenates and sputum supernatants were adjusted so that the active HNE concentration in the reaction medium was 10^{-8} M. Samples were incubated with a range of EPI-hNE-4 concentrations in PBS for 10 min at 28°C and the residual HNE activity assayed.

Interaction of EPI-hNE4 with pepsin

EPI-hNE4 (1.35×10^{-5} M final) was incubated with 5.6×10^{-6} M porcine pepsin in 100 mM citrate buffer, pH 4.1, at 37°C; aliquots were assayed at various times for their capacity to inhibit HNE ([E] = [I] = 3.6×10^{-8} M). Breakdown products were analyzed by reverse phase HPLC (Korkmaz et al., 2004).

Results

Resistance of EPI-hNE4 to proteolysis by protease 3 and cathepsin G

HNE is the best studied of the three neutral serine proteases stored in the neutrophil primary granules, but Pr3 and cat G are also secreted by activated cells and are present in lung secretions (Suter et al., 1988; Witko-Sarsat et al., 1999). Because HNE, Pr3 and cat G can compete for substrate and/or inhibitor binding physiologically, we first measured the inhibition of each of these purified proteases by EPI-hNE4 at different inhibitor / protease molar ratios. HNE (10^{-8} M) was fully inhibited by an equimolar amount of EPI-HNE4, in agreement with the low Ki for this interaction (Delacourt, 2002 #23), but Pr3 and cat G were not significantly inhibited in these conditions, even by a 100-fold molar excess of inhibitor (Figure 1).

Since Pr3 and cat G were not inhibited by EPI-hNE4, we checked that they did not interfere with inhibitor binding to HNE when all three protease were present in CF sputum. We measured the inhibition of HNE by EPI-hNE4 in the presence of equimolar amounts of Pr3 and cat G (Figure 2), adjusting the concentrations of HNE and EPI-hNE4 to obtain 75% inhibition. The inhibitory capacity of EPI-hNE4 was not significantly changed by Pr3 or cat G. Neither of these proteases hydrolyzed the inhibitor, even after a prolonged incubation; the HPLC profile of the inhibitor remained unchanged after 5h at 37°C (not shown). Thus, Pr3 and cat G do not alter the capacity of EPI-hNE4 to inhibit HNE when all three proteases are present in an inflammatory biological fluid.

Resistance of EPI-hNE4 to endogenous metalloproteases and to pseudolysin, a metalloprotease of *Pseudomonas aeruginosa*

The proteolytic inactivation of natural inhibitors contributes to the protease-antiprotease imbalance in lung inflammation (Moraes et al., 2003). The neutrophil metalloproteases MMP-

8 and MMP-9, epithelial MMP-7 and the bacterial metalloprotease pseudolysin from *P. aeruginosa* are among the main proteases that may break down inhibitors in CF sputum. We incubated EPI-hNE4 (2×10^{-6} M) with 10^{-10} to 10^{-8} M concentrations of each of these metalloproteases for 30 min at 37°C, and measured the residual activity of HNE incubated with equimolar amounts (2×10^{-8} M final) of the treated inhibitor. EPI-hNE4 was totally resistant to all the proteases except pseudolysin, which degraded about 50% of the inhibitor when used at 10^{-8} M. Hypothesizing that this concentration of pseudolysin can be present in lung secretions, we incubated equimolar amounts of HNE and pseudolysin (10^{-8} M) before adding the inhibitor. EPI-hNE4 remained fully active under these conditions as a result of its rapid binding to HNE and the resistance of the complex to degradation by the metalloprotease. The same result was obtained using MMP-7, MMP-8 and MMP-9 as competing proteases.

Resistance to oxidation

Unlike natural protease inhibitors in lung secretions that are sensitive to oxidation because they all have an oxidizable methionyl residue at position P1 or P1' in their inhibitory loop (Rosenberg et al., 1984 ; Heinzel-Wieland et al., 1991; Schuster et al., 1996), EPI-hNE4 has no Met residue that could alter its inhibiting properties. Nevertheless, we checked its resistance to inactivation by oxidants by comparing its inhibitory properties with those of α1-PI after incubation with N-chlorosuccinimide. EPI-hNE4 remained fully active, whereas α1-PI had lost all its inhibitory capacity. The oxidative environment of lung inflammatory secretions is therefore unlikely to influence EPI-hNE4 activity.

Inhibition of the membrane-bound HNE of blood neutrophils by EPI-hNE4

Part of the serine proteases released by activated neutrophils at inflammatory sites remains bound to the surface of activated or dying cells (Owen et al., 1995; Campbell et al., 2000). Any anti-inflammatory therapy that uses inhibitors must ensure that the inhibitor reaches and

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inhibits all active proteases that might degrade components of the extracellular matrix or other target proteins. We measured the inhibition of membrane-bound HNE (mHNE) by EPI-hNE4 using purified blood neutrophils. Free HNE and mHNE (both at 2 x 10^{-9} M final) were inhibited at the same rate by stoichiometric amounts of EPI-hNE4 (Figure 3A). Thus mHNE is fully accessible to the inhibitor and there is no other target protease for EPI-hNE4 at the neutrophil surface. We also showed that mHNE did not remain at the cell surface after cells had been incubated with EPI-hNE4. Western blots of cell supernatants obtained after centrifugation of the inhibitor-containing neutrophil suspension (Figure 3B) show that EPI-hNE4 formed soluble complexes, suggesting that EPI-hNE4 removes the bound protease from the cell surface, as does α1-PI (Korkmaz et al., 2005).

Inhibition of HNE in CF sputum by EPI-hNE4

The HNE activity in whole sputum homogenates of 12 patients chronically colonized with *P. aeruginosa* in sputum supernatants, and in suspended pellets was measured using Abz-APEEIMRRQ-EDDnp as substrate (Korkmaz et al., 2004). Concentrations were 0.4-4.9 x 10^{-7} M (median 2.3 x 10^{-7} M), 0.2-3.6 x 10^{-7} M (median 1.3 x 10^{-7} M) and 0.4-4.6 x 10^{-7} M (median 2.9 x 10^{-7} M) respectively. The total HNE activity in fractionated sputum was always higher than that in crude sputum, suggesting that sequestered HNE molecules were released from the pellet when it was suspended in buffer. The HNE in the sputum supernatants of all samples was fully inhibited by stoichiometric amounts of EPI-hNE4, demonstrating that the inhibitor has no other target molecule in the sputum soluble fraction (Figure 4A). But inhibition in whole sputum varied from 50 to 100% depending on the sample used (Figure 4B). This suggests that either EPI-hNE4 was partially inactivated by unidentified component(s) in some samples, or that the partitioning of HNE in sputum samples varied, a part being trapped and resistant to inhibition. This partitioning agrees with the finding that the total HNE activity in the supernatant plus pellet fractions is higher than that of crude

homogenate. We also showed that EPI-hNE4 was stable in crude sputum homogenates: excess inhibitor was incubated with a homogenate whose HNE activity had been stoichiometrically inhibited by EPI-hNE4. Any excess EPI-hNE4 was then titrated with purified HNE. EPI-hNE4 remained fully active under these conditions (not shown), indicating that nothing in sputum alters its inhibitory properties and that it probably remains fully active *in vivo*.

Interaction of EPI-hNE4 with pepsin

CF patients suffer from exocrine pancreatic insufficiency, which impairs the digestion of fats and proteins. Drugs rapidly reach the digestive tract after aerosol administration. Thus, active EPI-hNE4 given by aerosol is probably exposed to the gastric proteases. We therefore analyzed its HNE inhibiting capacity after incubation with pepsin. The inhibitor (1.35×10^{-5} M) was totally inactivated by incubation with pepsin (5.6×10^{-6} M) for 30 min at 37°C (Figure 5 A). Fractionation of the corresponding mixture by reverse phase HPLC showed complete proteolysis of native EPI-hNE4 (Figure 5 B).

Discussion

CF is characterized by cycles of lung inflammation and infection, with progressive destruction of tissue and loss of pulmonary function. Because inflammation occurs early in life, possibly before any pathogenic infection (De Rose, 2002), its early control can be a key element in CF therapy. However classical anti-inflammatory molecules, including oral corticosteroids and high-doses of ibuprofen, have adverse effects that limit their use (Konstan and Davis, 2002). Restoration of the protease-antiprotease balance with proteases inhibitors (McElvaney et al., 1991; McElvaney et al., 1992; Griese et al., 2001) is a promising alternative because the massive influx of activated PMNs into the CF lung dramatically increases the concentrations of extracellular proteases, especially that of HNE (Birrer et al., 1994). These escape control by their natural inhibitors, α1-PI, SLPI and elafin, as the inhibitors are inactivated by proteolysis and oxidation (Suter and Chevallier, 1991; Birrer et al., 1994; Schuster et al., 1996). Natural and recombinant protein inhibitors have been tried in CF patients (McElvaney et al., 1991; McElvaney et al., 1992; Griese et al., 2001), but none of them fulfils the requirements for routine use in CF therapy. Previous attempts to investigate the potential of EPI-hNE4 as a therapeutic inhibitor have shown that intratracheal or intravenous administration protects the lungs of rats (Delacourt et al., 2002; Honore et al., 2004). EPI-hNE4 also resists the physical constraints of nebulization (Grimbert et al., 2003).

The present study shows that EPI-hNE4 is not degraded by the HNE-related proteases Pr3 and cat G that are present and active in CF sputum. Similarly, MMP-7, MMP-8 and MMP-9, which are all present in significantly above-normal amounts in CF (Dunsmore et al., 1998; Ratjen et al., 2002), do not cleave EPI-hNE4 under the experimental conditions used. But the bacterial metalloprotease and major antigen of *P. aeruginosa*, pseudolysin does degrade EPI-hNE4 when incubated alone with the inhibitor, but not when it competes with an equimolar

concentration of HNE. This is probably due to the rapid formation of HNE-inhibitor complexes that are resistant to further degradation by the metalloprotease. In keeping with previous results (Venaille et al., 1998), we measured no significant pseudolysin activity in the sputum of chronic CF patients, suggesting that the bacterial protease will not interfere with EPI-hNE4 activity in CF sputum. But degraded forms of natural antiproteases are present in CF sputum (Suter and Chevallier, 1991; Birrer et al., 1994). The proteases that are responsible for this degradation have not yet been identified, though *in vitro* experiments have shown that host and bacterial proteases can cleave α1-PI and SLPI, altering their inhibitory function (Sponer et al., 1991; Desrochers et al., 1992; Sires et al., 1994; Rapala-Kozik et al., 1999).

Drugs that are to be administered as aerosols must resist oxidation at inflammatory sites. Oxidation of the Met 358 at position P1 of the reactive inhibitory loop of α1-PI (Rosenberg et al., 1984) and of the Met 73 at P1' of SLPI (Heinzel-Wieland et al., 1991) and Met 25 at P1'of elafin (Zani et al., 2004) dramatically impair their antielastase activities. And oxidized forms of α1-PI and SLPI are more sensitive to proteolysis than are the native inhibitors (Rapala-Kozik et al., 1999). Thus, oxidation and proteolysis may work together to promote local inflammation. EPI-hNE4 remains fully active under oxidative conditions that completely abolish α1-PI activity because it has no Met residues in its primary structure.

The critical feature of any inhibitors to be used in therapy is whether the inhaled inhibitors can inhibit both gel phase-entrapped and membrane-bound proteases, as the proteases in lung secretions are present in the soluble and gel phases. Chan et al. (Chan et al., 2003) have shown that HNE in the soluble phase of mucous bronchial secretions of patients with idiopathic bronchiectasis is not present as the free enzyme but as a proteolytically active member of a supramolecular complex that includes heparan sulphates, syndecan-1 and its physiological regulators α1-PI and SLPI. HNE may also be entrapped in neutrophil extracellular traps (NET) (Brinkmann et al., 2004) or in complexes with macrophage-derived

lipids (Fujita et al., 1999). We found that EPI-hNE4 stoichiometrically inhibits HNE bound to the membranes of purified blood neutrophils, forming soluble stable EPI-hNE4-HNE complexes. This stoichiometric inhibition occurred in some but not all the crude sputum homogenates, suggesting that other, unidentified, factors are involved in the regulation of proteolytic activity in the CF sputum of some individuals.

Nevertheless, low molecular mass protein inhibitors seem to be better than high M_r inhibitors at reaching proteases in lung secretions. This is emphasized by the observation that SLPI inhibits the HNE in the supramolecular complexes found in soluble fraction of sputum, while α1-PI does not (Chan et al., 2003). While aerosolized α1-PI however, significantly reduces the active HNE concentration in the epithelium lining fluid (McElvaney et al., 1991) and overall proteolysis in the bronchoalveolar lavage fluid (Griese et al., 2001) of CF patients, its effect on the progression of lung disease in CF is not yet known. Oxidation-resistant variants (Met 358→Val of α1-PI (Rosenberg et al., 1984) and of rSLPI (Met 73 → Leu and Met 73, 82, 94, 96→Leu) (Heinzel-Wieland et al., 1991) have also been constructed to counteract the deleterious effect of oxidation, but they have not yet been tried in humans. Synthetic chemical inhibitors could be attractive alternatives for controlling unwanted proteolysis but most clinical trials have been ended because of their toxicity and side effects (Chughtai and O'Riordan, 2004). Small protein inhibitors should therefore be preferred for CF therapy.

Protease inhibitors for therapeutic use are administered intravenously or as aerosols. Aerosolized inhibitors may be more efficient at reaching pulmonary tissues provided they resist the physicochemical conditions of nebulization, are well deposited on the epithelium surface of airways and do not aggravate any pancreatic insufficiency of CF patients when the excess reaches the digestive tract. Recombinant EPI-hNE4 therefore appears to be a promising molecule - it resists proteolysis, oxidation and the physical constraints imposed by administration as an aerosol. It also inhibits most of the active HNE in whole CF sputum and

is rapidly broken down by pepsin, which prevents its accumulation following the repeated administrations required to treat CF.

Acknowledgements

We thank Dr. Françoise Varaigne and Christèle Barbarin-Costes for CF patient recruitment, Dyax Corp. (Cambridge, Ma, USA) for allowing studies on EPI-hNE4, Pr Jean Wallach for generous gift of pseudolysin and Owen Parkes for editing the English text.

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Footnotes

This work was supported by the Association “Vaincre La Mucoviscidose” (France) and Debiopharm SA (Lausanne, Switzerland).

Figure Legends

Figure 1. Residual HNE, Pr3 and cat G peptidase activities after incubation with various amounts of EPI-hNE4.

10^{-8} M concentrations HNE (■), Pr3 (◊) and cat G (O) were incubated with various amounts of EPI-hNE4 for 10 min at 37°C. Proteases were assayed with 15 μM of their specific fluorogenic substrate (Abz-APEEIMRRQ-EDDnp, Abz-VADCADQ-EDDnp and Abz-TPFSGQ-EDDnp respectively). Data are means ± SD of 3 individual experiments, each performed in duplicate.

Figure 2. Inhibition of HNE by EPI-hNE4 in the presence of Pr3 and cat G.

HNE (4.7×10^{-8} M) and HNE plus Pr3 and cat G (each at 4.7×10^{-8} M) were incubated with EPI-hNE4 at an elastase/inhibitor molar ratio of 1: 0.75. The residual HNE activity in the presence (c and d) or absence (a and b) of the other proteases was measured using the specific HNE substrate, Abz-APEEIMRRQ-EDDnp. Data are means ± SD for 3 individual experiments, each performed in duplicate.

Figure 3. Inhibition of HNE bound to neutrophil membranes (mHNE) (A), and fate of the mHNE-EPI-hNE4 complexes (B).

(A) Purified blood neutrophils, whose membrane-bound HNE activity (a) had been adjusted to that of a 2×10^{-9} M free HNE solution (b), were incubated with EPI-hNE4 (2×10^{-9} M) and the progress curves of inhibition recorded (c, d). Free HNE and mHNE were inhibited at the same rate by equimolar amounts of EPI-hNE4. (B) Supernatants of the neutrophil-EPI-hNE4 mixtures prepared at a 50 fold higher concentration were fractionated by SDS-PAGE under non-reducing conditions and analyzed by Western blotting using rabbit anti-EPI-hNE4

antibodies (c). (a) Free EPI-hNE4 (M_r = 6231) control; (b) EPI-hNE4 incubated with an equimolar amount of free HNE (10^{-7} M).

Figure 4. Inhibition of the HNE in a representative sputum supernatant (A) and in 4 representative crude sputum homogenates (B), by EPI-hNE4.

Homogenates and supernatants were adjusted so that total HNE concentration was 10^{-8} M; they were incubated with increasing amounts of EPI-hNE4.

Figure 5. EPI-hNE4 inactivation by pepsin.

EPI-hNE4 (1.35 x 10^{-5} M) was incubated with pepsin (5.6 x 10^{-6} M) in citrate buffer, pH 4.1 at 37°C. Aliquots were removed during 30 min and their capacity to inhibit a stoichiometric amount of HNE (3.6 x 10^{-8} M) was measured (A). HPLC profile of native EPI-hNE4 (grey line) and EPI-hNE4 after incubation with pepsin for 30 min (black line) (B).

JPET Fast Forward. Published on April 20, 2006 as DOI: 10.1124/jpet.106.103440
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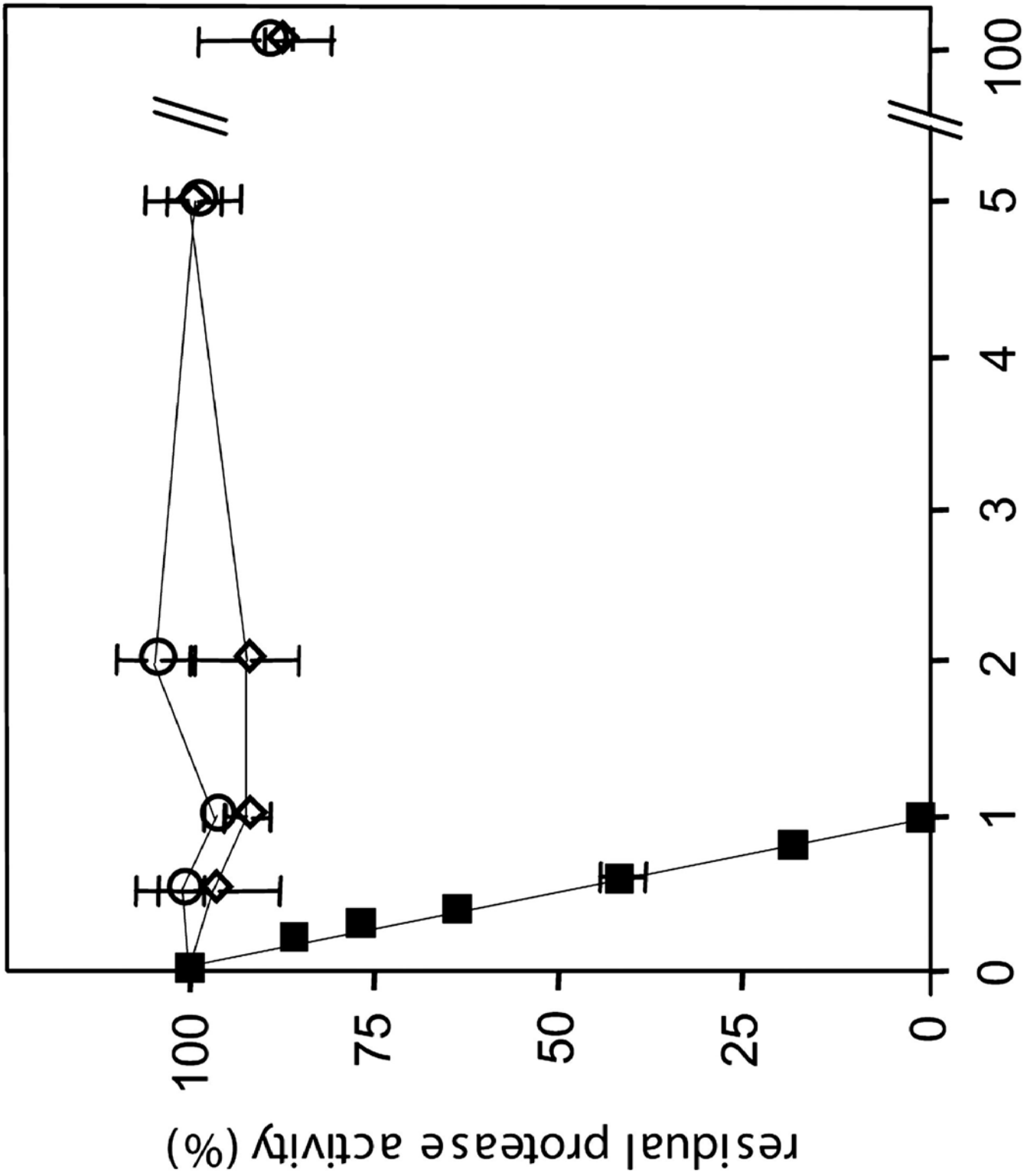

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