Molecular principles for heparin oligosaccharide–based inhibition of neutrophil elastase in cystic fibrosis

Cystic fibrosis (CF) is a multifactorial disease in which dysfunction of protease–antiprotease balance plays a key role. The current CF therapy relies on dornase α, hypertonic saline, and antibiotics and does not address the high neutrophil elastase (NE) activity observed in the lung and sputum of CF patients. Our hypothesis is that variants of heparin, which potently inhibit NE but are not anticoagulant, would help restore the protease–antiprotease balance in CF. To realize this concept, we studied molecular principles governing the effectiveness of different heparins, especially 2-O,3-O-desulfated heparin (ODSH), in the presence of sputum components and therapeutic agents. Using spuTA from CF patients and an NE activity assay, we found that heparins are ineffective if used in the absence of dornase. This is true even when mucolytics, such as DTT or N-acetylcysteine, were used. Computational modeling suggested that ODSH and DNA compete for binding to an overlapping allosteric site on NE, which reduces the anti-NE potential of ODSH. NE inhibition of both DNA and ODSH is chain length dependent, but ODSH chains exhibit higher potency per unit residue length. Likewise, ODSH chains exhibit higher NE inhibition potential compared with DNA chains in the presence of saline. These studies suggest fundamental differences in DNA and ODSH recognition and inhibition of NE despite engaging overlapping sites and offer unique insights into molecular principles that could be used in developing antiprotease agents in the presence of current treatments, such as dornase and hypertonic saline.

Dysregulation in mutant CFTR4 anion conductance results in mucus accumulation, which promotes bacterial infections, thereby initiating a cascade of events, including failure of innate immunity to clear infections and release of inflammatory mediators that cause lung injury and ultimately respiratory failure. Although improvements in CF therapy over the past 2 decades have increased life expectancy to about 41 years (1), much remains to be accomplished.

The CF therapy currently recommended by the United States Cystic Fibrosis Foundation includes inhaled dornase α to reduce sputum viscosity, antibiotics to kill bacteria, inhaled 7% saline to enhance sputum hydration, and immunomodulatory therapies (ibuprofen and azithromycin) to decrease lung injury (1). Other agents recommended for use include ivacaftor for patients with class 3 CFTR mutations and Orkambi for patients homozygous for phe508del to rescue CFTR function (2). It is instructive to note that for resolving cystic fibrosis, no agent has been approved for clinical use to inhibit neutrophil serine proteases (NSPs), a major cause of airway injury in CF.

Innate immunity in CF is mediated to a large extent by polymorphonuclear neutrophils. These are attracted to sites of bacterial infection in CF by IL-8 (CXCL8) and, following activation, release inflammatory mediators including NSPs, such as elastase, proteinase 3, and cathepsin G, and release DNA and reactive oxygen species (3). However, in the CF airway milieu, neutrophils release extracellular traps (NETs) (4), a primary source of extracellular DNA polymers and NE in the CF airway. The load of NE in the airway overwhelms the antiprotease activities provided by α1-proteinase inhibitor and serine leukoprotease inhibitor.

An attractive, yet unrealized, pathway to control lung injury in CF is re-establishment of protease–antiprotease balance through inhibition of NE (5). NE activates other proteases, such as matrix metalloprotease-9, degrades antiproteases, such as tissue inhibitor of metalloproteases, up-regulates neutrophil

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This article contains Tables S1–S4 and Figs. S1 and S2.

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4 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; CVLS, combinatorial virtual library screening; NAC, N-acetylcysteine; NE, neutrophil elastase; fNE, fluorescently labeled NE; NET, neutrophil extracellular trap; NSP, neutrophil serine proteases; ODSH, 2-O,3-O-desulfated heparin; RMSD, root mean square deviation; UFH, unfractionated heparin; IL, interleukin; HS, hypertonic saline.
chemokines, such as IL-8 and tumor necrosis factor α, and thereby augments inflammation (6). Additionally, NE impairs ciliary motility; up-regulates mucin expression and secretion; causes epithelial injury, apoptosis, and senescence; and blocks epithelial repair (5). As such, NE is a well-established biomarker of lung disease progression in patients with CF (7, 8), yet there are no approved therapies that block NE activity for patients with CF.

Although many different types of inhibitors of NE have been reported (9), an agent that exhibits considerable promise is heparin. In addition to potently inhibiting NE (1–20 nM), heparin inhibits cathepsin G, another neutrophil serine protease present in high concentrations in the CF airway (10). Additionally, heparin has other anti-inflammatory activities, including inhibiting activation of the receptor for advanced glycation end-products and blocking the release of its ligand, high mobility group box 1; inhibiting binding to L-selectin and P-selectin; and inhibiting NF-κB (10). Heparin also binds to pro-inflammatory chemokines, such as IL-8 and IL-6 (10), which implies that soluble heparin administration may also reduce neutrophil chemotaxis by providing competitive sites. However, heparin’s high anticoagulant activity may limit its value as a therapy.

We have proposed 2-O,3-O-desulfated heparin (ODSH), a modified heparin displaying potent anti-inflammatory and antiprotease activity with minimal anticoagulant effect, as an anti-NE agent for use in CF (10, 11). ODSH blocks airway neutrophilic inflammation in mice administered NE intratracheally (11), and in animals with *Pseudomonas aeruginosa* pneumonia, ODSH recovers impaired macrophage function, promotes bacterial clearance, and improves survival (12). To understand the molecular principles of ODSH action in CF-related samples, we studied its inhibition of NE activity in CF sputum. This work shows that NE is not inhibited by ODSH or heparin if only mucolytics are used, including N-acetylcysteine (NAC), Sputolysin (0.1% DTT), or hypertonic saline. More importantly, this work reveals that either ODSH or heparin inhibits NE in native CF sputum pretreated with dornase. The molecular basis for this favorable combinational outcome involves fundamental mechanistic biochemistry and leads to a putative therapy for treatment of CF that can be of value irrespective of the patient’s genetic status.

### Results

**ODSH did not inhibit NE in CF sputum**

Heparin is a polysulfated polysaccharide that is clinically used as an anti-coagulant and also has potent antiprotease activity against serine proteases, including NE (13) and cathepsin G, and furthermore, heparin is not degraded by exposure to proteases. However, heparin anticoagulant activity may potentially increase the risk for hemoptysis in CF. Therefore, we evaluated the anti-NE activity of a modified heparin, ODSH, that has minimal anti-coagulant activity. We first determined the potency of ODSH in inhibiting NE by the S1384 hydrolysis assay and measured an IC₅₀ of 13.6 nM (Fig. 1A), which was consistent with its reported antiprotease activity (10). We then solubilized CF sputum with normal saline (1:1, w/v) to test whether ODSH could inhibit NE in the typical airway milieu of CF patients. Unexpectedly, we found that ODSH did not reduce NE activity at all in CF sputum at concentrations as high as 10 μM, which is ~735-fold higher than its IC₅₀ in pH 7.4 buffer (Fig. 1B).

### Effect of mucolytics on NE activity in CF sputum

The observation that ODSH did not inhibit NE in solubilized CF sputum suggests that it is not able to effectively bind to NE, perhaps due to competition for binding sites. At least two major anionic polymers, DNA and mucin polymers, are present at high concentrations in CF sputum and potentially bind to and inhibit NE (14–18). To determine the potential NE-binding activity of these polymers, we first chemically or enzymatically degraded de-identified CF sputum samples to prepare sputum supernatants. For chemical degradation, we used either NAC (19) or DTT (8), typical reducing agents that degrade disulfide bonds. For enzymatic degradation, we treated sputum with bovine DNase-I. For all three agents, appropriate concentration ranges were chosen that matched either pharmacologic concentrations or research reports (8, 19). Following sputum supernatant preparation, NE activity was measured using the S1384 hydrolysis assay, as described under “Experimental procedures.” Interestingly, Sputolysin as well as DNase-I significantly increased NE activity of treated sputum supernatants (Fig. 2). However, although Sputolysin dose-dependently

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**Figure 1. Inhibition of NE by ODSH.** A, NE inhibition was measured using an S1384 hydrolysis assay in 0.125 M HEPES buffer, pH 7.4, at 37 °C. Solid lines, sigmoidal fits to obtain the IC₅₀, HS, YM, and Y₀. B, NE activity determined in native CF sputum treated with ODSH. Sputum supernatants (n = 3–5 individuals) solubilized with normal saline (1:1, w/v) were pretreated with ODSH from 0.1 to 10 μM and incubated for 2 h at 37 °C, and then supernatant was collected post-centrifugation for the NE activity assay. Results are expressed compared with the NE standard curve (nM enzyme). Values are expressed as mean ± S.D. (error bars).
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enhanced NE activity, its effect was much less than the nearly 300% increase in NE activity with DNase-I.

We then studied whether ODSH could decrease NE activity, measured using a substrate hydrolysis assay, in mucolytic-pretreated CF sputum. We found that ODSH only inhibited NE activity following DNase-I treatment and had no effect on sputum degraded with NAC or Sputolysin (Fig. 3). These results imply that disulfide bond reduction does not help ODSH to bind to NE. On the contrary, enzymatic degradation of polymeric DNA, to presumably smaller strands, allowed ODSH to bind to NE and reduce its catalytic activity. Nevertheless, the concentration at which ODSH started to inhibit NE in sputum is much higher than in pH 7.4 buffer. A plausible explanation for this is the possibility of competition between ODSH and DNA, both negatively charged polymers, for binding to NE. This explanation is also in line with results with disulfide-reducing agents, which are not expected to significantly reduce electrostatically bound DNA from NE.

**Inhibition of NE activity in DNase-I–degraded CF sputum is chain length–dependent**

The evidence that DNase I is needed for ODSH to exhibit its anti-NE activity suggested a critical point. Polymeric DNA chains impeded ODSH from binding to NE, whereas oligomeric DNA sequences appeared to do so less effectively. We then asked whether heparins exhibit chain length–dependent inhibition of NE. Fig. 4 shows NE activity in CF sputum with and without DNase-I pretreatment as well as with and without heparins of different chain lengths (Table S1) at a fixed concentration of 100 μM each. The best inhibitor was found to be unfraccionated heparin (UFH; $M_r$ 15,000), which was followed by ODSH ($M_r$ 10,000) and low-molecular weight heparin ($M_r$ 4,500). Fondaparinux ($M_r$ 1,772) had no anti-NE activity in DNase-I–treated CF sputum. These results indicate that a minimum saccharide length is critical for heparins to bind to and inhibit NE.

**Comparison of DNA and heparin inhibition of NE in vitro**

It is known that heparin and DNA each inhibit NE (18, 20). Nearly all studies have employed full-length polymeric DNA and heparin preparations. Considering that heparin chains and DNA chains also exhibit a similar chain length dependence. Thus, we studied three DNA variants (6-, 12-, and 24-mer), which are homogeneous variants of unique chain lengths (Table S2). With regard to heparins, we studied three clinically used variants, including UFH (~50-mer), ODSH (~35-mer), and enoxaparin (low-molecular weight heparin; ~15-mer), which are heterogeneous preparations containing ~30% variation in chain length on either side of the mean. We also studied fondaparinux, a clinically used anticoagulant, which has a defined structure and chain length (5-mer).

The dose dependence of NE inhibition by each inhibitor is shown in Fig. 5. Whereas corresponding longer chains of each class display almost equal IC50 and inhibition efficacy, significant differences were observed for shorter chains (Table 1). In fact, the 6-mer DNA displayed essentially no inhibition, whereas 5-mer fondaparinux displayed a reasonable IC50 of 1.2 μM and an efficacy of nearly 70%. Although the number of residues for corresponding DNA and heparin chains may be similar, the linear molecular sizes are not expected to be identical. For example, computational modeling shows that the molecular length of a 6-mer DNA in its extended conformation was ~42 Å, whereas it was ~22 Å for fondaparinux (not shown). This implies that the formal negative charge density of the two polymers is ~0.25 and ~0.40 negative charges per Å. Thus, a priori, the electrostatic interaction of NE with heparins is expected to be stronger than that with DNA of equal chain length.

**Comparison of salt dependence of DNA and heparin inhibition of NE in vitro**

Hypertonic saline (7% (w/v) NaCl) is clinically used to aid mucus clearance in CF patients. Considering that NE interactions are electrostatically mediated, we reasoned that DNA and heparin inhibition of NE might be adversely impacted in the presence of salt. To test the comparative inhibition potential of the two classes of inhibitors, we studied 24-mer DNA and 35-mer heparin (ODSH). Fig. 6 shows the dose dependence of DNA and ODSH inhibition of NE at varying Na+ levels (62.5–362.5 mM or 0.36–2.1% (w/v)). The results show that as Na+ concentration increased from 62.5 to 162.5 mM, the IC50 of 24-mer DNA increased ~6-fold (Table 2). A more striking effect was noted with respect to inhibition efficacy (ΔY), which

Figure 2. Mucolytic treatment of CF whole sputum and release of free NE activity. Incubation of CF sputum supernatant with N-acetylcysteine (A), Sputolysin (B), and bovine DNase-I (C) resulted in a statistically significant increase in the concentration of free, active NE in a concentration-dependent manner (*, p < 0.05; **, p < 0.005; ***, p < 0.0005, compared with normal saline control). Data are summarized as mean ± S.D. (error bars). n = 6–8 individuals, representing 2–3 separate experiments.
decreased from 87 to 35%. At 262.5 mM Na\(^+\), the 24-mer DNA was essentially noninhibitory. In contrast, ODSH displayed a significantly different profile. At 162.5 mM Na\(^+\), the 35-mer heparin was only 2-fold less active than its potency at 62.5 mM Na\(^+\). More importantly, ODSH retained almost full NE inhibition efficacy (85%) at this high Na\(^+\) level (0.94% (w/v)). This efficacy was reduced to 52% at 262.5 mM Na\(^+\) and almost negligible at 362.5 mM Na\(^+\) (Table 2).

Table 1

| Corresponding chain lengths | DNA chain (nM) | Heparin chain (%) | Inhibition (IC\(_{50}\)) | DNA chain (nM) | Heparin chain (%) |
|----------------------------|----------------|-------------------|------------------------|----------------|-------------------|
| 50 ± 10-mer                | NA             | 7.5 ± 0.4         | NA                     | 95 ± 2         |                   |
| 24-mer or 35 ± 8-mer       | 10.5 ± 1.2     | 13.6 ± 0.5        | 87 ± 2                 | 91 ± 1         |                   |
| 12-mer or 15 ± 5-mer       | 22.3 ± 1.3     | 25.4 ± 2.2        | 101 ± 5                | 88 ± 3         |                   |
| 6-mer or 5-mer             | >10,000        | 1,178 ± 260       | ND                     | 71 ± 6         |                   |

Figure 3. NE activity in CF sputum after mucolytic therapies and ODSH. CF sputum (n = 6–8 individuals) was pretreated with NAC (100 mM) (A), Sputolysin (20%; 0.2 mM DTT) (B), or DNase-I (0.3 mg/ml) (C), for 2 h, before treatment with ODSH or control vehicle. Data are presented as mean ± S.D. (error bars). NE activity in mucolytic-treated samples was significantly different from that in control-treated samples (**, p < 0.005; ***, p < 0.001), and NE activity in ODSH-treated samples was significantly different from that in control-treated samples for DNase-pretreated sputum samples only (+, p < 0.005).

Figure 4. Inhibition of NE activity in CF sputum by heparin depends on polymer chain length. In sputum that was not pretreated with DNase-I (0.3 mg/ml) (A), heparins (final concentration of 100 \(\mu\)M) did not inhibit NE activity (n = 6). Data are summarized as mean ± S.D. (error bars), representing two separate experiments. Pretreatment with DNase I released active NE (#, p < 0.01, compared with normal saline control). ODSH and UFH significantly inhibited NE activity (**, p < 0.005; ***, p < 0.001, compared with DNase-I alone), low-molecular weight heparin (LMH) had intermediate antiprotease activity, and pentasaccharide heparin (fondaparinux) had no antiprotease activity (n = 7) (B).

Figure 5. Inhibition of NE by DNA (A) and heparins (B) of different chain lengths. NE inhibition was measured using an S1384 hydrolysis assay in a 0.125 M HEPES buffer, pH 7.4, at 37 °C. Solid lines, sigmoidal fits to obtain the IC\(_{50}\), HS, Y\(_{M}\), and Y\(_{0}\). ENOX, enoxaparin; FOND, fondaparinux.
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These results suggest an atomistic level difference in the nature of interactions of DNA and heparin with NE. Both depend on electrostatic forces as Na$^+$ reduces DNA and ODSH inhibition potency. For comparable chain lengths, DNA lost its NE inhibition potential faster in salt-containing buffers compared with ODSH. This implies that ODSH is a better inhibitor of NE than DNA at comparable chain lengths. More importantly, ODSH appeared to exhibit reasonable NE inhibition at Na$^+$ levels reaching 1.5% (w/v), which is likely to be reached in the lung of a CF patient when inhaling 7.0% (w/v) hypertonic saline.

DNA and ODSH potentially bind to overlapping sites on NE

The above results show that heparin was effective only in the presence of DNase-I, which presumably depolymerizes polymeric DNA into smaller chains liberating bound NE for subsequent inhibition by heparins, including ODSH. A simple model that explains this phenomenon is that ODSH and DNA bind to an overlapping site on NE, thereby competing with each other. Because a huge amount of DNA, most probably in the form of NETs, was present in the sputum of CF patients, ODSH was unable to compete, resulting in its failure to inhibit NE in sputum.

To assess whether DNA and heparins compete for an overlapping site on NE, we used combinatorial virtual library screening (CVLS) to identify sites of heparin binding on proteins (21–23). The algorithm predicts the binding site residues as well as the most preferred heparin sequence for the target site. We built a library of 1,728 sequences, which represent the most common hexameric chains likely to be present in a sample of ODSH. Application of the CVLS algorithm showed that many sequences bound NE with high GOLD score and high consistency, suggesting a potentially high-affinity interaction (Table S3). Calculations using Chimera (23) showed that three of these sequences were predicted to bind to NE at a site encompassed by Gln-34, Leu-35, Arg-36, Gly-38, His-40, Phe-41, Cys-42, and Arg-147 (Fig. 7). This implies that many of the 19 arginines on the NE surface are predicted to be uninvolved in binding to ODSH. More importantly, the site of ODSH binding did not include NE’s active site residues (Fig. 7), which are located ~6 Å away from the heparin chain. To assess whether longer ODSH sequences would bind to NE and interact, the most common octa- and decasaccharide sequences were studied using our CVLS approach. The results showed that the preferred binding orientations of both octa- and decasaccharide sequences were similar to that of these heparin sequences bound to NE (see Fig. S1).

To assess the nature of DNA-NE interactions, we used a HADDOCK-based docking and scoring strategy (24), which is routinely used to determine the site of DNA binding on proteins (25). HADDOCK study of 6-, 12-, and 24-mer DNA showed that that the two latter oligomers bound avidly to NE (see Table S4), whereas the 6-mer was ineffective. Both 12- and 24-mer bound to NE with a root mean square deviation (RMSD) of <2.5 Å from the overall lowest energy structure, which supports the inhibition results discussed above. Chimera analysis (23) of the predicted DNA–NE complexes showed that the oligomer preferred to interact with residues Gln-34, Leu-35, Arg-36, Gly-38, Gly-39, Asn-61, Ala-64, Tyr-94, Arg-177, Arg-217, and Tyr-224; these are in the same region that interacts with ODSH hexasaccharide sequences (Fig. 7). Thus, both DNA and ODSH are predicted to bind to overlapping sites on NE.

DNA and ODSH inhibited NE by a mixed mechanism

The computational studies show that neither ODSH nor DNA interacted with the active site of NE, suggesting that a

Figure 6. Salt dependence of DNA and ODSH inhibition of NE. 24-Mer DNA (A) and ODSH inhibition of NE (B) were measured using a chromogenic substrate hydrolysis assay in a 0.125 M HEPES buffer, pH 7.4, containing different levels of Na$^+$ at 37 °C. Solid lines, sigmoidal fits to obtain the IC$_{50}$ and percentage inhibition (see “Experimental procedures” for additional details).

Table 2
Salt dependence of NE inhibition by 24-mer DNA and 35-mer ODSH

| [Na$^+$] (mM (% w/v)) | DNA chain IC$_{50}$ (µM) | DNA chain Inhibition (%) | Heparin chain IC$_{50}$ (µM) | Heparin chain Inhibition (%) |
|-------------------------|--------------------------|--------------------------|-----------------------------|-----------------------------|
| 62.5 (0.36)             | 10.5 ± 1.2               | 14.1 ± 0.9               | 87 ± 2                      | 91 ± 6                      |
| 112.5 (0.65)            | 10.1 ± 2.0               | 13.5 ± 1.1               | 81 ± 6                      | 90 ± 5                      |
| 162.5 (0.94)            | 66.6 ± 24.9              | 26.7 ± 2.2               | 35 ± 5                      | 85 ± 4                      |
| 262.5 (1.5)             | >5,000                   | 261 ± 33                 | ND                          | 52 ± 4                      |
| 362.5 (2.1)             | ND                       | >50,000                  | ND                          | ND                          |

S.D. ND, not determined.
small peptide substrate can access the active site without much impedance from the two anionic polymers. To test this, Michaelis–Menten kinetics of chromogenic substrate hydrolysis was performed in the presence of the two polymers at various concentrations. Characteristic hyperbolic profiles of substrate hydrolysis by NE were obtained in the presence of varying concentrations of ODSH as well as DNA (Fig. 8). The data were analyzed using the traditional hyperbolic mixed inhibition equation described in the literature (26). For both DNA and ODSH, the $V_{\text{max}}$ decreased nearly 3–4-fold as the inhibitor concentration increased, whereas the $K_m$ increased about ~2-fold for ODSH and ~4-fold for DNA. More importantly, the nonlinear regression yielded a nonzero, nonunitary $\alpha$ value for both DNA and ODSH ($\alpha_{\text{DNA}} = 1.16; \alpha_{\text{ODSH}} = 1.36$), which suggests the operation of a mixed inhibition mechanism (i.e., competitive as well as noncompetitive). Overall, these results support the expectation that the active site remains fairly accessible to small peptidic substrates, while also indicating that ODSH and DNA engaged an allosteric site.

6-Mer DNA and ODSH competed for direct binding to NE

Although the substrate kinetics studies described above show that ODSH and DNA interact with an allosteric site on NE, in principle, possibilities exist for ODSH and DNA to bind to distinct allosteric sites. To test whether DNA and ODSH compete directly for binding to an overlapping binding site, as suggested by the CVLS studies, we utilized spectrofluorimetric titrations with fluoresceinylated NE (fNE). In preliminary studies, we found that both ODSH and DNA induce parallel and similar changes in fluorescence of fNE (not shown), which caused significant loss of signal in competitive titrations. This difficulty was reduced a little when 6-mer DNA was used as a competitor, thereby affording a window for monitoring differential fluorescence changes. Fig. S2 presents fNE titrations of ODSH in the presence three different concentrations of 6-mer DNA. Analysis of data using the saturation ligand binding equation showed that the apparent affinity of ODSH ($K_{D,\text{app}}$) increased linearly from 25.4 to 184 nM as the concentration of

Figure 7. In silico molecular modeling of NE interaction with DNA and ODSH. A, molecular model of NE showing highly electropositive residues present across most of the NE surface. A and B, the positions of basic residues (cyan color by atom) and catalytic site residues (green color by atom) are shown as spheres. C, CVLS-based identification of site of binding of ODSH hexasaccharides on NE. D, DNA (12-mer) bound to NE predicted by HADDOCK docking. E, overlay of both ODSH and 12-mer DNA binding domains for NE to show the occupancy of the same binding site on NE. The protein molecule is shown as a surface (white), DNA as a cartoon (12-mer green), and ODSH sequences as sticks (magenta color by atom).
6-mer DNA increased from 0 to 2.0 μM (Fig. 9A). Likewise, the maximal change in fluorescence decreased from 15.7 to 5.1% as more 6-mer DNA was present as a competitor (Fig. 9B). Both these observations indicate that DNA and ODSH compete for the same site and induce similar conformational change. Thus, the two inhibitors directly compete for the allosteric site of binding on NE.

Discussion

NE and proteases are a focus for CF therapy to prevent early lung injury. Heparin and ODSH inhibit serine proteases (10) and, unlike other antiproteases, are not degraded by sputum proteases (27) and are not susceptible to oxidation (28). This report suggests that heparins, especially ODSH, and DNA share overlapping binding sites on NE. Mechanistic studies revealed that both ODSH and DNA function as allosteric mixed inhibitors of NE. More importantly, we found that heparins are more potent at inhibiting NE than DNA. However, the massive load of DNA (10% of organics) (14) in CF sputum outcompetes ODSH for binding to NE, explaining its almost nonexistent inhibitory activity.

Inhaled dornase α is an established therapy for patients with CF (19). Although dornase has been shown to decrease sputum viscoelasticity and decrease pulmonary exacerbations, it also releases active NE that can potentially injure airways (29). We show that exposure of sputum to DNase-I reduces this competitive effect because smaller chains of DNA are unable to bind to NE with high potency. This means that free NE could be inhibited by a combination of DNase-I and ODSH to yield an anti-inflammatory effect.

A key finding of this work confirms the importance of polymer length for antiprotease activity demonstrated earlier (13). Both DNA and ODSH must possess a minimum chain length to effectively inhibit NE. DNA chains of 6-mer or shorter did not inhibit NE. Likewise, ODSH chains should be some ~15-residues long, as shown by the activity of enoxaparin, to effectively inhibit NE. This is important for therapy development because these considerations determine the amount of dornase to use to reduce DNA length to less than 6-mer as well as determine the minimal length of the ODSH chain to reach full inhibition potency.

Another mainstay of CF maintenance therapy is inhaled 7% hypertonic saline (HS). This is most probably because HS increases sputum hydration, which improves mucociliary clearance. Our data on the salt dependence of NE inhibition suggest that ODSH retains stronger interactions with NE at all concentrations of NaCl tested; as high as 2.1% (w/v) was tested.
Given that the half-life of inhaled heparin in the alveoli is 28 h (30), inhaled ODShS may retain significant anti-NE efficacy even following inhaled HS. Considering that inhaled HS quickly disperses in the lung and increases airway surface liquid volume (31), it can be expected that ODShS will function well as an anti-NE agent with HS.

We and others have previously reported that NE and other proteases are critical targets to prevent impairment of innate immunity. Inhibition of NE would also reduce the load of other proteases (e.g., matrix metalloproteases), neutrophilic cytokines/chemokines, oxidative stress, and airway iron, which promote bacterial proliferation and biofilm generation. Recently, we have shown that NE also increases HMGB1 release in vivo, which activates the RAGE receptor, resulting in increased inflammation and mucin (32). Thus, ODShS is likely to have diverse anti-inflammatory activities, including blocking HMGB1, RAGE, L- and P-selectin, and NF-κB activation (10). The current results lay out the molecular principles for developing heparin-based anti-protease therapy in CF.

**Experimental procedures**

**Materials**

Human NE purified from sputum (catalogue no. SE563) and fNE (catalogue no. FS563) were obtained from Elastin Products Co. (Owensville, MO). N-Succinyl-Ala-Ala-Val-p-nitroanilide (S1384), HEPES buffer (H0887), DMSO (D2650), and DNase Co. (Owensville, MO).

**Sputum collection**

The protocol for sputum collection was approved by the institutional review board at Virginia Commonwealth University (HM12402). Written informed consent from each subject was obtained before enrollment into the study. Participants were recruited from the CF Care Center at VCU Medical Center. Spontaneously expectorated sputum was collected from each subject during spirometry as part of their routine evaluation in clinic for the VCU Sputum Biorepository. Within 2 h of collection, sputum was visually separated from saliva and oral detritus and stored at −80 °C for analysis later.

**Sputum processing with mucolytics**

Sputum samples from CF subjects (n = 40) were thawed on ice, weighed, and mixed with either normal saline alone, at a ratio of 1:1 (w/v), or normal saline containing an individual mucolytic agent and processed as described below. Mucolytic reagents included NAC (20% buffered acetylcysteine), 0–100 μM solution added at a ratio of 1:2 (w/v), Sputolysin (0–20%; 0.1% DTT is equivalent to 10% sputolysin) added at a ratio of 1:2 (w/v), and bovine DNase-I (0–0.3 mg/ml), a substitute for dornase, added at a ratio of 1:1 (w/v). NAC- and DNase-I–treated samples were mixed thoroughly via gentle vortexing and incubated at 37 °C for 2 h. Next, the homogenized sputum samples were centrifuged at 25,000 rpm for 30 min at 4 °C, and the supernatant was used for measuring NE activity.

Sputolysin-treated samples were mixed gently via vortexing and incubated at 37 °C for 15 min, followed by the addition of an equal volume of normal saline and incubation at 37 °C for 10 min. Last, homogenized sputum samples were centrifuged at 25,000 rpm for 30 min at 4 °C, and the supernatant was used for measuring NE activity.

**Neutrophil elastase activity assay**

NE activity in sputum supernatants was measured using a chromogenic 96-well microtiter plate assay (33). Briefly, NE was reconstituted in 1:1 glycerol/0.02 M sodium acetate (pH 5) buffer, and serial dilutions were included in the assay to create a standard curve. NE standards were prepared from 0.23 to 30 μg/ml (0.01–1.0 μM) in 125 mM HEPES buffer (pH 7.4, 0.125% Triton X-100). Sputum supernatant or NE standards, diluted 1:1 (100 μl) in HEPES buffer, were loaded on a microtiter plate and incubated at room temperature for 15 min with gentle agitation, and the reaction was initiated by the addition of the S1384 chromogenic substrate (3 μl in 50% DMSO, 50 μl). The OD was measured at 30-s intervals for 5 min. NE activity was calculated from the slope of the time-dependent increase in OD at λ = 405 nm. The maximum change in OD per minute (Vmax/min) was derived, and the corresponding elastase concentration was determined from the standard curve.

**Treatment of sputum with ODShS**

Frozen sputum samples were thawed on ice, weighed and mixed with either normal saline or normal saline containing an appropriate amount of each respective mucolytic, and incubated at 37 °C, as described above. After incubation, ODShS (1–100 μM) was added, and samples were incubated for a further 2 h at 37 °C. Next, samples were centrifuged at 25,000 rpm for 30 min at 4 °C, and the supernatant was used for measuring NE activity.

**Inhibition of NE activity by heparins and DNA in buffer**

Direct inhibition of NE was measured using a chromogenic substrate (S1384) hydrolysis assay using a microplate reader (FlexStation III, Molecular Devices), as reported earlier (34, 35). NE inhibition assays were performed in a 96-well plate with a final volume of 100 μl with a 0.125 mM HEPES buffer, pH 7.4, containing 0.125% Triton X-100. To each well of the 96-well plate containing 88 μl of the buffer was added 4 μl of 2 μM NE (effective concentration ~80 nM) and 0–100 μM heparins including ODShS (Mw 10,000), fondaparinux (Mw 1,772), enoxaparin (Mw 4,500), or unfractionated heparin (Mw 15,000). After a 10-min incubation, 3 μl of 25 mM S1384 (effective concentration ~750 μM) was rapidly added, and the residual enzyme activity was measured from the initial rate of increase in the absorbance at 405 nm. For DNA polymers, three random oligonucleotide sequences (6-, 12-, and 24-mer, all with 50% GC
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content) with complementary sequences for double-strand formation were annealed to create dsDNA oligomers (sequence information is provided in the supporting material). The final concentration of double-stranded oligonucleotides was 50 μM. The rate of NE-catalyzed S1384 hydrolysis remained linear over the assay conditions. The relative residual enzyme activity at each concentration of the polymers was calculated from the ratio of the enzyme activity in the presence and absence of the polymers. Logistic Equation 1 was used to fit the dose dependence of residual protease activity to obtain the potency (IC$_{50}$) and efficacy (ΔY) of inhibition. In this equation, Y is the ratio of residual NE activity in the presence of inhibitor to that in its absence (fractional residual activity); $Y_M$ and $Y_0$ are the maximum and minimum possible values of the fractional residual proteinase activity, respectively; IC$_{50}$ is the concentration of the inhibitor that results in 50% inhibition of enzyme activity; and HS is the Hill slope. Nonlinear curve fitting resulted in calculation of $Y_M$, $Y_0$, and IC$_{50}$.

\[
Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(\log IC_{50} - \log C_{inhibitor}) \times HS}} \quad \text{(Eq. 1)}
\]

Michaels–Menten kinetics of S1384 hydrolysis

The initial rate of substrate hydrolysis by 80 nM NE was monitored from the linear increase in absorbance at 405 nm corresponding to less than 10% consumption of the substrate. The initial rate was measured as a function of various concentrations of S1384 (0–3 mM) in the absence and presence of fixed concentrations of ODSH or DNA 24-mer (5–20 nM) following a 15-min incubation in a 0.125 M HEPES buffer containing 0.125% Triton X-100. Nonlinear regression was performed to derive one set of parameters that globally fit all profiles at varying levels of the two inhibitors (ODSH and DNA) using the traditional mixed inhibition Equation 2 described in the literature (26).

\[
V_i = \frac{V_{max,app}[S]}{[S] + K_{m,app}} \quad \text{(Eq. 2)}
\]

where

\[
V_{max,app} = \frac{V_{max}}{1 + \frac{[I]}{\alpha K_I}} \quad \text{(Eq. 3)}
\]

and

\[
K_{m,app} = \frac{K_m(1 + \frac{[I]}{\alpha K_I})}{1 + \frac{[I]}{\alpha K_I}} \quad \text{(Eq. 4)}
\]

Salt dependence of NE inhibition by ODSH and 24-mer DNA

The dependence of NE inhibition by ODSH and 24-mer on Na$^+$ concentration was studied using the S1384 hydrolysis assay, described above. The assay was performed in a 96-well plate with a final volume of 100 μl in a 0.125 M HEPES buffer containing 0.125% Triton X-100 and varying Na$^+$ levels in the range of 65–265 mM. The relative residual NE activity at each concentration of the inhibitors at respective Na$^+$ levels was calculated from the ratio of NE activity in the presence and absence of the inhibitors. The data were fitted using Equation 1 to obtain the potency (IC$_{50}$) and efficacy (ΔY) of ODSH and 24-mer DNA at the different Na$^+$ concentrations.

Computational studies on the interaction of ODSH and DNA with NE

The coordinates for human NE were first extracted from the crystal structure 1HNE from the Protein Data bank (36). Hydrogen atoms were added using SYBYL X2.1 (Tripos Associates, St. Louis, MO), and the structure was minimized with fixed heavy-atom coordinates using the Tripos force field for a maximum of 10,000 iterations subject to a termination gradient of 0.05 kcal/(molÅ). The structure of unbound dsDNA molecules was built using SYBYL X2.1 with the “build biopolymer DNA helix” option. The residues of each complementary chain were numbered consecutively with identical chain identification. For this study, the models were generated in B-DNA conformation, as it is the naturally occurring form of DNA in the living cells. DNA sequences of multiple lengths (6-, 12-, and 24-mer) were built. Molecules built were capped on the termini, and AMBER charges were added and energy-minimized for 10,000 iterations with a termination gradient of 0.05 kcal/(molÅ). A library of 1,728 ODSH hexasaccharide sequences was constructed based on earlier reports (21, 22, 37, 38) and energy-minimized using Tripos force field with Gasteiger–Hückel charges, a fixed dielectric constant of 80, and a non-bonded cutoff radius of 8 Å.

The HADDOCK (high ambiguity-driven protein–protein docking) web server (39) was used to predict the binding and interaction of dsDNA of various lengths (6-, 12-, and 24-mer) with NE. In NE–DNA docking using HADDOCK, the residues of the interface were defined as all basic residues that were surface-exposed. The ambiguous interaction restraints, which consist of active and passive residues, were defined primarily based on the above approximation. Each DNA oligomer was repeatedly docked in triplicate to NE to check for consistency in binding. The best cluster complex was ranked based on the type of interactions, such as hydrogen bonds, hydrophobic and electrostatic interactions, stability scores, the binding energy, and HADDOCK scores. The lower the score for a particular cluster, the higher is the probability of its complex formation. For NE–ODSH docking, a library of hexasaccharide sequences were selected and docked using GOLD version 5.2.2 (40), which uses a genetic algorithm to place ligands into the binding site. The tentative binding site for ODSH was defined based on the literature (13). Each ODSH structure was docked using 100 genetic algorithm runs, each consisting of 100,000 iterations. A 16-Å docking radius was used to define the binding site. The genetic algorithm runs were allowed to terminate early if the top three solutions had an RMSD of 2.5 Å or lower. The two best poses were stored and analyzed at the end of the docking experiment. Experiments were carried out in triplicate, which would yield at least six solutions for each sequence. GOLD-Score and RMSD were used to assess the fitness of the docked poses.
Binding affinity of ODSH and DNA for NE

Fluorescence experiments were performed using a PTI QM 400 fluorospectrophotometer (HORIBA Scientific, Edison, NJ) in 20 mM Tris–HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂, and 0.1% PEG 8000 at 37 °C. The affinities of fNE for ODSH and DNA were measured using the change in fluorescence of fNE (λex = 492 nm, λem = 516 nm) as a function of ligand concentrations [L], as described earlier (41). Titrations were performed by adding aliquots of aqueous stock solutions of ligand to 200 μL of fNE (150 nM) and monitoring emission intensity. To assess competition between ODSH and 6-mer DNA, labeled fNE (300 nM) was incubated with specified concentrations of 6-mer DNA, followed by the addition of aliquots of ODSH. Excitation and emission slits were set to 1.0 mm. The observed change in fluorescence (ΔF) relative to initial fluorescence (F₀) was fitted using a standard single-site binding model with a nonspecific component to obtain the apparent dissociation constant (K'D_app) and the maximal change in fluorescence (ΔFmax) at saturation. Each measurement was performed at least in duplicate.

Statistical analysis

Data are expressed as mean ± S.D. Statistical analyses were performed using a statistics program (Statistix version 8.0, Analytical Software, Tallahassee, FL). The Kruskal–Wallis one-way, nonparametric analysis of variance test, followed by post hoc comparisons by the Wilcoxon rank sum test, was used to determine significant differences between treatment groups. Statistically significant differences between groups were pre-defined as p < 0.05.

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