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On the Process of Discovering Leads that Target the Heparin-Binding Site of Neutrophil Elastase in the Sputum of Cystic Fibrosis Patients

Shravan Morla^{1,2}, Nehru Viji Sankaranarayanan^{1,2}, Daniel K. Afosah^{1,2}, Megh Kumar^{1,2}, Apparao B. Kummurapurugu³, Judith A. Voynow³, Umesh R. Desai^{1,2}

¹Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, Virginia 23298,

²Institute for Structural Biology, Drug Discovery and Development, Virginia Commonwealth University, Richmond, Virginia 23219

³Children's Hospital of Richmond at Virginia Commonwealth University, Richmond, Virginia 23298

Abstract

Cystic fibrosis (CF) is a disease of dysregulated salt and fluid homeostasis that results in massive accumulation of neutrophil elastase resulting in lung degradation and death. The current CF therapy, which relies on mucus modifying agents, inhaled deoxyribonuclease, and hypertonic saline, improves quality of life but does not target neutrophil elastase, a key mediator of chronic lung inflammation and an established biomarker of disease progression. It is highly challenging to identify small anti-neutrophil elastase agents that would work in sync with current therapy. In fact, several orthosteric inhibitors, including some in clinical trials, have suffered setbacks. We reasoned that small, synthetic, allosteric agents that target the heparin-binding site of neutrophil elastase without being affected by components of current therapy would offer a new therapeutic paradigm. Screening a focused library of non-saccharide glycosaminoglycan mimetics (NSGMs), we discovered a group of 23 promising inhibitors of neutrophil elastase. We developed a protocol based on fundamental computational, biochemical, mechanistic, and adverse effects studies to identify one promising NSGM that worked simultaneously with current anti-CF agents. This NSGM fully neutralized neutrophil elastase present in the sputum of six CF patients only in the presence of deoxyribonuclease with sustained activity under high salt conditions. Our work develops the process for discovering potent heparin-binding site-based allosteric anti-CF agents

CORRESPONDING AUTHOR INFORMATION: Institute for Structural Biology, Drug Discovery and Development, Virginia Commonwealth University, Richmond, VA 23219. Tel.: 804-828-7575; Fax: 804-827-3664; urdesai@vcu.edu.

AUTHOR CONTRIBUTIONS

SM performed library screening, inhibition and mechanistic studies, analysed the results and wrote the first draft of the manuscript; NVS performed and analysed computational experiments and wrote that section; DKA and MK performed inhibition experiments; ABK performed experiment on human sputum samples; JAV supervised human sputum studies and edited the manuscript; URD directed the project, analysed the results and wrote the manuscript.

Additional tables and figures showing chemical structures of the 60 NSGMs, computational GOLDScores, interactions between NSGMs and HNE, salt-dependence on the inhibition of HNE by NSGMs, plasma clotting and selectivity studies, inhibition of cathepsin G and proteinase 3 by NSGM 32, and sputum studies. Molecular strings formula of the 60 NSGMs is also available.

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and presents a novel molecule for further development in animal models of CF with disease manifestations similar to humans.

Graphical Abstract

INTRODUCTION

Cystic fibrosis (CF) is an orphan indication with about 100,000 patients worldwide, for whom the quality of life is not good because of complex, time-intensive and inadequate treatment options.^{1–4} The median age at death is approximately 40 years, during which a majority of patients have to bear hospitalizations and a demanding daily regimen to maintain health.⁵ Although multiple organs are affected by CF, lung disease progression to respiratory failure is the primary cause of death.

CF is a disease caused by functional deficiency of a trans-membrane protein that transports chloride and bicarbonate ions, called the CFTR protein. Mutations in the *CFTR* gene results in dysregulation of fluid homeostasis, which triggers thickening of mucus, thereby clogging airways and trapping bacteria leading to infections. This activates the immune system, which recruits and activates neutrophils that release oxidants and proteases, especially elastase. In fact, elastase is detected very early in CF infants and is now classified as a biomarker of disease progression.^{6,7} Frequent and prolonged infections cause the death of large numbers of neutrophils, which release their cellular contents, e.g., DNA, that form neutrophil extracellular traps (NETs) for sequestering pathogens.⁸ But this also releases massive amounts of elastase, which degrades elastin, the fibrous connective lung tissue that injures the airways resulting in bronchiectasis, respiratory failure and death.

Currently, there are three approved CFTR targeted therapies (ivacaftor, lumacaftor and tezacaftor) for people with particular mutations types, including the most common Phe508del.⁹ For people with other types of mutations, the Cystic Fibrosis Foundation (CFF) recommends a combination therapy including an inhaled deoxyribonuclease to reduce sputum viscosity, hypertonic saline to enhance hydration and clearance of sputum, and antibiotics to treat exacerbations of bronchitis.³ The combination therapy addresses several factors associated with disease severity and has been successful in improving quality of life; however, there are no approved therapies that mitigate inflammation. In fact, “*no other lung disease is known to induce such an early, sustained and intense inflammatory process*”.¹⁰ Considering that neutrophil elastase, a mediator of chronic inflammation, is an established biomarker, inhibition of its activity could be expected to improve patient outcomes.^{11,12}

Human neutrophil elastase (HNE) is a serine protease belonging to the chymotrypsin family that preferentially recognizes small aliphatic amino acids.¹³ Although the discovery of

synthetic inhibitors of HNE has been pursued for some 50 years,^{14,15} only alpha-1 HC, alvelestat,¹⁶ and POL6014 from the thousands of inhibitors designed and investigated have reached phase II studies as CF therapeutics.¹⁷ Alvelestat, a reversible, potent and selective inhibitor of HNE, did not affect sputum neutrophil count, HNE activity, lung function or clinical outcomes for CF patients.¹⁸ Despite this major setback, development of active site inhibitors for resolving HNE insult to the CF lung is continuing.¹⁹

We reasoned that agents that allosterically inhibit HNE, while simultaneously administering the cocktail of sputum modifiers (DNAse and hypertonic saline) used in the clinic today, may succeed better than the oral, active site inhibitors developed so far. Yet, discovering such agents is challenging because the process has not been developed. Also, small allosteric inhibitors of HNE are not known. Although allosteric inhibition by polymeric, highly charged biopolymers, such as DNA and heparin, has been studied, these are unlikely to succeed clinically because of the use of DNAse and hypertonic saline in therapy. DNAse would cleave DNA and eliminate anti-HNE activity, while high salt would reduce the affinity of heparin for HNE and drastically reduce its efficacy.

In this work, we describe the development of a process for discovering small, highly water soluble agents that could be instilled directly into the lung with DNAse and hypertonic saline and induce inhibition of HNE. We present that non-saccharide glycosaminoglycan mimetics (NSGMs) offer a major class for the discovery of allosteric, anti-HNE inhibitors. NSGMs are small, homogeneous, highly water-soluble agents that are untouched by DNAse. Although the interaction of HNE with NSGMs can be susceptible to high salt, our work shows that it is possible to identify agents that function in this hostile environment. Our work has identified NSGM **32** as a promising allosteric, selective anti-HNE agent with good potency in human CF sputum. Finally, our work opens up a new therapeutic approach for treatment of inflammatory conditions wherein a high level of elastase is the primary driver of pathology but is also aided by secondary factors such as DNA and NETs.

RESULTS AND DISCUSSION

Rationale behind synthetic mimetics of glycosaminoglycans (GAGs) as inhibitors of HNE.

Because of the critical role of HNE in a number of inflammatory lung pathologies such as CF, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), pneumonia, and bronchiectasis, GAGs have been heavily studied as inhibitors of HNE.^{20–26} In fact, this inhibition of HNE has been suggested to be fairly selective with heparin being the most potent GAG. However, GAGs are highly heterogeneous polymers, which carry considerable risks owing to their interaction with a host of proteins in blood, lung and other organs.^{26,27} This is especially important because under inflammatory conditions in the lung, the levels of HNE jump several 100-fold, which necessitates high dosing of GAGs, thereby possibly inducing enhanced adverse consequences, especially bleeding.

In contrast, small, synthetic, mimetics of GAGs possess characteristics that are essentially devoid of concerns besetting the natural biopolymers. These agents, which are called non-saccharide GAG mimetics (NSGMs), are fully synthetic and homogeneous, completely

unlike GAGs.²⁸ They are much smaller than GAGs and therefore devoid of the extensive sequence of negative charges. They also are more prone to bind to enzymes selectively, thereby displaying reduced non-specific binding in comparison to GAGs.^{29–35} Thus, we hypothesized that an optimal anti-HNE agent that works in tandem with DNase and hypertonic saline should be possible to discover from a library of synthetic NSGMs. Such an agent may be more appropriate for management of CF.

Recently, we discovered that an NSGM called G2.2 (Figure 1) was a structural and functional mimetic of the hexasaccharide sequence of heparin (HS06).^{36,37} Comparative molecular docking of G2.2 and HS06 onto HNE showed that the NSGM could bind to HNE with higher affinity than the oligosaccharide (Figure 1). In fact, the predicted pose of G2.2 bound to HNE overlaid well with that of HS06, which allosterically inhibits the protease, while also competing with a DNA sequence binding to the same allosteric site.^{21,38} Moreover, G2.2 is known to display minimal anticoagulant properties suggesting the absence of putative bleeding risk in the lung.³⁷ We, therefore, hypothesized that screening a library of small, synthetic NSGMs related to G2.2 would open up a new route to unique, allosteric inhibitors of HNE that are completely different from the traditional orthosteric inhibition route that has not worked so far. Such an agent would be homogeneous, relatively easier to synthesize, and compete more efficiently against the substantial amounts of DNA released in the CF lung.

Structure – activity relationship for the library of NSGMs.

We screened a library of 60 NSGMs (Figure S1, Supplementary Information) that has been known to mimic different functions of GAGs such as anticoagulation, anticancer (colon, breast, and pancreatic), and antifibrinolysis.^{30–32,39–41} The 60 NSGMs belonged to 5 different chemical scaffolds with a wide range of sulfation (1 to 12 moieties), which ensures high probability of resembling the variable sulfation pattern of GAGs. The different chemical scaffolds engineer diversity in three-dimensional orientation of sulfate groups and aromatic rings, which are key to recognition of a complementary protein surface. The rationale was that ionic and hydrogen-bonding interactions, originating from sulfate groups, plus hydrophobic and π interactions, originating from the aromatic scaffold, will lead to selective recognition of HNE.

Screening the NSGM library against HNE using a chromogenic substrate hydrolysis assay at high inhibitor concentration (200 μ M) showed a wide range of inhibitor efficacies (0 – 98%; Figure 2A). As would be expected, HNE inhibition seemed to be highly sensitive to the chemical scaffold of NSGMs. For example, a majority of the benzofuran monomers (**1** – **11**) and benzofuran dimers (**18** – **28**) displayed marginal inhibition (< 40 %), while apigenin (**29** – **35**) and quercetin dimers (**36** – **46**) showed significant inhibition (> 80%) (see structures in Supplementary Figure S1). Interestingly, although HNE inhibition generally increased with the level of sulfation, there were exceptions. NSGM **15** with 5 sulfate groups displayed lower HNE inhibition (~50%) when compared to NSGM **34** (98%), which carried only 4 sulfate groups.

Of the 60 NSGMs, 23 members showing >80% inhibition (Figure 2B) of HNE were processed for IC₅₀ profiling. These molecules showed a very broad range of IC₅₀s from 40

nM – 160 μ M (Figure 3; Table 1). The results show exquisite structural dependence of inhibition with large changes in potency (IC_{50}) arising from small changes in the functional groups. For example, the apigenin series of NSGMs, i.e., **29** – **35**, was generally more than 100-fold less potent than the quercetin series (**36** – **45**), except for NSGM **32**. Within the quercetin series, several agents displayed very promising activity, e.g., **36** – **41**. Interestingly, contraction of the linker between two quercetins from a propyl chain to an ethyl chain decreased IC_{50} nearly 28-fold (**41** v/s **42**, Table 1). Likewise, introducing rigidity in the linker (**43** v/s **45**) reduced potency by a factor of 24. Changing the scaffold from morin to quercetin was detrimental to HNE recognition (**41** v/s **46**, 12.8-fold loss), an impact also replicated upon changing from a dimeric to a trimeric scaffold (**41** v/s **58**). Overall, the screening brought forth a group of several sub-micromolar NSGM inhibitors, which represented an impressive hit ratio of ~13%.

Computational analysis of the preferred site of NSGMs binding to HNE.

We resorted to *in silico* studies of the 23 promising NSGMs to elucidate their potential mode of binding onto HNE. We have earlier utilized a genetic algorithm-based docking protocol to identify the preferred binding site, pose and interaction scores of GAGs and NSGMs with proteins including HNE.^{27,38,39} Exhaustive search of the HNE surface revealed that an allosteric site ~13.5 Å away from the active site (center point of binding site and the center point of the catalytic triad) is the preferred site of binding for NSGMs, GAGs and DNA. For the 23 inhibitors, analysis of the binding poses showed that most NSGMs bound HNE with high consistency and high binding score (see Supplementary Table S1). When pIC_{50} ($= -\log IC_{50}$) was plotted against the predicted *in silico* ‘affinity’, an excellent linear relationship with an R^2 of 0.81 was observed (Figure 4B). This represents a strong structure–function correlation across different scaffolds and sulfation diversity for NSGMs.

At an atomistic level, the NSGMs utilized hydrogen bonding, electrostatic (charge and π –charge) and hydrophobic (π – π ; π –alkyl) forces in recognizing the site on HNE (Table S2, Supplementary Information). Interestingly, analysis of binding poses indicated that several members, e.g., **32**, **34**, and **44**, preferred to bind in the allosteric site in an orientation that facilitated interaction with active site residues H57 and S195 (Figure S2, Supplementary Information). Such dual interaction is unusual and arises from the juxtapositioning of the two sites, the active site and allosteric site. In this context, the NSGM–HNE co-complex appears to be a relatively rare system. Of the 23 members, NSGM **32** was especially interesting because it exhibited extensive interactions with a large number of electropositive, polar as well as non-polar residues resulting in a fairly high *in silico* score of 106.3, although it has only four sulfate groups (Figure 4C). Further analyses of NSGM **32**’s binding to HNE suggests that makes key interactions with residues in the subsites S3’–S2 (Schechter and Berger nomenclature; Figure S3, Table S2, Supplementary Information). The *in silico* analysis explains its unusually low IC_{50} (0.7 μ M) in comparison to its nearest neighbors (**29** – **31** and **33** – **35**), while also presenting it as a putative agent of high promise. Further, loss of its four sulfate groups, as in unsulfated precursor **32a** (Figure S3, Supplementary Information) completely eliminated HNE inhibition potential suggesting that sulfates are key for **32**’s inhibition of HNE.

Biochemical mechanistic support for computational prediction on NSGM 32.

To assess whether the unusual binding mode discovered above could be experimentally supported, we performed substrate kinetic studies with NSGM **32**. The Michaelis-Menten parameters were measured from the hyperbolic dependence of enzyme activity on chromogenic substrate concentration in the absence and presence of the NSGM (Table 2). Whereas V_{MAX} and K_M in the absence of an inhibitor were 40.13 mAU/min and 650.8 μ M, respectively, the presence of **32** depressed V_{MAX} by ~2.5-fold mAU/min and enhanced K_M by ~3-fold (Figure 5). This profile is classically called a mixed inhibition profile and arises from a conformational change as well as steric blockade in the active site induced by an inhibitor. Thus, **32** utilizes a mixed inhibition profile confirming the predictions of computational studies.

Multiple NSGMs retain potent inhibition of HNE in high salt.

Considering that the current CF therapy utilizes hypertonic saline to relieve mucus build up in patients, we studied HNE inhibition potential of the nine most promising NSGMs (**32**, **36** – **38**, **40**, **41**, **58** – **60**) in the presence of high salt levels. Although it is difficult to estimate the concentration of salt around mucus in a CF lung upon instillation of hypertonic saline (~7% v/v), it could undergo significant dilution. Hence, we studied the dependence of HNE inhibition at varying salt concentrations (62.5 – 462.5 mM) (see Supplementary Information, Figure S4, Table S3). The profiles of the measured IC_{50} s as a function of salt concentration show very interesting trends (Figure 6). Although all agents retained good anti-HNE activity at salt levels as high as 362.5 mM (2.1% w/v), **37**, **41**, and **58** displayed a dramatic loss in potency at 462.5 mM salt, which implied that these may not be suitable with hypertonic saline. The remaining NSGMs showed some loss in the inhibitory potential with salt, except for **32**, **36**, and **60**, which displayed essentially invariant potencies through 2.1% v/v NaCl. This implied that the interaction of these NSGMs with HNE relies on strong non-ionic forces with some ionic component.

NSGMs 32 and 60 inhibit cleavage of macromolecular substrate elastin.

Although HNE cleavage of small peptidic substrates is the obvious assay used in initial lead identification, inhibition of cleavage of macromolecule elastin, which forms the connective tissue of the lung, is much more relevant to *in vivo* function. Additionally, it is not necessary that a small molecule inhibitor should continue to exhibit inhibition of macromolecular cleavage because the binding energy gained upon complexation with a macromolecule, which is typically high, may help disengage the small inhibitor from its allosteric binding site. Thus, identifying NSGMs that maintain prolonged inhibition of elastin degradation is of particular interest for CF therapy.

To assess whether NSGMs inhibit cleavage of elastin, we used an elastin-congo red assay,⁴² in which the insoluble macromolecular substrate releases congo red in solution upon cleavage. Thus, we measured soluble congo red concentration following incubation of HNE, elastin-congo red and NSGM at various time points (Figure 7). Of the four most potent NSGMs identified above, **32** and **60** displayed >90% inhibition as compared to NSGMs **36** and **58**, which displayed only partial inhibition (60 – 70%). More importantly, although each NSGM inhibited HNE well (>50%) for a reasonable time period (4 hr), **36** and **58** had begun

losing their HNE inhibitory activity by ~12 hours. In sharp contrast, NSGMs **32** and **60** retained their full inhibition potential for over 48 hr, which could turn out to be important *in vivo*.

NSGM 32 is not a potent anticoagulant.

Although heparin inhibits HNE very potently, it is not suitable for reducing inflammation associated with HNE because of its powerful anticoagulant activity.¹⁴ For an NSGM to be useful in CF, it would be important to ensure the absence of an anticoagulant property. The anticoagulant properties of NSGMs **32** and **60** were studied by measuring the concentration of these agents required to double the two clotting parameters, activated partial thromboplastin time (APTT) and prothrombin (PT), in human plasma. Whereas **32** did not affect either PT and APTT at concentrations as high as 855 μ M (Figure 8A), NSGM **60** had major effects on both APTT and PT (Figure 8B). The concentration of **60** required to double APTT and PT was 49 μ M and 152 μ M, respectively (Table S4, Supplementary Information).⁴⁰ These results indicate that NSGM **32** lacks anticoagulant activity in human plasma and would be an excellent HNE inhibitor for further functional studies.

To confirm these results, we studied inhibition of human coagulation serine proteases, factor Xa, factor XIa, thrombin, and plasmin by **32** (Figure 8C). None of the coagulation proteases studied were particularly targeted by **32**. For example, **32** was 28 times more selective for HNE than factor XIa, while the selectivity increased >50-fold against factor Xa, thrombin, and plasmin (Table S5, Supplementary Information). Thus, NSGM **32** could be expected to have lower bleeding consequences in comparison to heparin when instilled in the lung.

NSGM 32 is also an inhibitor of cathepsin G, another inflammatory protease.

Apart from HNE, cathepsin G and proteinase 3 are known to be significantly upregulated contributing to the pathogenesis of lung inflammatory conditions.⁴³ Earlier work has shown that polymeric GAGs or GAG-like molecules⁴⁴ and/or peptides⁴⁵ inhibit one or both of these neutrophil serine proteases (NSPs). Yet, no small molecule has been identified to allosterically target these agents. We tested the ability of **32** to inhibit cathepsin G and proteinase 3 using standard chromogenic substrate hydrolysis assays. NSGM **32** inhibited cathepsin G with an IC₅₀ of 1.1 μ M, but did not inhibit proteinase 3 (IC₅₀ = 54 μ M) (Figure S5, Supplementary Information). Thus, **32** was found to be a dual HNE/cathepsin G inhibitor. We expect this dual inhibition property to be especially useful for its potential in re-establishing protease–anti-protease balance in inflammatory conditions such as CF. Yet, the biochemical mechanism of this NSGM's inhibition of cathepsin G would need to elucidated.

NSGM 32 inhibits high levels of HNE in human sputum from CF patients.

Sputum from CF patients is thick viscous material and contains diverse types of species such as DNA, mucin polymers, HNE, cytokines, salt, inflammatory cells, bacteria, degraded neutrophil fragments, etc.⁴⁶ Much of the HNE in sputum exists in sequestered form bound to the massive amounts of DNA released as NETs by dying neutrophils. Our earlier work has shown that this CF sputum HNE cannot be inhibited by heparins without enzymatic degradation of DNA with DNase.³⁸ Likewise, in the absence of DNase, no reduction in HNE

activity was observed for sputum samples from 6 CF patients even with 2 mM **32**. Hence, the 6 sputum samples were first incubated with NSGM **32** and DNase so as to solubilize the mucus. Following centrifugal removal of non-soluble debris, HNE activity was measured using chromogenic hydrolysis assay. In the presence of DNase, the HNE activity of human sputum samples was reduced with increasing concentrations of **32** (Figure 9A). Although significant decrease in the HNE activity could not be observed until concentrations of **32** reach >250 μ M, a dose-dependent decrease was noted with subsequent increases in levels such that **32** reduced HNE activity to basal levels. More specifically, ~2 mM **32** was necessary to fully inactivate HNE.

It was immediately not clear why such concentrations of **32** are needed to inhibit HNE. We initially reasoned that the human sputa carries high levels HNE, especially after treatment with DNase, which correspondingly requires high concentrations of **32**. Hence, we studied the introduction of high salt (400 mM) to sputum samples containing DNase and NSGM **32**. Figure 9B shows the HNE inhibition profile under these conditions. Interestingly, high salt enhanced the release of more (~15-fold) HNE as compared to that in its absence. Yet, the HNE inhibition profile followed a trend similar to that at normal salt levels. Thus, once again 2 mM **32** was necessary to reduce the HNE activity of human CF sputum to basal levels.

Considering that the affinity of **32** for HNE is in the high nM range, it was disconcerting to note that ~2 mM **32** was needed in the presence of both DNase and high salt. We reasoned that because human sputum is a highly heterogeneous, thick fluid that carries a large number of components including biopolymers and bacteria, not all NSGM **32** was perhaps becoming available for inhibiting HNE. Hence, we used spectrofluorimetry to measure the levels of **32** in the sputum supernatant, which was used to measure HNE activity. We found that the concentrations of **32** were approximately 10-fold lower (see Figure S6, Supplementary Information) for both conditions (DNase alone and DNase + high salt). Thus *in vitro*, a significant proportion of **32** is captured by the highly viscous component of sputum necessitating high levels of the inhibitor. We expect that *in vivo*, the flow and movement within lungs would help with dispersal of **32** resulting in a much improved inhibition profile.

CONCLUSIONS

The key conclusions from this work are i) it is possible to identify potent small molecule inhibitors of HNE that function through the heparin-binding site in the presence of two clinically used anti-CF therapeutics; ii) this *in vitro* process is fairly extensive and cannot just rely on high-throughput screening-based identification of inhibitors, which would fail because of the challenges of working with sputum; and iii) the process also turns out to be more involved than a traditional drug discovery campaign because of involvement of DNase and salt, both of which are necessary to clear the lung of mucous, bacteria and other releasates due to inflammation. Despite these challenges, the process of successive implementation of high-throughput screening, computational modeling, enzyme kinetics, and bleeding-related enzyme assays led to identification of one promising NSGM that worked simultaneously with current anti-CF agents. NSGM **32** fully neutralized neutrophil elastase present in the sputum of six CF patients ONLY in the presence of

deoxyribonuclease with sustained activity under high salt conditions. Thus, this work put forward a novel process for discovering a completely new type of anti-HNE agents for CF.

NSGM **32** presents major advantages. It is a small, highly water soluble agent, which implies that it can be formulated with both DNase and hypertonic saline with ease at virtually any desired dosage level for direct instillation into the lung. The structure, potency and mechanism of NSGM **32** are unique. In terms of selectivity of targeting, **32** does not potently target coagulation proteases, which also possess heparin-binding domains. This is important because direct instillation in the lung could induce bleeding in CF lung, which would be a serious consideration for developing a GAG-based anti-CF therapy.³⁸

Yet, **32** inhibits cathepsin G also with good potency. In addition to HNE, cathepsin G is also upregulated in CF and dual inhibition may turn out to be beneficial. This work does not elaborate on this interaction, especially on the nature of cathepsin G inhibition by **32** in the presence of high salt, which could drastically reduce the potency. Thus, a key line of structure-guided drug discovery in the future could be developing dual inhibitors that function in the presence of DNase and high salt.

Perhaps the most valuable property of **32** is its ability to potently target HNE under high salt conditions. Although the use of hypertonic saline in current CF therapy is to hydrate and aid clearance of mucus, with respect to a potential CF therapy based on NSGM **32**, high salt would have the additional function of reducing **32**'s non-specific interactions with lung proteins. It is well-established that highly sulfated GAGs bind to many proteins through non-specific interactions, which are greatly reduced as the ionic strength of the medium increases.^{27,47}

The *in vitro* success, favourable potency and low to nil high bleeding risk of NSGM **32** implies that the next step could be evaluating its *in vivo* profile. Several animal models, including ferret,⁴⁸ pig⁴⁹ or sheep⁵⁰, offer disease manifestations similar to humans.⁵¹ Finally, this work could have implications beyond CF too. Increased levels of HNE are observed in several other inflammatory conditions of the lung such as α 1-antitrypsin deficiency (AATD), COPD, acute lung injury, and adult respiratory distress syndrome.^{1,2} Although the etiology of inflammation in these disorders is not identical to CF, NSGM **32** may find use in reducing HNE activity in these clinical indications too.

EXPERIMENTAL SECTION

Materials.

Human sputum leukocyte elastase was obtained from Elastin Products Company (Owensville, MO). Human thrombin, factor Xa, factor XIa, and plasmin were obtained from Haematologic Technologies (Essex Junction, VT). Human leukocyte cathepsin G, human neutrophil proteinase 3, *N*-succinyl-Ala-Ala-Val-*p*-nitroanilide (S1384), *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (S7388), *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (M4765), elastin-congo red, and bovine pancreatic DNase (DN25) were obtained from Sigma (St. Louis, MO). Low-binding polypropylene 96-well plates for HNE activity assay were from (Greiner Bio One, Monroe, NC). Spectrozyme TH (H-*D*-cylohexylalanyl-Ala-

Arg-*p*-nitroanilide) and Spectrozyme factor Xa (methoxycarbonyl-*D*-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide) were from American Diagnostica (Greenwich, CT). Factor XIa chromogenic substrate (S-2366, *L*-PyroGlu-Pro-Arg-*p*-nitroaniline·HCl) was from Diapharma (West Chester, OH). APTT reagent containing ellagic acid (APTT-LS), thromboplastin-D, and 25 mM CaCl₂ were obtained from Fisher Diagnostics (Middletown, VA). The library of 60 NSGMs were synthesized with >95% purity using reported schemes^{30–32,39–41} and were characterized by NMR and UPLC-MS.

Sputum samples from Cystic Fibrosis patients.

The standard operating procedure for sputum collection was approved by the Institutional Review Board (# HM12402) at Virginia Commonwealth University (VCU). Written informed consent from each subject was obtained before enrolment 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 the clinic. Within 1–2 h of collection, sputum was processed to remove the salivary contamination and oral detritus, and stored at –80°C for further analysis.

Stocks and buffer conditions.

HNE (1 mg) was reconstituted with 100 µL of 1:1 glycerol:200 mM sodium acetate buffer, pH 5, which was diluted using HEPES buffer, pH 7.4, containing 125 mM HEPES, 0.125% Triton-X 100, and 100 mM NaCl to give stocks of desired concentrations. NSGMs were dissolved in ddH₂O to make stocks of 0.5 – 20 mM concentrations. Coagulation protease stocks were prepared in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂, 0.1% PEG8000, and 0.02% Tween 80.

Molecular modeling studies.

The coordinates of HNE were retrieved from the PDB (ID: 1HNE, 1.84 Å resolution) and prepared for modeling studies using Tripos Sybyl-X v2.2 (www.tripos.com/sybyl).⁵² Hydrogen atoms were added and 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/(mol·Å). The putative site of NSGM binding was identified using three independent approaches including a) primary sequence analysis for the presence of consensus sequence(s) that could bind to glycosaminoglycans (GAGs), b) calculation of the electrostatic surface potential (Figure 4A) using APBS tool in PyMOL (Molecular Graphics System, v1.5.0.4, Schrödinger, New York, NY), and c) review of literature on heparin binding to HNE.^{36,52} Based on this information, a 16 Å radius around Arg36 residue was defined as the putative site of NSGM binding. The structure of each NSGM was optimized in Sybyl and docked using GOLD v5.2, as described earlier.^{30,39} A GOLD algorithm relying on triplicate experiments, each with 300 genetic algorithm runs and 100,000 iterations, was employed. Each binding pose from the experiments performed in triplicate was scored using GOLDScore and the best two poses from each run were retained for calculation of consistency of binding in the form of root-mean-square-difference (RMSD) between the six docked poses.

Direct inhibition of HNE.

NSGMs were screened for inhibition of HNE using the S1384 hydrolysis assay in a 96-well plate format, as described earlier.³⁸ Briefly, 4 μ L of HNE and 1 μ L NSGM (or reference buffer) were added at 37 °C to 92 μ L of HEPES buffer to give 80 nM enzyme and 0 – 200 μ M inhibitor. After 5 min, 3 μ L of 25 mM S1384 was added and the residual enzyme activity was measured from the initial rate of increase in A_{405} . Inhibition studies at varying levels of ionic strengths were performed in HEPES buffer, pH 7.4, containing 62.5 to 462.5 mM NaCl. The IC_{50} was calculated using logistic equation

$$1 \left(Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(\log[I]_0 - \log IC_{50}) \times HS}} \right), \text{ in which } Y \text{ is ratio of residual enzyme activity in the}$$

presence of NSGM to that in its absence, Y_0 and Y_M are the minimal and maximal values of Y , respectively, obtained following regression, and HS is the Hill slope of inhibition.

Kinetics of substrate hydrolysis.

The initial rate of S1384 hydrolysis by ~80 nM HNE was monitored from the linear increase in absorbance at 405 nm corresponding to less than 10% consumption of the substrate.³⁸

The initial rate was measured as a function of various concentrations of S1384 in the presence of a fixed concentration of NSGM (0–200 μ M) in HEPES buffer, pH 7.4, at 37 °C. The data were analyzed using the standard Michaelis–Menten equation to determine K_M and V_{MAX} .

NSGM inhibition of HNE cleavage of a physiologic substrate.

The inhibitory activity of NSGMs for HNE degradation of elastin was measured using an elastin-congo red assay, as described earlier.⁴² Briefly, 272 μ L of 2 μ M HNE was incubated with 10 μ L of 4 mM NSGM (or vehicle) at 37 °C. After 30 min, 100–200 μ L of 50 mg/mL elastin-congo red in 100mM Tris-HCl buffer, pH 8, was added and the reaction diluted to 1 mL with buffer. The samples were placed on a shaker and incubated at 37 °C for 48 hours. At different time points, an aliquot of reaction mixture was withdrawn, centrifuged at 13000 rpm for 10 min, and the absorbance of the supernatant at 497 nm measured. The difference in A_{497} between NSGM containing samples and corresponding blank was used to calculate percentage inhibition of HNE.

Inhibition of related serine proteases by NSGM 32.

Inhibition of serine proteases, cathepsin G, proteinase 3, factor Xa, factor XIa, thrombin, and plasmin by NSGM **32** was determined using chromogenic substrate hydrolysis assays, as described earlier.^{29,30,43} The assays were essentially identical to that used for HNE, except for the use of substrates corresponding to each protease. Assays for NSPs were performed in HEPES buffer, pH 7.4 at 37 °C, and those for coagulation serine proteases were performed in 20 mM Tris-HCl buffer, pH 7.4, at either 25 °C (thrombin) or 37 °C (factor Xa, factor XIa, and plasmin). The final protease concentrations were 20 nM (cathepsin G), 100 nM (proteinase 3), 6 nM (thrombin), 1.09 nM (factor Xa), 0.75 nM (factor XIa) and 25 nM (plasmin), whereas chromogenic substrate concentrations were 2 mM (S7388 for cathepsin G), 5 mM (M4765 for proteinase 3), 0.25 mM (Spectrozyme FXa), 0.17 mM (S-2366), 0.1

mM (Spectrozyme TH) or 0.1 mM (Spectrozyme plasmin). IC_{50} was calculated from the variation of the relative residual enzyme activity as a function of **32** using logistic equation 1 (as above).

Human plasma coagulation studies.

APTT and PT assays were performed in the standard one-stage format (STA PT-Neoplastin CI, STA PTT- Automate, respectively) on the STA Compact Analyzer (Diagnostica Stago, Parsippany, NJ, USA), according to manufacturer's instructions.⁴³ An aqueous solution of target NSGM (0 – 1 mM final concentration) was added to 300 μ L of pooled human plasma from Valley Biomedical (Winchester, VA), incubated at 37°C for 5 min and the instrument operated in automated mode until collection of all clotting times. Experiments were performed in triplicate.

Inhibition of HNE activity in human sputum by NSGM.

Sputum HNE activity was measured, as described earlier.³⁸ Briefly, frozen sputum samples from CF subjects were thawed on ice, weighed and mixed with either low salt buffer (154 mM NaCl) or high salt buffer (400 mM NaCl) alone or with buffer containing DNase-1 (0.3 mg/mL) at the ratio of 1:1 (w:v). Following gentle vortexing, the mixture was incubated at 37°C for 2 h, followed by addition of NSGM **32** (0.5 – 2000 μ M), mixed and then incubated further for 2 h. Following incubation, samples were centrifuged at 25,000 rpm for 30 min at 4°C and the HNE activity in the supernatant was measured using chromogenic substrate hydrolysis assay, as described above. For re-calculation of concentrations of NSGM **32** in the supernatant of each sputum sample, its intrinsic fluorescence at 460 nm (λ_{ex} =250 nm) was measured and quantified against a standard preparation of the NSGM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CF	cystic fibrosis
NSGM	non-saccharide glycosaminoglycan mimetics
NET	neutrophil extracellular traps
CFF	cystic fibrosis foundation
HNE	human neutrophil elastase

APTT	Activated partial thromboplastin time
PT	prothrombin time
GAG	glycosaminoglycan
NSP	neutrophil serine proteases
ARDS	acute respiratory distress syndrome
HS06	heparin hexasaccharide

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Figure 1. Comparative modeling of G2.2 and a sulfated hexasaccharide sequence binding to human neutrophil elastase (HNE).

Using advanced genetic algorithm-based docking, G2.2 was found to bind to the same binding site on HNE as that recognized by a heparan sulfate hexasaccharide sequence and DNA. **(A)** shows the chemical structure of G2.2 **(B)** shows molecular overlay of the docked poses of G2.2 and **(B)** displays overlays of both hexasaccharide and DNA (12-mer). HNE is shown as white surface; G2.2 (in B) and hexasaccharide (in B) as sticks; and DNA as green ribbon. The active site of HNE is shown in van der Waals sphere rendering. The image of part B is taken with permission from *J. Biol. Chem.* **2018**, *293*, 12480–12490.

Figure 2. Discovery of NSGM inhibitors of HNE.

(A) Screening of 60 NSGMs against HNE using chromogenic substrate hydrolysis assay in 125 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 0.125% Triton-X 100 at 37°C. The concentrations of NSGM and HNE were 200 μ M and 0.1 μ M. Measurements were performed at least in duplicate and error bars represent ± 1 SD. (B) Structures of the 23 'hit' NSGMs identified from the library of 60 molecules.

Figure 3. Direct HNE inhibition profiles of NSGMs.

Dose-response profiles of the 23 promising NSGMs (structures shown in Figure 1B). Solid lines represent fits of the data to standard sigmoidal dose-response equation 1 to calculate IC_{50} , Y , and HS. Groups were chosen to avoid overlap of curves. (A) shows profiles of NSGMs 13 & 29 – 33; (B) shows 34 – 39; (C) presents 40 – 45; and (D) shows profiles of 46 and 54 – 60.

Figure 4. In silico studies identify the preferred site of NSGM binding on HNE.

(A) Surface electrostatics of HNE (PDB ID = 1HNE) calculated using APBS tool in PyMol.

Blue and red represent electropositive and electronegative surfaces, respectively. (B)

Relationship between pI (= -log IC₅₀ (μM)) and in silico 'affinity' (in terms of

GOLDScore). Similar colors attempt to show NSGMs of same scaffolds. (C) NSGM 32

interacts with basic, polar as well as non-polar residues of HNE. The preferred pose shows

binding to an allosteric site adjacent to the active site such that H57 is also engaged.

Figure 5. Kinetics of HNE inhibition.

Michaelis-Menten kinetics of chromogenic substrate hydrolysis by HNE in the presence of NSGMs 32. Solid lines represent the nonlinear regression fit to data, using Michaelis-Menten equation, to obtain K_M and V_{max} .

Figure 6. Parsing NSGMs for their HNE inhibition potential in high salt conditions.

The IC₅₀s of nine promising NSGMs were measured in buffer, pH 7.4, containing 62.5 to 462.5 mM NaCl. Symbols: **32** (black square), **36** (ochre circle), **37** (green triangle), **40** (yellow triangle), **41** (red square), **38** (green diamond), **58** (white circle), **59** (green circle), **60** (red diamond). Inset shows data for 262.5 and 362.5 mM NaCl concentrations.

Figure 7. Inhibition of HNE cleavage of macromolecular substrate elastin.

Inhibition by NSGMs **32**, **36**, **58**, and **60** was studied using elastin-congo red hydrolysis assay at pH 8. Congo red released upon elastin degradation is quantified by measuring absorbance at 497 nm. Experiments were carried out in triplicate. Error bars correspond to one standard deviation.

Figure 8. Anticoagulant properties of NSGMs 32 and 60.

Effect of (A) 32 and (B) 60 on human plasma clotting times (PT and APTT). Experiments were carried out in triplicate. Error bars correspond to one standard deviation. (C) Direct inhibition of HNE, factor Xa, factor XIa, thrombin, and plasmin by NSGM 32 at pH 7.4 and 37°C. Solid lines represent the sigmoidal fits to data to obtain IC_{50} using standard dose-response relationship.

Figure 9. NSGM 32 inhibition of HNE in sputum samples from CF patients.

HNE activity was measured after treatment with varying levels of **32** in the presence of 0.3 mg/mL DNase-1 under both (A) low salt (150 mM NaCl) {##, $p < 0.009$ vs NS control; **, $p < 0.015$ vs 0.3 mg/ml DNase-1} and (B) high salt conditions (400 mM NaCl) {##, $p < 0.002$ vs HS control; **, $p < 0.087$ vs 0.3 mg/ml DNase-1}. Note the difference in basal HNE activity (several orders of magnitude) of sputum with addition of salt and DNase-1. NS or HS represents HNE activity in the absence of both DNase-1 and NSGM **32**. Graph represents data from sputum samples from 6 patients.

Table 1.

Direct inhibition of HNE by NSGMs using chromogenic substrate hydrolysis assay.

NSGM	IC ₅₀ (μM)	Y (%) ^a	HS ^b
13	21.7 ± 4.7	98 ± 10	0.9
29	47.6 ± 12.7	103 ± 13	1.4
30	64.8 ± 10.4	88 ± 8	2.3
31	19.1 ± 2.6	97 ± 3	1.6
32	0.69 ± 0.12	90 ± 5	1.7
33	91.3 ± 7.5	99 ± 2	2.6
34	51.9 ± 6.3	95 ± 11	2.6
35	157 ± 8.4	93 ± 3	2.7
36	0.23 ± 0.02	96 ± 4	1.2
37	0.81 ± 0.14	93 ± 9	1.1
38	0.32 ± 0.05	95 ± 8	1.3
39	2.65 ± 0.52	95 ± 10	1.2
40	0.64 ± 0.14	98 ± 4	0.7
41	0.25 ± 0.03	94 ± 4	0.7
42	7.0 ± 1.8	89 ± 12	1.0
43	1.56 ± 0.26	98 ± 7	1.5
44	6.3 ± 0.9	94 ± 5	0.8
45	35.8 ± 5.7	89 ± 3	0.9
46	3.23 ± 0.96	90 ± 9	1.0
54	10.1 ± 2.1	97 ± 5	1.5
58	2.19 ± 0.55	76 ± 2	1.7
59	0.14 ± 0.01	98 ± 2	2.4
60	0.04 ± 0.01	96 ± 7	0.9

^aEfficacy (Y) and^bHill slope (HS) were obtained by the non-linear regression analysis of direct inhibition of HNE by NSGMs.

Table 2.

Michaelis-Menten kinetics of chromogenic substrate hydrolysis by HNE in the presence of NSGM **32**.

[NSGM 32] nM	V _{max} (mAU/min)	K _m (μM)
0	40.1 ± 0.5	650 ± 24
250	38.6 ± 1	908 ± 65
500	30.3 ± 0.9	788 ± 66
750	19.0 ± 0.7	1219 ± 117
1000	15.3 ± 1.6	1673 ± 373