Published in final edited form as:

ACS Infect Dis. 2019 July 12; 5(7): 1129–1138. doi:10.1021/acsinfecdis.9b00004.

Small-molecule inhibitors of *Haemophilus influenzae* IgA1 protease

Livia Shehaj^{†,‡}, Santosh K. Choudary^{†,‡}, Kamlesh M. Makwana^{†,‡}, Mary C. Gallo^{‡,§}, Timothy F. Murphy^{‡,§,||}, and Joshua A. Kritzer^{†,*}

[†]Department of Chemistry, Tufts University, 62 Talbot Ave, Medford, Massachusetts 02155, United States

[‡]Department of Microbiology and Immunology, University at Buffalo, Jacobs School of Medicine and Biomedical Sciences, 3435 Main St., Buffalo, NY 14203, United States

[§]Clinical and Translational Research Center, 875 Ellicott St., University at Buffalo, Jacobs School of Medicine and Biomedical Sciences, Buffalo, NY 14203, United States

^{||} Division of Infectious Disease, Department of Medicine, 875 Ellicott St., University at Buffalo, Jacobs School of Medicine and Biomedical Sciences, Buffalo, NY 14203, United States

Abstract

Newly identified, non-typable *H. influenzae* strains represent a serious threat to global health. Due to the increasing prevalence of antibiotic resistance, virulence factors have emerged as potential therapeutic targets that would be less likely to promote resistance. IgA1 proteases are secreted virulence factors of many Gram-negative human pathogens. These enzymes play important roles in tissue invasion as well as evasion of the immune response, yet there has been limited work on pharmacological inhibitors. Here we report the discovery of the first small molecule, non-peptidic inhibitors of *H. influenzae* IgA1 proteases. We screened over 47,000 compounds in a biochemical assay using recombinant protease, and identified a hit compound with micromolar potency. Preliminary SAR produced additional inhibitors, two of which showed improved inhibition and selectivity for IgA protease over other serine proteases. We further showed dose-dependent inhibition against four different IgA1 protease variants collected from clinical isolates. These data support further development of IgA protease inhibitors as potential therapeutics for antibacterial-resistant *H. influenzae* strains. The newly-discovered inhibitors also represent valuable probes for exploring the roles of these proteases in bacterial colonization, invasion, and infection of mucosal tissues.

Graphical Abstract

*Corresponding Author: joshua.kritzer@tufts.edu.

[†]These authors contributed equally to this work

The authors declare no competing financial interest.

Supporting Information

Supplement 1 Small molecule libraries used for HTS, general HTS strategy, HTS assay development, gel-based assays for positive controls, ¹H NMR data, compound stability over time as measured by ¹H NMR, trypsin and IgA1P active site homology, and additional compounds evaluated as part of structure-activity relationships.

Keywords

antibacterial resistance; high-throughput screening; *H. influenzae*; IgA1 Protease; FRET assay; protease inhibitors

Although antibiotic therapy is an effective treatment for many infections, it causes considerable stress to the healthy microbiome and also selects for resistance.¹ Emerging alternatives to traditional antibiotics include anti-virulence strategies, which focus on inhibiting and neutralizing virulence factors. This approach is thought to reduce selection pressure, potentially providing more resistance-proof modes of antibacterial treatment.^{2,3} Anti-virulence strategies include several therapies FDA-approved or currently in clinical trials.⁴ These include Bezlotoxumab, a monoclonal antibody against *Clostridium difficile* Toxin B, and Raxibacumab and Obiltoxaximab that bind the protective antigen of anthrax toxin; these agents block virulence of the pathogen without direct bacteriocidal effects.^{5,6,7} Although toxins have been more heavily explored as targets for anti-virulence therapies, these successes point to the potential for targeting other virulence factors.

In its most recent report on antibiotic resistance, the World Health Organization included ampicillin-resistant *Haemophilus influenzae* as one of the twelve highest-priority bacterial threats to human health.^{8,9} *H. influenzae* is an exclusively human pathogen responsible for several acute and chronic diseases including meningitis, pneumonia, bacteremia, and otitis media. It is also prominent among pathogens that cause lower respiratory infections in individuals with chronic obstructive pulmonary disease.^{10,11} The introduction of the *H. Influenzae* type b (Hib) vaccine about 30 years ago led to a rapid reduction of Hib infections in the United States, but Hib infection remains a global health problem. In addition, the past few years have seen the emergence of antibiotic-resistant strains of non-typeable *H. influenzae* (NTHi), pointing to a need for new approaches to combat this pathogen.¹²

H. influenzae is a commensal bacterium found in the upper respiratory tract. The switch from commensal to pathogenic *H. influenzae* relies on several virulence factors that promote invasion of host tissue, evasion and suppression of host immune responses, and intercellular communication. One such virulence factor produced by *H. influenzae* is the secreted immunoglobulin A1 protease (IgA1P). IgA1P cleaves human immunoglobulin A1 (IgA1), the predominant antibody on human mucosal surfaces. The actions of IgA1Ps impede IgA1

from neutralizing the bacteria (Figure 1A).^{13,14} Specifically, IgA1P cleaves IgA1 at a unique “hinge” region, separating the Fc and Fab fragments of the immunoglobulin. This impairs its Fc-mediated effector response (Figure 1), and can even allow bacteria to retain the Fab fragment on their surface in order to mask their polysaccharide capsule and other surface antigens.¹⁵ The cloaking of dominant antigens aids in evasion of the host’s immune response.¹⁶ IgA1P cleavage of IgA1 also promotes adherence to host respiratory epithelial cells.^{13,17,18} Additional substrates for bacterial IgA1Ps include LAMP-1, cleavage of which aids in survival of bacteria in epithelial cells by avoiding lysosomal degradation, and TNF-RII, cleavage of which aids in evasion of the host immune response by preventing TNF α -induced apoptosis.^{19,20} Finally, pathogenic strains of *H. influenzae* and other Gram-negative bacteria have consistently shown to have much higher IgA1P activity than their non-pathogenic counterparts, highlighting IgA1P’s clinical relevance and making it an attractive anti-virulence target.^{21–23}

The investigation of *H. influenzae* IgA1P as an antibacterial target has been hindered by the lack of a convenient substrate for high-throughput screening (HTS).²⁴ IgA1Ps are very specific for the hinge region of human IgA1, and do not cleave other human antibodies or non-primate IgAs. Early development of synthetic substrates based on the IgA1 hinge region did not produce robust substrates for HTS,²⁵ and recent work has suggested that distal interactions with IgA1 are necessary for substrate recognition of the IgA1 hinge.²⁶ SDS-PAGE, ELISA and LC-MS can be used to measure protease activity using human IgA1 as a substrate, but they are too time-consuming, low throughput, and/or low-sensitivity to be used for HTS. The most recent HTS approach for IgA1Ps was published by Garner *et al.* in 2013. They reported an assay that measured the aggregation of negatively charged nanoparticles in the presence of positively charged Fab fragments, which are a product of IgA1 cleavage.²⁷ Long incubation times (over 18 hours) and large amounts of human IgA1 were required, limiting this prior screen to only 96 compounds.

The limitations of existing drug discovery efforts targeting IgA proteases led us to explore alternative substrates as potential starting points for HTS. In prior work, we developed the first synthetic Förster resonance energy transfer (FRET) probe for monitoring IgA1P activity, molecule **F2.2** (Fig. 1c).³⁰ **F2.2** incorporates a peptide sequence from an autoproteolytic site of *Neisseria gonorrhoeae* type 2 IgA1P. **F2.2** is cleaved by a variety of IgA1Ps from Gram-negative pathogens, including *H. influenzae*.³⁰ We previously demonstrated the compatibility of **F2.2** with a 384-well HTS format using *Neisseria meningitidis* IgA1P. With that protease, we observed good signal-to-noise ratio and reproducibility, with a Z'-factor of 0.70. In this work, we optimized a HTS assay using **F2.2** with recombinant *H. influenzae* type 1 IgA-A1P. We used this assay to screen 47,839 compounds from diverse commercial libraries. Then, we selected a promising series of inhibitors, confirmed activity in follow-up assays, and obtained preliminary structure-activity relationships. This initial effort has produced the first small-molecule, non-peptidic inhibitors of Gram-negative IgA1 proteases.

Results and Discussion

High-Throughput Screening

Recombinant *H. influenzae* type A1 IgA1P was expressed and purified as previously described.³¹ The assay was optimized in terms of reaction volume, concentration of **F2.2**, and concentration of IgA1P to consistently produce a signal window of at least 3-fold between positive control (**F2.2** alone) and negative control (**F2.2** with IgA1P). The optimized assay used a final reaction volume of 20 μ L in a 384-well plate, with 1.1 μ M **F2.2** and 300 nM IgA1P. Compounds (100 nL) were added to the IgA1P, then probe was added, and the plates were incubated for 3 h at 37 °C before measuring fluorescence.

Using these conditions, a total of 47,839 compounds from several commercial libraries (including known bioactives; Figure S1) were screened against recombinantly produced *H. influenzae* IgA1P. Compounds were screened in duplicate. The Z' -factor values ranged between 0.65–0.9 throughout screening, demonstrating excellent signal-to-noise (Figure S2).

Our HTS workflow is shown in Figure S2. 97 compounds showed 50% inhibition or more at a final concentration of 25 ng/ μ L. We removed redundant compounds and compounds with poor reproducibility between the two screening replicates. We also removed compounds that had been identified as hits in many other, unrelated HTS campaigns; these were likely to have a nonspecific mode of action, or to be PAINS compounds.³² This left 85 potential hits, which were cherry-picked from screening stocks to generate dose-response data. 30 compounds with the most potent inhibition and the clearest dose response were selected for purchase and independent validation. Among these were compound **1** and several analogs (Figure 2).

Follow-up assays

We used two different follow-up assays to verify hit compounds were inhibitors of *H. influenzae* IgA1P. Since our primary HTS assay was a FRET assay, we chose secondary assays with a different substrate and a different readout to rule out artifacts. Both assays monitored cleavage of purified human IgA1 instead of a synthetic peptide substrate. One used SDS-PAGE to quantitate cleavage of IgA1, and the other used an ELISA. In both instances, serial dilutions of inhibitor were incubated with IgA1P prior to introduction of IgA1.

We first sought a positive control inhibitor for IgA1 cleavage assays. We tested several broad-spectrum serine protease inhibitors including Pefablock, PMSF, and 3,4-dichloroisocoumarin (DCIC).^{33–35} Notably, Pefablock and PMSF did not inhibit recombinant *H. influenzae* IgA1P up to 100 μ M (Figure S3). DCIC inhibited IgA1P with an IC_{50} of 0.006 μ M in the ELISA and 0.41 μ M in the gel-based assay (Figure 3). The discrepancy in these values is a result of the difference in IgA1 and IgA1P concentrations needed for each assay, with the gel-based assay requiring higher concentrations of both. While the IC_{50} values in these two assays differ, the trends between the assays remained consistent in these and subsequent experiments.

These two follow-up assays identified the most promising hit, compound **1**, with an IC₅₀ of $5.9 \pm 0.8 \mu\text{M}$ in the ELISA and $22.7 \pm 2.1 \mu\text{M}$ in the gel-based assay (Figure 3A–C). These values are for freshly diluted compound **1**, and over time the activity of **1** in aqueous solution decreased. **1** is a benzoic ester, so we surmised the ester was being hydrolyzed. This was confirmed using NMR spectroscopy. In DMSO, **1** broke down into benzoic acid and 4-morpholin-4-yl-1,2,5-thiadiazol-3-ol (compound **1a**) over the course of several days, presumably due to moisture present after transient exposure to air. About 50% of **1** was hydrolyzed in 10 days, and by 30 days it was completely hydrolyzed (Figure S17). In aqueous solution under assay conditions, we found that **1** was stable for at least 2 h (Figure S18). The fragmented compound had no effect on IgA1P, suggesting that the intact ester was required for the observed inhibition of IgA1P. To reduce hydrolysis, we stored aliquots of **1** in DMSO at -20°C , which allowed stable long-term storage of the compound (Figure S19).

Preliminary structure-activity relationships

We characterized and tested several commercially available analogs of **1** to understand and improve its potency. **1** can be separated into three regions: the benzylic ester, the thiadiazole, and a morpholine ring attached to the thiadiazole. Compounds **1a–1d** (Figure 4, Table 1) are all inactive, demonstrating that all three major components are required for activity. Compound **1d**, which replaced the ester bond with an ether, was inactive, and several other ether-containing analogs were also inactive (Figure S23). These data confirmed the importance of the ester for IgA1P inhibition. Compound **2**, which has a carbamate in place of the ester, exhibited similar inhibition as compound **1** (Figure 4, Table 1).

Carbamates are common groups in drug and prodrug design, particularly for protease inhibitors.³⁶ Carbamates are not as easily hydrolyzed as esters. As expected, **2** was stable over the course of 5 days in DMSO (Figure S20) and showed no signs of degradation in aqueous conditions for 7 h (Figure S21). Carbamate compounds **3–5** were also stable for days in DMSO (Figure S20).

Next, we tested analogs of compound **2** with modifications to the two morpholine rings (Table 1). Compound **3** replaced the morpholine attached to the thiadiazole with piperidine, and this single-atom change led to no change in inhibitory potency. By contrast, replacement of the morpholine carbamate with a piperidine carbamate (compound **4**) diminished inhibitory potency by 10-fold. Similarly, replacing the morpholine carbamate with an azolidine carbamate (compound **5**) resulted in a roughly 6-fold decrease in inhibitory potency. These results highlight the importance of the morpholine carbamate group for the activity of **2** and its analogs.

Specificity of selected inhibitors

We next examined the selectivity of **1**, **2** and **3** against representative serine hydrolases trypsin and β -lactamase. These assays provided three measures of selectivity: first, they would further rule out pan-assay interference compounds (PAINS);³⁷ second, they would identify non-specific inhibitors of serine hydrolases; and third, the active site of trypsin has about 60% homology to the active site of *H. influenzae* IgAP (Figure S22), so they would provide a measure of selectivity among trypsin-like serine proteases.

Trypsin inhibition was tested in a gel-based assay with denatured BSA as the substrate.^{38–40} 100 nM trypsin was added to denatured BSA and serial dilutions of inhibitor, and these reactions were incubated for 2 h at 37°C. Proteolytic fragments of BSA were analyzed by SDS-PAGE (Figure 5a). **1** showed some inhibition against trypsin with an IC₅₀ of 16.9 ± 1.0 μM, while **2** did not inhibit trypsin activity at concentrations up to 100 μM.

β-lactamase uses a mechanism analogous to serine proteases, and β-lactamase inhibition assays have been used as a counter-screen to help identify colloidal aggregators.⁴¹ Clavulanic acid was used as a positive control in a standard colorimetric β-lactamase assay. **1** showed some inhibitory potency against β-lactamase, with an IC₅₀ of 5.1 ± 0.3 μM (Figure 5b). Overall, **1** displayed about 3-fold lower activity against trypsin and β-lactamase as compared to IgA1P, suggesting weak selectivity. Carbamates **2** and **3** showed no inhibition of trypsin or β-lactamase up to 300 μM, revealing a high degree of selectivity for IgA1 proteases over these other serine hydrolases.

Compound **3** shows activity against different IgAP variants from clinical isolates

Next, we examined whether treatment of clinical isolates of non-typeable *H. influenzae* (NTHi) with compound **3** would inhibit NTHi-associated IgA1P activity. NTHi bacteria express four different variants of IgA proteases. IgA1P A1 and A2 are produced from two different alleles of the *igaA* gene, and IgA1P B1 and B2 are produced from two different alleles of the *igaB* gene; these variants have different relative activities and cleavage properties (Figure 1b). The *igaA* gene is present in all strains of *H. influenzae*,^{22,42} and the *igaB* gene is present in approximately 30 to 40% of NTHi.^{10,43,44} The recombinant protease used for high-throughput screening and follow-up assays was NTHi IgA1P variant A1.^{31,45} To measure NTHi-associated IgAP activity, we used a quantitative ELISA similar to the ELISA described above but in the presence of broth culture supernatants, which contain secreted IgA protease from cultures of NTHi. Since each individual strain produces only one IgA1P variant, we compared results among four different NTHi strains, one for each variant. In the absence of any IgA1P inhibitors, we observed that strains that express variants B1 and B2 produce roughly 3-fold higher IgA1P activity than strains that express variants A1 and A2 (Figure 6). This observation matches prior work that showed that expression levels of B1 and B2 variants are higher than expression levels of A1 and A2 variants.⁴⁴

Inhibitory activity of compound **3** was investigated by serially diluting the compound, pre-incubating it in broth culture supernatant from one of four NTHi strains, adding human IgA1, and then performing an ELISA to detect remaining intact IgA1. The results showed dose-dependent inhibition of proteases produced by all NTHi-derived strains (Figure 6). Due to the lower protease activity produced by A1 and A2 variants, supernatants from strains A1 and A2 were incubated with up to 10 μM of compound **3**, while B1 and B2 variants were incubated with a maximum of 100 μM. IgA1P A1 and A2 activities were inhibited to a similar extent by **3**, with nearly complete inhibition at 10 μM. **3** also showed dose-dependent inhibition of IgA1P B1 and B2, but at higher inhibitor concentrations, consistent with the higher expression levels of these variants. Nearly complete inhibition of IgA1P B1 and B2 was observed at 50 μM, with complete inhibition at 100 μM.

Acute toxicity of compound 3

Compound **3** was tested in an acute toxicity assay using the human bronchial mucocypidermoid carcinoma cell line H292, a cell line previously used to examine interactions of *H. influenzae* and other pneumonia-causing pathogens with bronchial tissue.⁴² We examined the effect of serial dilutions of **3** on H292 monolayers using a trypan blue exclusion assay to quantitate toxicity. Cell viability remained high (97% or greater) when treated for 3 hours with up to 100 μ M compound **3** (Table S1). This indicates that, at concentrations and time points that effectively inhibit native IgA1P activity, compound **3** is not acutely toxic to H292 cells.

Conclusions

Since their discovery forty years ago, research on IgA1 proteases and their roles in infection and disease has been limited. The slow pace of progress has primarily been due to the lack of synthetic substrates and HTS-ready assays. Using a previously reported synthetic substrate, **F2.2**, we performed a high-throughput screen and identified small-molecule inhibitors of recombinant *H. Influenzae* IgA protease. These include an ester with limited aqueous half-life (**1**), and two carbamates that are stable in aqueous solution (**2** and **3**). The ester or carbamate is necessary for activity, suggesting a key role in inhibition.³⁶ The carbamates **2** and **3** show promising inhibitory potency and good selectivity for *H. influenzae* IgAP compared to other serine hydrolases. Compound **3** blocked IgA1P activity of all 4 IgA protease variants produced by four different clinical isolates.

Compound **3**, also known as Lalistat, is a known inhibitor of lysosomal acid lipase (LAL).^{46,47} LALs are enzymes found in late endosomes and lysosomes. They catalyze the hydrolysis of cholesterol esters and triglycerides, freeing cholesterol and fatty acids. Lalistat is thought to act by transiently carbamylating the active serine of LAL and inhibiting its activity.⁴⁷ Lalistat has also been reported to inhibit growth of *Mycobacterium tuberculosis* by inhibiting the activity of its lipases and hydrolases.⁴⁸ The potency trends for compounds **2**, **3** and **4** as LAL inhibitors are divergent from our data.⁴⁷ Most notably, going from **2** to **4** is a one-atom change but has a ten-fold difference in activity against *H. Influenzae* IgA1P, while **2** and **4** show comparable inhibition as LAL inhibitors.⁴⁷ These results imply that further specificity for IgAPs over LALs, if desired, could be achieved by altering the ring attached to carbamate moiety.

In future work, we will explore additional structure-activity relationships of **2** and test the most promising compounds in more sophisticated models of human infection. The tools and assays we have developed can be adapted to screen for inhibitors of other IgA1 proteases from human pathogens. IgA1P-producing pathogens include drug-resistant *N. gonorrhoeae* and *S. pneumoniae*, two pathogens among the most urgent of global health threats.¹² Through the discovery and testing of IgA1P inhibitors, we will continue exploring the hypothesis that inhibiting IgA1 proteases represents a viable anti-virulence strategy to treat human infections caused by mucosal Gram-negative and Gram-positive pathogens.

Methods

Protease expression

The plasmid for production of recombinant *H. influenzae* IgA-A1P from *H. influenzae* strain Rd was kindly provided by Todd Holyoak.³¹ BL21(DE3) *E. coli* cells (New England Biolabs) were transformed and cells were grown in LB broth containing 50 µg/mL kanamycin for 14–18 h at 37 °C. Overnight cultures were then grown for 20 h at 17 °C in sterile ZYP-5052 rich autoinduction medium with vitamin B complex (B₁, B₂, B₃, B₆). Bacterial pellet was resuspended in Buffer A containing 25 mM Tris and 150 mM NaCl, pH 7.4. Lysozyme (1mg/mL of bacterial pellet) and universal nuclease (1 µL/L of bacterial pellet) were then added and the suspension was allowed to incubate on ice for 15–20 minutes, followed by a 10 min sonication at 65% amplitude with 10 s cycles. The lysate was then cleared by centrifugation at 8000 x g for 20 min. The supernatant was applied to a Ni-NTA Fast Flow column (ThermoFisher) and eluted in Buffer A with 400 mM imidazole. The protease-containing fractions were further purified using a Superdex S200 column (GE) in buffer A. Purified fractions were then tested for activity in gel-based assay, flash frozen and stored at –80°C.

High-throughput screening

The HTS was performed at the ICCB-Longwood Screening Facility. Using a Combi liquid handling system (Thermo-Fisher), 10 µL of IgA1P was added to each well of a low-volume 384 well plates (final assay concentration was 300 nM). Then, 100 nL of compound (or DMSO vehicle) was pin transferred into the assay plates; DMSO concentration in this and all other assays were kept constant and below 3%, below which DMSO concentration showed no effect on enzyme activity. Finally, using the same liquid handling system, 10 µL of the FRET substrate **F2.2** was added to each well (final assay concentration was 1.1 µM). The plates were briefly spun at 1000 rpm to get rid of any air bubbles, then they were tightly sealed and incubated at 37 °C for 3 h. After incubation, the plates were read using an Envision plate reader (excitation 340 nm, emission 480 nm).

Compounds

All the purchased compounds were from Vitascreen, MolPort or TimTec. Compounds were judged to be at least 95% pure as analyzed by HPLC. Compounds were dissolved in DMSO to make a stock concentration of 10–30 mM and stored at –80 °C.

NMR

Purity and stability of all compounds were tested using proton NMR spectroscopy. All the NMR spectra were recorded in Bruker 500 MHz spectrometer. Spectra recorded in DMSO-*d*₆ and in TBS buffer (10% D₂O + 90% TBS) were referenced with TMS and DSS respectively, as internal standard, set to 0.0 ppm. All the spectra were recorded at 291 K.

ELISA using recombinant protein

IgA1 protease activity was measured using a modified sandwich ELISA, previously described by Rehinoldt et al.⁴⁹ 96-well plates (Nunc Maxisorp) were coated overnight at

4 °C with 100 µL of 1/16000 dilution anti-Kappa Light chain antibody (Abcam) in coating buffer (50 mM sodium bicarbonate/sodium carbonate, pH = 9.6). The following day, plates were washed three times with TBST (25 mM Tris, 150mM NaCl with 0.05% tween-20, pH 7.4) and blocked with 2% BSA (Sigma Aldrich) in TBST for 3 h. IgA1P (3 nM final concentration) and serial dilutions of the inhibitors were incubated for 1 h. Then, IgA1 substrate (5 nM final concentration) was added to all reactions and was incubated for 2 h at 37 °C. After washing the plate three times with TBST, the reaction was then transferred into plate and incubated for 30 min. After washing, 100 µL of Anti-Fc IgA-HRP (ThermoFisher) was added at a dilution of 1/1000, and incubated for 30 min. The plate was then washed and developed using TMB ELISA substrate (ThermoFisher). The reaction was stopped using 0.5 M sulfuric acid and the ELISA signal was recorded by measuring the absorbance of the wells at 450 nM using a TECAN Infinity M200 Pro plate reader. All incubations were performed at room temperature unless otherwise noted. IC₅₀ values were derived from fitting to a standard, variable-slope dose-response curve using KaleidaGraph software (Synergy Software).

Gel-based inhibition assay

In this assay, recombinant IgA1P (final concentration 100 nM) was incubated with serial dilutions of the compounds for 1 h at room temperature in TBS buffer (25mM Tris pH 7.4, 150 mM NaCl). After incubation, human IgA1 (Abcam, final concentration 200 nM) was added to the reaction and incubated for 2 h at 37 °C. The reaction was then stopped by adding 2% SDS, and samples were run on SDS-PAGE. The results were then visualized using SYPRO Ruby (ThermoFisher). Quantification of the band intensities was performed using a ChemiDoc XRS+ system (BioRad). Inhibition of IgA1P was directly proportional to amount of heavy band of IgA1 remaining. IC₅₀ values were derived from fitting to a standard, variable-slope dose-response curve using KaleidaGraph software (Synergy Software).

β-lactamase assay

Assay was performed in 50 mM potassium phosphate, pH 7.0, at room temperature. Serial dilutions of compounds were incubated for 15 min with recombinant β-lactamase protein (Abcam, 1 nM final concentration) in 90 µL in a 96 well plate. Nitrocefin (300 µM final concentration) was added to each well and incubated for 1 h. Control wells included nitrocefin alone, and β-lactamase with nitrocefin and no inhibitor. Absorbance was measured at 340 nm on a TECAN Infinity M200 Pro plate reader. IC₅₀ values were derived from fitting to a standard, variable-slope dose-response curve using KaleidaGraph software (Synergy Software).

Trypsin assay

This assay was carried out similar to the gel-based IgA1P inhibition assay. The substrate used against trypsin in this assay was denatured BSA prepared in TBS buffer containing 2 mM DTT and heated for 5 min at 100 °C to monitor its activity and inhibition. Each compound was incubated with 100 nM trypsin for 1 h at room temperature and then incubated with 200 nM BSA substrate for 2 h at 37°C. The final products were loaded for SDS-PAGE and visualized with SYPRO Ruby staining. IC₅₀ values were derived from

fitting to a standard, variable-slope dose-response curve using KaleidaGraph software (Synergy Software).

Bacterial strains

NTHi strains were originally collected from deidentified subjects enrolled in a 20-year prospective study of adults with COPD that was conducted at the Buffalo Veterans Affairs Medical Center from 1994 to 2014.^{11,49} NTHi were identified using standard microbiology techniques, and a P6-specific monoclonal antibody was used to distinguish NTHi from *H. haemolyticus*.

Preparation of IgA1P from NTHi clinical isolate

A small loopful of bacteria from overnight chocolate agar plates was inoculated into 20 mL brain heart infusion supplemented with hemin and nicotinamide adenine dinucleotide (sBHI) and grown at 37°C until the culture reached an O.D₅₅₀ of between 0.7 and 0.8. Liquid cultures were centrifuged at 14,000 x g for 10 minutes at room temperature and the supernatant was filter-sterilized through a 0.2 µm syringe filter. Supernatant was stored in 1 mL aliquots at -20°C to avoid freeze-thaw cycles.

Measuring activity of IgA1P from NTHi strains

A modified version of the ELISA described above was used for measuring IgA1P from NTHi clinical isolates. Polystyrene microtitration plates (Immulon 2HB, ThermoFisher) were coated overnight at 4°C with rabbit anti-human Kappa light chain antibody (Sigma-Aldrich) in coating buffer. Plates were blocked with 3% bovine serum albumin (BSA) in wash buffer (0.5M NaCl, 0.015 M KH₂PO₄, 0.065 M anhydrous 0.5M Na₂HPO₄, 0.05% Tween 20) for 1 hour at 37°C. IgA1 substrate (3.2 µg/mL) in substrate buffer (wash buffer + 0.5% bovine serum albumin) was added to the plates and incubated for 2 hours at 37 °C. Freshly thawed broth culture supernatant or sterile culture medium (negative control) was added and the plate was incubated for 1 hour at 37°C. After washing, peroxidase-conjugated rabbit anti-human IgA (1:3000 dilution) in substrate buffer was applied and incubated for 2 hours at 37 °C. When testing activity in the presence of the inhibitor, the supernatant was pre-incubated with inhibitor for 1 hour at room temperature before addition to the ELISA plate. Plates were developed with 100 µL TMB ELISA substrate for 15 minutes. The reactions were terminated by addition of 100 µL 4N sulfuric acid, and absorbance at 450 nm was recorded using an iMark Bio-Rad model 3550-UV microplate reader. Activity was calculated as the difference in signal intensity at 450 nm (A_{450}) between the negative control wells containing sterile sBHI and the wells containing broth culture supernatant in the presence or absence of inhibitor. Values were then normalized to the amount of IgA1P produced by dividing A_{450} by the absorbance of the NTHi cultures at OD₅₅₀. Each trial was the average of three technical replicates, and reported data is the average of three independent trials.

Acute toxicity studies

Toxicity was tested using H292 respiratory epithelial cells and the trypan blue exclusion assay. NCI-H292, a human bronchial mucoepidermoid carcinoma cell line, was acquired

from the American Type Culture Collection. Cells were grown at 37°C in 5% CO₂ in supplemented RPMI media (Life Technologies) with 10% fetal bovine serum. H292 cells were seeded at 0.5×10^5 cells/ well in 24-well plates and allowed to approach confluency overnight. Nearly-confluent monolayers of H292 cells were first washed with warm sRPMI before adding fresh media with varying concentrations of inhibitor and incubating at 37°C with 5% CO₂ for 3 hours. At the end of the incubation, media was aspirated and fresh sRPMI was added and incubated at 37°C with 5% CO₂ for 1 hour. Monolayers were then washed with PBS, incubated with trypsin for 10–15 minutes to lift cells, and further diluted with PBS before being subjected to the trypan blue exclusion assay. Equal volumes of lifted cell solution and trypan blue stain (0.4%, Life Technologies) were combined, and cell count was performed using an automatic counter. Percent viability was defined as the number of live cells divided by the total cell count for each well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Todd Holyoak (University of Waterloo) for providing us with the plasmid for expressing *H. influenzae* IgA1P. We would also like to thank Dr. Jennifer Smith at Harvard ICCB for her help with HTS, and the University of Illinois School of Chemical Sciences Mass Spectrometry Laboratory for high-resolution mass spectrometry. This work was funded by the National Institutes of Health (R01GM111042 and R01AI19641).

References

- (1). Hauser AR; Mecsas J; Moir DT Beyond Antibiotics: New Therapeutic Approaches for Bacterial Infections. Clin. Infect. Dis 2016, 63 (1), 89–95 DOI 10.1093/cid/ciw200. [PubMed: 27025826]
- (2). Allen RC; Popat R; Diggle SP; Brown SP Targeting Virulence: Can We Make Evolution-Proof Drugs? Nat. Rev. Microbiol 2014, 12 (4), 300–308 DOI 10.1038/nrmicro3232. [PubMed: 24625893]
- (3). Rasko DA; Sperandio V Anti-Virulence Strategies to Combat Bacteria-Mediated Disease. Nat. Rev. Drug Discov 2010, 9 (2), 117–128 DOI 10.1038/nrd3013. [PubMed: 20081869]
- (4). Dickey SW; Cheung GYC; Otto M Different Drugs for Bad Bugs: Of Antibiotic Resistance. Nat. Publ. Gr 2017, 16 (7), 457–471 DOI 10.1038/nrd.2017.23.
- (5). Migone T-S; Subramanian GM; Zhong J; Healey LM; Corey A; Devalaraja M; Lo L; Ullrich S; Zimmerman J; Chen A; et al. Raxibacumab for the Treatment of Inhalational Anthrax. N. Engl. J. Med 2009, 361 (2), 135–144 DOI 10.1056/NEJMoa0810603. [PubMed: 19587338]
- (6). Greig SL Obiltoxaximab: First Global Approval. Drugs 2016, 76 (14), 1403–1412 DOI 10.1007/s40265-016-0634-8. [PubMed: 27577550]
- (7). Wilcox MH; Gerding DN; Poxton IR; Kelly C; Nathan R; Birch T; Cornely OA; Rahav G; Bouza E; Lee C; et al. Bezlotoxumab for Prevention of Recurrent Clostridium Difficile Infection. N. Engl. J. Med 2017, 376 (4), 305–317 DOI 10.1056/NEJMoa1602615. [PubMed: 28121498]
- (8). Watkins RR; Bonomo RA Overview: Global and Local Impact of Antibiotic Resistance. Infect. Dis. Clin. North Am 2016, 30 (2), 313–322 DOI 10.1016/j.idc.2016.02.001. [PubMed: 27208761]
- (9). Center for Disease Control and Prevention; Department of Health and Human Services. Antibiotic Resistance Threats in the United States, 2013. 2013, <http://www.cdc.gov/drugresistance/threat-report-2013> DOI:10.1181/239559-B.
- (10). Murphy TF; Kirkham C; Jones MM; Sethi S; Kong Y; Pettigrew MM Expression of IgA Proteases by Haemophilus Influenzae in the Respiratory Tract of Adults With Chronic

Obstructive Pulmonary Disease. *J. Infect. Dis* 2015, 212 (11), 1798–1805 DOI 10.1093/infdis/jiv299. [PubMed: 25995193]

- (11). Murphy TF; Brauer AL; Schiffmacher AT; Sethi S Persistent Colonization by *Haemophilus Influenzae* in Chronic Obstructive Pulmonary Disease. *Am. J. Respir. Crit. Care Med* 2004, 170 (3), 266–272 DOI 10.1164/rccm.200403-354OC. [PubMed: 15117742]
- (12). Tacconelli E; Magrini N; Kahlmeter G; Singh N Global Priority List Of Antibiotic-Resistant Bacteria To Guide Research, Discovery, And Development Of New Antibiotics. *World Heal. Organ* 2017, 1–7.
- (13). Kilian M; Reinholdt J; Lomholt H; Poulsen K; Frandsen EV Biological Significance of IgA1 Proteases in Bacterial Colonization and Pathogenesis: Critical Evaluation of Experimental Evidence. *APMIS* 1996, 104 (5), 321–338. [PubMed: 8703438]
- (14). Plaut AG The IgA1 Proteases of Pathogenic Bacteria. *Annu Rev Microbiol* 1983, 37, 603–622. [PubMed: 6416146]
- (15). Janoff EN; Rubins JB; Fasching C; Charboneau D; Rahkola JT; Plaut a G.; Weiser JN Pneumococcal IgA1 Protease Subverts Specific Protection by Human IgA1. *Mucosal Immunol.* 2014, 7 (2), 249–256 DOI 10.1038/mi.2013.41. [PubMed: 23820749]
- (16). Kilian M Bacterial Immunoglobulin-Evading Mechanisms: Ig-Degrading and Ig-Binding Proteins In Bacterial Evasion of Host Immune Responses; Henderson B, Oyston PCF, Eds.; The Press Syndicate of the University of Cambridge, 2009; pp 81–102.
- (17). Bogaert D; De Groot R; Hermans PWM *Streptococcus Pneumoniae* Colonisation: The Key to Pneumococcal Disease. *Lancet Infect. Dis* 2004, 4 (3), 144–154 DOI 10.1016/S1473-3099(04)00938-7. [PubMed: 14998500]
- (18). St. Geme JW; Maria L de la M; Falkow S A *Haemophilus Influenzae* IgA Protease-like Protein Promotes Intimate Interaction with Human Epithelial Cells. *Mol. Microbiol* 1994, 14 (2), 217–233 DOI 10.1111/j.1365-2958.1994.tb01283.x. [PubMed: 7830568]
- (19). Lin L; Ayala P; Larson J; Mulks M; Fukuda M; Carlsson SR; Enns C; So M The *Neisseria* Type 2 IgA1 Protease Cleaves LAMP1 and Promotes Survival of Bacteria within Epithelial Cells. *Mol. Microbiol* 1997, 24, 1083–1094 DOI 10.1046/j.1365-2958.1997.4191776.x. [PubMed: 9220014]
- (20). Beck SC; Meyer TF IgA1 Protease from *Neisseria Gonorrhoeae* Inhibits TNF α -Mediated Apoptosis of Human Monocytic Cells. *Febs Lett.* 2000, 472 (2–3), 287–292. [PubMed: 10788628]
- (21). Pohlner J; Halter R; Meyer TF *Neisseria Gonorrhoeae* IgA Protease. Secretion and Implications for Pathogenesis. *Antonie Van Leeuwenhoek* 1987, 53 (6), 479–484 DOI 10.1007/BF00415506. [PubMed: 3130785]
- (22). Vitovski S; Dunkin KT; Howard AJ; Sayers JR Nontypeable *Haemophilus Influenzae* in Carriage and Disease A Difference in IgA1 Protease Activity Levels. *J. Am. Med. Assoc* 2004, 291 (5), 2457–2461 DOI 10.1128/JA.291.5.2457.
- (23). Vitovski S; Read RC; Sayers JR Invasive Isolates of *Neisseria Meningitidis* Possess Enhanced Immunoglobulin A1 Protease Activity Compared to Colonizing Strains. *FASEB J.* 1999, 13 (2), 331–337. [PubMed: 9973321]
- (24). Qiu J; Brackee GP; Plaut AG Analysis of the Specificity of Bacterial Immunoglobulin A (IgA) Proteases by a Comparative Study of Ape Serum IgAs as Substrates. *Infect. Immun* 1996, 64 (3), 933–937. [PubMed: 8641803]
- (25). Burton J; Wood SG; Lynch M; Plaut AG Substrate Analogue Inhibitors of the IgA1 Proteinases from *Neisseria Gonorrhoeae*. *J. Med. Chem* 1988, 31 (8), 1647–1651 DOI 10.1021/jm00403a027. [PubMed: 3135406]
- (26). Chi Y-C; Rahkola JT; Kendrick AA; Holliday MJ; Paukovish N; Robers TS; Janoff EN; Eisenmesser EZ *Streptococcus Pneumoniae* IgA1 Protease: A Metalloprotease That Can Catalyze in a Split Manner in Vitro. *Protein Sci.* 2016, 22 (6), 1–50 DOI 10.1002/pro.
- (27). Garner AL; Fullagar JL; Day J. a.; Cohen SM; Janda KD Development of a High-Throughput Screen and Its Use in the Discovery of *Streptococcus Pneumoniae* Immunoglobulin A1 Protease Inhibitors. *J. Am. Chem. Soc* 2013, 135 (27), 10014–10017 DOI 10.1021/ja404180x. [PubMed: 23808771]

- (28). Senior BW; Woof JM The Influences of Hinge Length and Composition on the Susceptibility of Human IgA to Cleavage by Diverse Bacterial IgA1 Proteases. *J. Immunol* 2005, 174 (12), 7792–7799 DOI 10.4049/jimmunol.174.12.7792. [PubMed: 15944283]
- (29). Senior BW; Batten MR; Kilian M; Woof JM Amino Acid Sequence Requirements in the Human IgA1 Hinge for Cleavage by Streptococcal IgA1 Proteases. *Biochem. Soc. Trans* 2002, 71 (3), 1462–1469 DOI 10.1042/BST0300516.
- (30). Choudary SK; Qiu J; Plaut AG; Kritzer JA Versatile Substrates and Probes for IgA1 Protease Activity. *ChemBioChem* 2013, 14 (15), 2007–2012 DOI 10.1002/cbic.201300281. [PubMed: 24038810]
- (31). Johnson T.; Qiu J; Plaut A.; Holyoak T Active-Site Gating Regulates Substrate Selectivity in a Chymotrypsin-Like Serine Protease The Structure of Haemophilus Influenzae Immunoglobulin A1 Protease. *J.Mol.Biol* 2009, 389, 559–574. [PubMed: 19393662]
- (32). Panaceas IM The Ecstasy and Agony of Assay Interference Compounds. DOI 10.1021/acs.jmedchem.7b00229.
- (33). Markwardt F; Drawert J; Walsmann P Synthetic Low Molecular Weight Inhibitors of Serum Kallikrein. *Biochem. Pharmacol* 1974, 23 (16), 2247–2256 DOI 10.1016/0006-2952(74)90554-1. [PubMed: 4859519]
- (34). James GT Inactivation of the Protease Inhibitor Phenylmethylsulfonyl Fluoride in Buffers. *Anal. Biochem* 1978, 86 (2), 574–579 DOI 10.1016/0003-2697(78)90784-4. [PubMed: 26289]
- (35). Harper JW; Powers JC Reaction of Serine Proteases with Substituted 3-Alkoxy-4-Chloroisocoumarins and 3-Alkoxy-7-Amino-4-Chloroisocoumarins: New Reactive Mechanism-Based Inhibitors. *Biochemistry* 1985, 24 (25), 7200–7213 DOI 10.1021/bi00346a028. [PubMed: 3910097]
- (36). Ghosh AK; Brindisi M Organic Carbamates in Drug Design and Medicinal Chemistry. *J. Med. Chem* 2015, 58 (7), 2895–2940 DOI 10.1021/jm501371s. [PubMed: 25565044]
- (37). Seidler J; McGovern SL; Doman TN; Shoichet BK Identification and Prediction of Promiscuous Aggregating Inhibitors among Known Drugs. 2003, 4477–4486 DOI 10.1021/jm030191r.
- (38). Shee C; Sharma A Purification and Characterization of a Trypsin Inhibitor from Seeds of *Murraya Koenigii*. *J. Enzyme Inhib. Med. Chem* 2007, 22 (1), 115–120 DOI 10.1080/14756360601027332. [PubMed: 17373557]
- (39). Kleiner RE; Hang LE; Molloy KR; Chait BT; Kapoor TM A Chemical Proteomics Approach to Reveal Direct Protein-Protein Interactions in Living Cells. *Cell Chem. Biol* 2017, 1–11 DOI 10.1016/j.chembiol.2017.10.001. [PubMed: 28107651]
- (40). Seidler J; McGovern SL; Doman TN; Shoichet BK Identification and Prediction of Promiscuous Aggregating Inhibitors among Known Drugs. *J. Med. Chem* 2003, 46 (21), 4477–4486 DOI 10.1021/jm030191r. [PubMed: 14521410]
- (41). Feng BY; Simeonov A; Jadhav A; Babaoglu K; Inglese J; Shoichet BK; Austin CP A High-Throughput Screen for Aggregation-Based Inhibition in a Large Compound Library. 2007, 2385–2390 DOI 10.1021/jm061317y.
- (42). Clementi CF; Håkansson AP; Murphy TF Internalization and Trafficking of Nontypeable Haemophilus Influenzae in Human Respiratory Epithelial Cells and Roles of IgA1 Proteases for Optimal Invasion and Persistence. *Infect. Immun* 2014, 82 (1), 433–444 DOI 10.1128/IAI.00864-13. [PubMed: 24218477]
- (43). Fernaays MM; Lesse AJ; Sethi S; Cai X; Murphy TF Differential Genome Contents of Nontypeable Haemophilus Influenzae Strains from Adults with Chronic Obstructive Pulmonary Disease. *Infect. Immun* 2006, 74 (6), 3366–3374 DOI 10.1128/IAI.01904-05. [PubMed: 16714566]
- (44). Fernaays MM; Lesse AJ; Cai X; Murphy TF Characterization of IgaB, a Second Immunoglobulin A1 Protease Gene in Nontypeable Haemophilus Influenzae. *Infect. Immun* 2006, 74 (10), 5860–5870 DOI 10.1128/IAI.00796-06. [PubMed: 16988265]
- (45). Lamm ME; Emancipator SN; Robinson JK; Yamashita M; Fujioka H; Qiu J; Plaut AG Microbial IgA Protease Removes IgA Immune Complexes from Mouse Glomeruli in Vivo: Potential Therapy for IgA Nephropathy. *Am. J. Pathol* 2008, 172 (1), 31–36 DOI 10.2353/ajpath.2008.070131. [PubMed: 18165266]

- (46). Hamilton J; Jones I; Srivastava R; Galloway P A New Method for the Measurement of Lysosomal Acid Lipase in Dried Blood Spots Using the Inhibitor Lalistat 2. *Clin. Chim. Acta* 2012, 413 (15–16), 1207–1210 DOI 10.1016/j.cca.2012.03.019. [PubMed: 22483793]
- (47). Rosenbaum AI; Cosner CC; Mariani CJ; Maxfield FR; Wiest O; Helquist P Thiadiazole Carbamates: Potent Inhibitors of Lysosomal Acid Lipase and Potential Niemann - Pick Type C Disease Therapeutics. 2010, 5281–5289 DOI 10.1021/jm100499s.
- (48). Lehmann J; Vomacka J; Esser K; Nodwell M; Kolbe K; Rämer P; Protzer U; Reiling N; Sieber SA Human Lysosomal Acid Lipase Inhibitor Lalistat Impairs Mycobacterium Tuberculosis Growth by Targeting Bacterial Hydrolases. *Medchemcomm* 2016, 7 (9), 1797–1801 DOI 10.1039/C6MD00231E.
- (49). Sethi S; Evans N; Grant BJB; Murphy TF New Strains of Bacteria and Exacerbations of Chronic Obstructive Pulmonary Disease. *N. Engl. J. Med* 2002, 347 (7), 465–471 DOI 10.1056/NEJMoa012561. [PubMed: 12181400]
- (50). Murphy TF; Brauer AL; Sethi S; Kilian M; Cai X; Lesse AJ Haemophilus Haemolyticus: A Human Respiratory Tract Commensal to Be Distinguished from Haemophilus Influenzae. *J. Infect. Dis* 2007, 195 (1), 81–89 DOI 10.1086/509824. [PubMed: 17152011]

Figure 1.

a) Immunoglobulin A1 proteases (IgA1Ps) are virulence factors produced by several Gram-negative and Gram-positive pathogens. IgA1Ps cleave the hinge region of human Immunoglobulin A1 (IgA1), with very few other known substrates. b) IgA1Ps from different bacteria have convergently evolved to cleave the IgA1 hinge region, but at different sites. Specific cleavage sites within the hinge region are labeled with an asterisk, as well as the species and strain that produces the IgA1P that cleaves at that position.^{14,28,29} c) **F2.2**, the FRET probe used in the high-throughput screen (chemical structure provided in Fig. S2d). Enzymatic activity of the IgA1P was monitored as an increase in fluorescence emission from the EDANS FRET donor. Prior work showed that IgAPs from five different Gram negative bacterial strains cleaved **F2.2** at the site labeled with an asterisk.³⁰

Figure 3.

Activity of compound **1** in gel-based assays and ELISA. a) Structures of positive control DCIC and **1**. b) Representative results from the gel-based assay monitoring cleavage of the IgA1 heavy chain. Serial dilutions of inhibitor were first incubated with 100 nM IgA protease at room temperature for 1 h. Then, 200 nM human IgA1 was added and the reaction was further incubated at 37°C for 2 h. The resulting protein was run on an SDS-PAGE gel and stained with Sypro Ruby, allowing quantitation of the 58 kDa band (full-length heavy chain) and the 25 and 32 kDa bands (cleaved pieces of heavy chain). c) Quantitated results from integration of the 58 kDa band (full-length heavy chain). These data represent at least three independent replicates, each of which was the average of three technical replicates. Error bars show standard error of the mean. Positive control DCIC inhibited IgA1P activity with an IC_{50} of $0.4 \pm 0.1 \mu M$ and **1** inhibited with an IC_{50} of $22.0 \pm 4.7 \mu M$. d) ELISA data. Serial dilutions of inhibitor were first incubated with 3 nM IgA protease at room temperature for 1 h. Then, 5 nM human IgA1 was added. After incubation at 37°C for 2 h, the level of uncleaved IgA1 was measured using a sandwich ELISA. In the ELISA, DCIC inhibited IgA1P with a IC_{50} of $0.004 \pm 0.001 \mu M$ and **1** inhibited the IgA1P with a IC_{50} of $4.3 \pm 0.9 \mu M$. These data represent at least three independent replicates, each of which was the average of three technical replicates. Error bars show standard error of the mean.

Figure 4. Inhibitory potency of carbamates 2-5. Compounds were tested in a sandwich ELISA assay that detects intact human IgA1 (see Figure 3 and Methods for details). Data represents at least three independent replicates, each of which was the average of three technical replicates. Error bars show standard error of the mean.

Figure 5.

Inhibition of other serine hydrolases by IgA1P inhibitors. a) Gel-based assay for trypsin activity with denatured BSA as substrate. The indicated compounds were incubated with 100 nM trypsin for 2 h, then combined with 200 nM denatured BSA for 1 h. The results were visualized using SYPRO Ruby. b) Inhibition of β -lactamase activity was used as a second assay for specificity, with clavulanic acid as a positive control. β -lactamase (1 nM) was incubated with serial dilutions of compounds **1** and **2** for 15 min. The colorimetric substrate nitrocefin (300 μ M) was then added and incubated for 1 h. The plot shows IC_{50} curve fits, with associated IC_{50} values of 0.06 μ M for clavulanic acid and 16.9 μ M for **1**. Error bars represent standard error of the mean from three independent replicates, each of which was the average of three technical replicates.

Figure 6:

Effect of compound **3** on IgA1P activity from clinical isolates of NTHi. Plot shows raw ELISA data for IgA1P activity in broth culture supernatant from four different NTHi strains. Strains producing IgA1 protease A1 and A2 were incubated with Compound **3** in concentrations from 0 to 10 μM, while strains producing IgA1 protease B1 and B2 were incubated with inhibitor in concentrations from 0 to 100 μM. The NTHi strain used for each set of experiments is noted along the X-axis, with the variant of IgA protease in parentheses. Error bars represent standard error of the mean from three independent replicates, each of which was the average of three technical replicates.

Table 1:

Structure-activity relationships among derivatives of **1**. IC₅₀ values are from sandwich ELISA.

Compound	IC ₅₀ (μM)
DCIC	0.006 ± 0.001
1	5.9 ± 0.8
1a	>100
1b	>100
1c	>100
1d	>100
2	3.2 ± 0.2
3	3.7 ± 0.3
4	50.4 ± 4.9
5	41.3 ± 3.2