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Development of a High-Throughput Screen and Its Use in the Discovery of *Streptococcus pneumoniae* Immunoglobulin A1 Protease Inhibitors

Amanda L. Garner,[†] Jessica L. Fullagar,[‡] Joshua A. Day,[‡] Seth M. Cohen,[‡] and Kim D. Janda*[,†]

[†]Departments of Chemistry and Immunology and Microbial Science, The Skaggs Institute for Chemical Biology, and The Worm Institute for Research and Medicine, The Scripps Research Institute, and [‡]Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: *Streptococcus pneumoniae* relies on a number of virulence factors, including immunoglobulin A1 protease (IgA1P), a Zn^{2+} metalloprotease produced on the extracellular surface of the bacteria, to promote pathogenic colonization. IgA1P exhibits a unique function, in that it catalyzes the proteolysis of human IgA1 at its hinge region to leave the bacterial cell surface masked by IgA1 Fab, enabling the bacteria to evade the host's immune system and adhere to host epithelial cells to promote colonization. Thus, *S. pneumoniae* IgA1P has emerged as a promising antibacterial target; however, the lack of an appropriate screening assay has limited the investigation of this metalloprotease virulence factor. Relying on electrostatics-mediated AuNP aggregation, we have designed a promising high-throughput colorimetric assay for IgA1P. By using this assay, we have uncovered inhibitors of the enzyme that should be useful in deciphering its role in pneumococcal colonization and virulence.

Streptococcus pneumoniae is a commensal colonizer of the upper respiratory tract of humans, where it inhabits the mucosal surfaces lining the nasopharynx; however, the bacteria can spread from the upper respiratory tract to the sterile spaces of the lower respiratory tract, ear, or bloodstream to cause pneumococcal diseases such as pneumonia, otitis media, sepsis, and meningitis.[1,2] To promote pathogenic colonization, *S. pneumoniae* relies on a combination of virulence factors and immune modulators; in particular, the bacteria use up to four surface-presented, extracellular zinc metalloproteases to evade the host immune system and promote virulence.[1,2] Of these metalloproteases, *S. pneumoniae* immunoglobulin A1 protease (IgA1P) has emerged as the most promising antibacterial target.[3,4]

IgA1 proteases are a class of extracellular bacterial enzymes that act as post-proline endopeptidases and specifically cleave human IgA1 (Figure 1),[5] the predominant form of Ig in mucosal tissues and secretions.[6] Cleavage occurs in the unique hinge region of the α chain of IgA1 to yield intact Fab$_\alpha$ and Fc$_\alpha$ fragments,[6−8] leaving IgA1P-producing organisms coated with antibody fragments lacking Fc domains, thus enabling the bacteria to evade recognition by Fc receptors and complement to prevent production of an inflammatory response.[2]

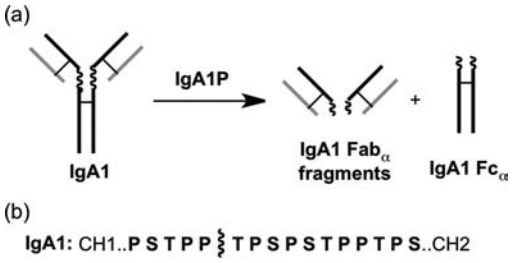

Figure 1. IgA1 cleavage by IgA1Ps. (a) IgA1P-catalyzed degradation of IgA1 (black = heavy chain, gray = light chain). (b) Sequence of amino acids in the hinge region of the α chain of IgA1 Fab and proposed site of cleavage by *S. pneumoniae* IgA1P.

Interestingly, in the case of *S. pneumoniae* IgA1P, upon cleavage of IgA1, the bacterium retains the Fab fragments on its surface to mask its capsular polysaccharide, the immunodominant surface antigen on pneumococci.[9] This event promotes, rather than inhibits, adherence to epithelial cells via electrostatic interactions between the positively charged Fab molecules coating the bacteria and negatively charged plasma membrane of the host's cells, thereby enhancing infection.[9] In addition to promoting adherence, *S. pneumoniae* IgA1P has also been linked to pneumococcal virulence in murine models of pneumonia[10,11] and sepsis[12] via mutation analyses; yet, there exists a disconnect with the targets of this protease between rodents and humans, as currently the enzyme is only known to cleave human[5] and primate[13] IgA1. Thus, it may be likely that IgA1P engages additional host substrates to promote virulence,[10] but this remains to be determined.

A number of factors have precluded the investigation of *S. pneumoniae* IgA1P as an antibacterial target. In addition to the lack of an appropriate animal model,[5] no high-throughput-amenable screening assays have been reported for IgA1P activity. ELISA-based tests have been described;[14,15] however, they have never been utilized for small molecule inhibitor screening. Moreover, these methods are not applicable to such efforts, as they require several expensive antibody reagents and a separate reaction mixture transfer step.[14,15] With respect to examining inhibition of IgA1P activity, only Western blot-based[16] and autoradiographic[17] assays have been reported. As a

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result, no *S. pneumoniae* IgA1P-specific inhibitors have been disclosed.[16] Thus, in order to identify IgA1P-specific inhibitors with the capacity to elucidate the role of *S. pneumoniae* IgA1P in pneumococcal colonization and virulence, we sought to design a user-friendly, potentially high-throughput screening assay for IgA1P.

As inspiration for our assay design, we chose to take advantage of the fact that, following IgA1P-mediated cleavage, positively charged Fab fragments are produced.[9] Thus, we reasoned that if a pro-colorimetric, negatively charged reagent could selectively bind to the positively charged Fab molecules to produce a measurable signal, then a simple, colorimetric high-throughput assay for IgA1P activity would be established. Fortunately, negatively charged gold nanoparticles (AuNPs) have previously been employed as a means to interrogate protein kinase and phosphatase activity *in vitro* and in cell lysate using a similar electrostatics-based principle.[18,19] Briefly, the charge differential between a phosphorylated and non-phosphorylated arginine- or lysine-containing, kinase- or phosphatase-recognized peptide substrate was monitored via aggregation of citrate-modified AuNPs,[18,19] where dispersed AuNPs exhibit a deep red color while aggregated AuNPs are blue, based on a shift in the wavelength of the surface plasmon resonance due to increased particle size.[20] Taking a similar approach, in the absence of IgA1P, AuNPs would be dispersed with no noticeable color change; however, in the presence of IgA1P, the enzyme would cleave human IgA1 to yield positively charged Fab fragments, which would electrostatically interact with negatively charged, citrate-adsorbed AuNPs to induce AuNP aggregation and a color change from red to blue, thereby establishing a colorimetric screening assay for IgA1P activity (Figure 2a).

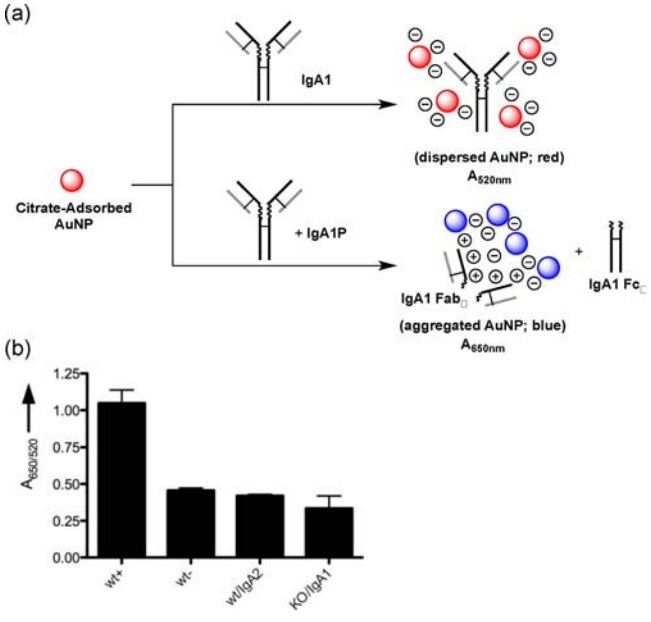

Figure 2. AuNP-based colorimetric IgA1P assay. (a) Assay design (black = heavy chain, gray = light chain). (b) Proof-of-concept data: wt + = D39 strain *S. pneumoniae* supernatant treated with secretory IgA; wt− = D39 strain *S. pneumoniae* supernatant without secretory IgA; wt/IgA2 = D39 strain *S. pneumoniae* supernatant treated with IgA2; KO/IgA1 = P1285 strain *S. pneumoniae* supernatant treated with secretory IgA; $A_{650/520}$ = raw ratiometric absorbance signal.

To test the applicability of the designed AuNP assay for *S. pneumoniae* IgA1P, D39 strain *S. pneumoniae* was grown to stationary phase (OD_{600} 0.4−0.5) in Todd−Hewitt broth supplemented with 0.5% yeast extract.[3] Bacterial supernatant was then obtained via centrifugation and used as the source of *S. pneumoniae* IgA1P. Although production of recombinant *S. pneumoniae* IgA1P has been reported, the activity and stability of this recombinant enzyme are poor.[21] For the assay, diluted IgA1P-containing *S. pneumoniae* supernatant (2× in 50 mM Tris-HCl, pH 7.0)[3] was incubated with human secretory IgA (0.15 mg/mL) at 37 °C for 18 h.[22] After a freeze−thaw cycle to disrupt protein−antibody interactions, AuNPs (20 nm) were added, and absorbance signals A_{650} and A_{520} were then measured. As Figure 2b shows, our assay design was successful, and under these conditions a 2.3-fold raw ratiometric absorbance enhancement was observed over samples not treated with IgA1. These positive results were further verified via specificity and knockout studies, and in the presence of IgA2, which is not cleaved by IgA1P,[5] and when using supernatant from an IgA1P-knockout *S. pneumoniae* strain (P1285),[22] no ratiometric absorbance signal was observed over background (Figure 2b). Western blot analysis was performed as previously described[16,22] for each of these samples to monitor IgA1 structural integrity and confirm cleavage status. Positive correlation was observed between our results using the AuNP assay and Western blot findings, demonstrating the accuracy of our assay for monitoring secretory IgA cleavage by IgA1P (see Figure S4 in the Supporting Information (SI)).

Given these successful results, we next characterized the IgA1P-catalyzed IgA1 cleavage reaction using our AuNP assay. We first examined the time dependence of IgA1 cleavage by IgA1P. As Figure 3a shows, the proteolysis of IgA1 was linear

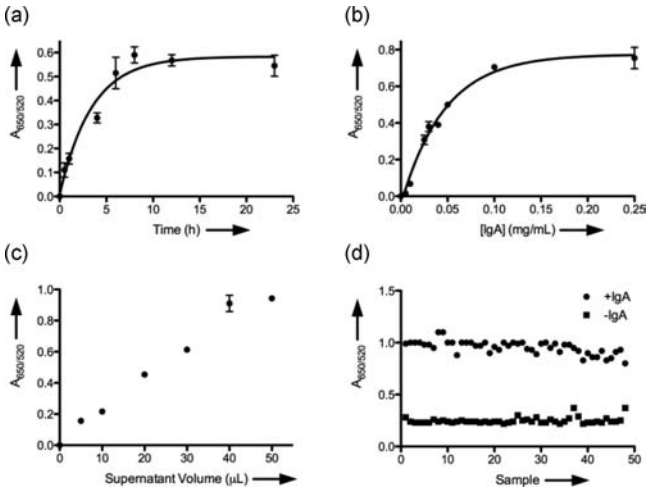

Figure 3. Characterization of AuNP assay for IgA1P-mediated IgA1 cleavage: $A_{650/520}$ = normalized ratiometric absorbance signal with respect to the negative control (wt−). (a) Time dependence. (b) Dependence on the concentration of secretory IgA. (c) Dependence on the volume of D39 strain *S. pneumoniae* supernatant containing IgA1P. (d) Z′ factor.

with time for ∼5 h. The assay was also linear with respect to the concentration of secretory IgA (up to ∼0.05 mg/mL) added to the wells (Figure 3b). Moreover, as Figure 3c shows, the assay exhibited saturation kinetics with respect to the volume of *S. pneumoniae* supernatant. In addition to reaction characterization, we also assessed the suitability of this AuNP assay for

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high-throughput screening. The signal-to-noise ratio (S/N) was 22.7, the signal-to-background ratio (S/B) was 3.8, and the Z' factor[23] was 0.60, indicating the potential of our AuNP assay for high-throughput screening, as assays with Z' values between 0.5 and 1.0 are typically regarded as excellent assays (Figure 3d).

Using our AuNP assay for IgA1P activity, we initiated a screening campaign to identify small molecule inhibitors of *S. pneumoniae* IgA1P. Libraries of substituted pyridines and naphthalenes,[24,25] natural products (Enzo Natural Product Library), and metal-chelating fragments (CFL1.1)[26,27] were analyzed. These efforts resulted in the identification of three natural products, remerine (**1**), norharmane (**2**), and berberine (**3**) (Figure 4a; see Figure S1 for IC_{50} curves), and one family

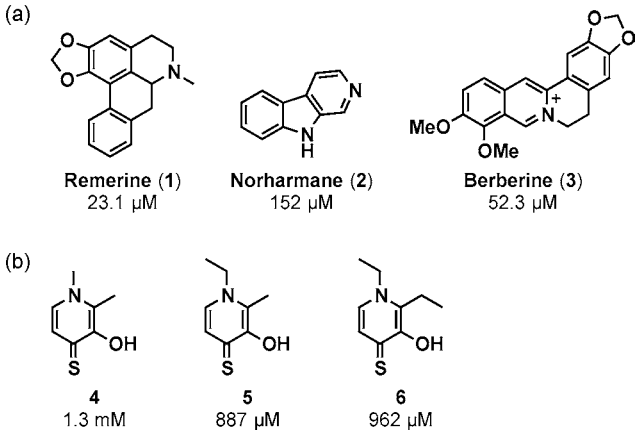
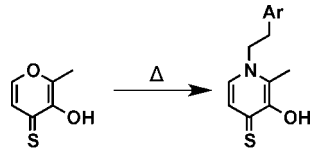


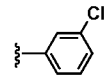
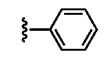
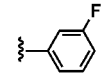
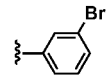
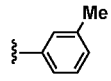
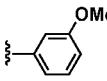
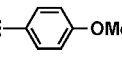
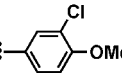
Figure 4. Identified inhibitors of *S. pneumoniae* IgA1P. (a) Natural product hits. (b) 3,4-HOPTO-based hits.

of chelator fragments, 3-hydroxy-1-alkyl-2-methylpyridine-4(1*H*)-thiones (3,4-HOPTO, compounds **4**–**6**, Figure 4b; see Figures S2 and S3 for the activity of other CFL1.1 members), exhibiting dose-dependent inhibition of *S. pneumoniae* IgA1P. The 3,4-HOPTO scaffold is amenable to medicinal chemistry, as previously demonstrated in the discovery of a potent inhibitor of another Zn^{2+} metalloprotease virulence factor, LasB of *Pseudomonas aeruginosa*.[28] Thus, we chose to focus our initial efforts on the 3,4-HOPTO scaffold for the development of more advanced lead compounds.

In order to identify 3,4-HOPTO derivatives with improved *in vitro* potency against *S. pneumoniae* IgA1P, a sublibrary of compounds based on fragments **4** and **5** was analyzed.[26,28] 3,4-HOPTO analogues **8** were constructed via a one-step condensation of a primary amine with thiomaltol **7** (Table 1)[26,28] and initially tested for inhibition at 100 μM. From this sublibrary and additional structure–activity relationship studies, one compound was discovered with >200-fold improvement in potency, compound **8a** with an IC_{50} value of 4.30 μM (Table 1; see Table S1 for the activity of other sublibrary members). With this inhibitor in hand, we then confirmed its inhibitory activity via Western blot analysis as described above, and complete inhibition was observed with 25 μM compound **8a** (see Figure S4). These results provide additional evidence that libraries of metal-chelating fragments can successfully be used to identify first-in-class inhibitors of bacterial Zn^{2+} metalloprotease virulence factors.[28,29] Future synthetic efforts will focus on the preparation of additional analogues to identify compounds that not only have enhanced affinity for IgA1P but are also highly specific for this virulence factor, as 3,4-HOPTO derivatives including **8a** have been shown to be active against

Table 1. Structures and IC_{50} Values for Select 3,4-HOPTO Analogue Hits



Compound	Ar	IC_{50} (μM)
8a		4.30 ± 0.16
8b		12.7 ± 0.08
8c		7.74 ± 0.23
8d		6.14 ± 0.18
8e		9.14 ± 0.13
8f		6.64 ± 0.04
8g		7.00 ± 0.08
8h		4.98 ± 0.18

other metalloenzymes (see Figure S5 for the metalloenzyme specificity of **8a**).[26,27,29]

In summary, we have described the first user-friendly, colorimetric screening assay for IgA1P from *S. pneumoniae*. By utilizing the enzyme's ability to modulate the surface charge of the bacteria, we have designed an electrostatics-based assay in which the resulting Fab fragments are able to induce the aggregation of citrate-adsorbed, negatively charged AuNPs to produce a colorimetric absorbance signal. Importantly, this assay approach offers many advantages over the current state-of-the-art for IgA1Ps, including amenability to high-throughput screening for the identification of small molecule inhibitors and elimination of the requirement of harmful or expensive reagents. Although the overall charges of IgA1 Fab fragments produced by other IgA1Ps have yet to be measured, the assay platform described herein may be amenable to monitoring the activity of these related proteases, such as the IgA1Ps produced by oral *Streptococcal* species, *Haemophilus influenzae*, and *Neisseria meningitidis*.[6] Finally, and most significantly, using our AuNP assay for *S. pneumoniae* IgA1P, we have uncovered the first inhibitors of the enzyme, and these compounds should enable the investigation of this pneumococcal virulence factor as a potential antibacterial target.

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■ ASSOCIATED CONTENT

Supporting Information

General methods and materials, IgA1P preparation, assay protocols, and supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

kdjanda@scripps.edu

Notes

The authors declare no competing financial interest.

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