JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

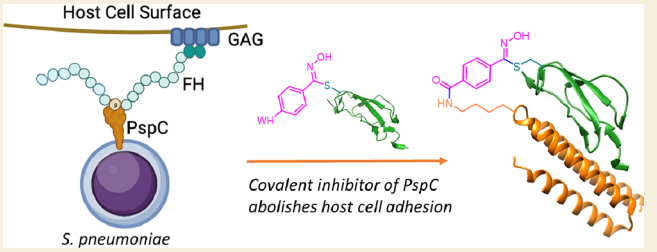
pubs.acs.org/jacsau
Article

Covalent Inhibition of a Host–Pathogen Protein–Protein Interaction Reduces the Infectivity of *Streptococcus pneumoniae*

Yuhan Lyu, Fan Yang, Bharathi Sundaresh, Federico Rosconi, Tim van Opijnen, and Jianmin Gao*

Cite This: *JACS Au* 2024, 4, 2484–2491
Read Online

ABSTRACT: The ever-expanding antibiotic resistance urgently calls for novel antibacterial therapeutics, especially those with a new mode of action. We report herein our exploration of protein–protein interaction (PPI) inhibition as a new mechanism to thwart bacterial pathogenesis. Specifically, we describe potent and specific inhibitors of the pneumococcal surface protein PspC, an important virulence factor that facilitates the infection of *Streptococcus pneumoniae*. Specifically, PspC has been documented to recruit human complement factor H (hFH) to suppress host complement activation and/or promote the bacterial attachment to host tissues. The CCP9 domain of hFH was recombinantly expressed to inhibit the PspC–hFH interaction as demonstrated on live pneumococcal cells. The inhibitor allowed for the first pharmacological intervention of the PspC–hFH interaction. This PPI inhibition reduced pneumococci's attachment to epithelial cells and also resensitized the D39 strain of *S. pneumoniae* for opsonization. Importantly, we have further devised covalent versions of CCP9, which afforded long-lasting PspC inhibition with low nanomolar potency. Overall, our results showcase the promise of PPI inhibition for combating bacterial infections as well as the power of covalent inhibitors.



KEYWORDS: *protein–protein interaction, covalent inhibitors, Streptococcus pneumoniae, PspC, human complement factor H*

INTRODUCTION

The antibiotic resistance problem has become a global concern with growing resistance observed for a wide range of bacterial pathogens.[1] This ever-growing threat urgently calls for novel antibiotics with a new mode of action. To this end, it is imperative to identify previously unexplored or underexplored targets against which novel antibiotic agents can be devised. Protein–protein interaction (PPI) inhibition has recently emerged as a rewarding mechanism for developing therapeutics, leading to a number of drugs under clinical evaluation.[2] This exciting new approach, however, has been heretofore minimally explored for developing antibacterials[3] even though numerous PPIs are known to be involved in bacterial pathogenesis and pertinent host defense mechanisms. With this contribution, we investigate the potential of inhibiting a host–pathogen PPI for disrupting bacterial pathogenic pathways.

Streptococcus pneumoniae, also referred to as pneumococcus, has been listed as a serious threat by the Centers for Disease Control and Prevention (CDC).[4] *S. pneumoniae* is a Gram-positive bacterium responsible for the majority of global cases of community-acquired pneumonia. Over 30% of pneumococcal infections are reported to show resistance against one or more antibiotics,[1] and novel strategies are being pursued to treat pneumococcal diseases.[5] Common to bacterial pathogens, *S. pneumoniae* expresses a battery of cell surface proteins that facilitate the bacterium's invasion of and proliferation within the human body. These bacterial proteins interact with various host proteins to enable their colonization of human epithelia and allow evasion of the host's immune surveillance mechanisms.[6] We envisioned that such host–pathogen PPIs could be an effective target for curbing bacterial pathogenesis. We test this hypothesis herein by targeting a pneumococcal surface protein PspC, which is known to recruit the human complement factor H (hFH), a critically important protein deemed the master regulator of the complement system.[7,8] We devised molecular inhibitors of the PspC–hFH interaction with nanomolar potency. Biochemical and cell biological characterization of these inhibitors revealed that disrupting the PspC–hFH interaction effectively inhibits the bacterium's adhesion to human lung epithelial cells and suppresses the immune evasion mechanism of selected bacterial strains. The results of this study collectively demonstrate the promise of PPI inhibition as a strategy to develop novel antibacterial agents.

Received: March 2, 2024
Revised: May 29, 2024
Accepted: May 31, 2024
Published: June 20, 2024

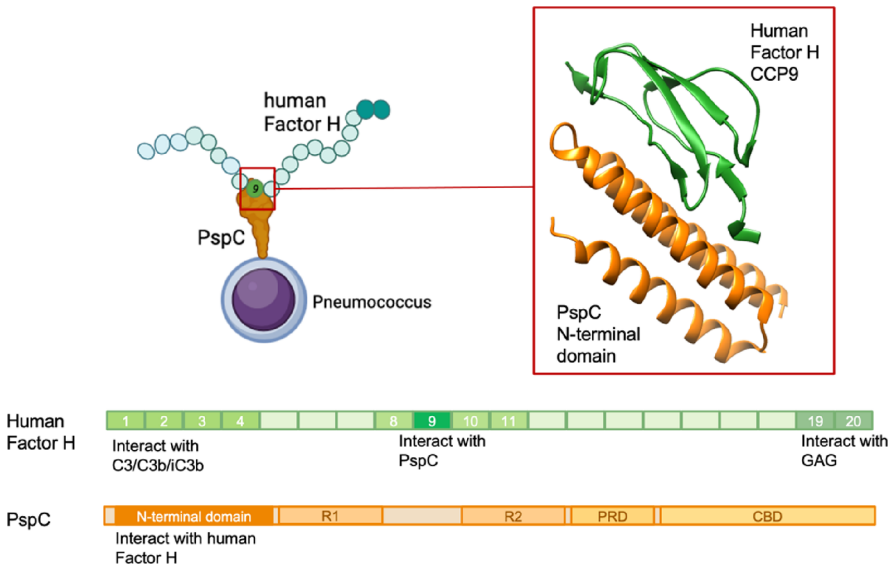


Figure 1. Illustration of the PspC−hFH interaction. The human complement factor H (hFH) has 20 short homologous domains (CCP1−CCP20). The PspC−hFH interaction is primarily mediated by CCP9 binding the N-terminal domain of PspC (PspCN). A cocrystal structure of CCP9-PspCN (PDB: 4k12) is shown on the right. PRD: proline rich domain. CBD: choline binding domain.

EXPERIMENTAL DESIGN AND RESULTS

The PspC−hFH Interaction

PspC, also termed CbpA or SpsA, is one of the cell wall anchored proteins of *S. pneumoniae* initially discovered in 1997 in the search for novel choline-binding proteins (CBPs).[9] This 75 kDa protein consists of multiple domains with a choline-binding motif on its C-terminus, similar to other choline binding proteins including PspA and LytA. PspC has been found in essentially all strains of *S. pneumoniae*,[10] although, in some strains, it is anchored to the cell wall via sortase-mediated peptide ligation.[11] Remarkably, pneumococci recovered from mouse blood after intravenous administration were found to overexpress PspC mRNA by 23-fold. Furthermore, an 870-fold increase of PspC expression was observed after intraperitoneal challenge of mice by *S. pneumoniae*.[12] This induced overexpression indicates the functional significance of this protein in pneumococcal pathogenesis. Indeed, multiple publications document the pathophysiological importance of PspC through studying PspC deletions and mutations. For example, a PspC-deficient mutant of *S. pneumoniae* elicited a 100-fold weaker nasopharyngeal colonization in mouse studies.[9] Similarly, pneumococcal strains with inactivated PspC were found to show significantly attenuated intranasal colonization.[13] Furthermore, these PspC deletion strains failed to infect and multiply within the lungs of mice. These findings are consistent with a report where PspC deletion resulted in 250-fold increase in LD_{50} (50% lethal dose) in a mouse model of bacteremia.[14]

The role of PspC in pneumococcal pathogenesis has been investigated on the molecular level. Specifically, PspC has been found to bind the ectodomain of the polymeric immunoglobin receptor (pIgR) on nasopharyngeal epithelial cells,[15] which is believed to facilitate the bacterium's adhesion to and then transcytosis across the nasopharyngeal epithelia. PspC has also been shown to associate with secretory IgA (sIgA), although the sIgA binding appears to be indirect and mediated by the secretory component of pIgR (SC, ectodomain cleaved of the cells).[16] Importantly, PspC serves as the primary pneumococcal protein to recruit the human complement factor H (hFH),[11,17] a critical component of the complement system with multiple validated functions. The bacterial recruitment of hFH is believed to exert protection against the human complement system. For example, trypsin digestion of the pneumococcal surface proteins elicited a much reduced resistance to complement activation and phagocytosis.[18] Further, a PspC deletion mutant was found to show greater vulnerability to phagocytosis and diminished C3b degradation by factor I, which uses hFH as a cofactor.[19] In animal studies, pneumococci pretreated with hFH showed increased proliferation in mouse blood,[12] consistent with the role of hFH in suppressing complement activities.

The hFH recruitment by *S. pneumoniae* has also been shown to enhance the bacterium's attachment to epithelial cells. Specifically, *S. pneumoniae* D39 preincubated with hFH displayed a 5-fold increase of adherence to human umbilical vein endothelial cells (HUVECs) and an 18-fold increase to lung epithelial cells (SK-MES-1).[20] A similar result was independently documented by another group studying different bacterium−host cell combinations.[21] Consistently, a mouse infection study showed that an hFH-treated *S. pneumoniae* resulted in greater bacterial counts in both blood and the animals' lungs.[20] The hFH-assisted cell adhesion can be perhaps best rationalized by the protein's role to mark the host's own cells to protect them against the attack of the complement system. On the molecular level, hFH is known to bind cell surface glycans including sialic acids, heparins, and glycosaminoglycans.[22] Certain cell surface receptors have also been indicated to contribute to the hFH-mediated bacterial invasion of host epithelial cells.[23]

The biological importance of the PspC−hFH interaction suggests that inhibiting this PPI may be an effective strategy to curb pneumococcal pathogenesis. We herein report miniprotein based PspC−hFH inhibitors, which are found to block the pneumococcal adhesion to lung epithelial cells and mitigate the bacterium's evasion of the human complement system as well.

CCP9 as a PspC−hFH Inhibitor

Both PspC and hFH are multidomain and multifunction proteins (Figure 1). Literature reports have consistently shown that the N-terminal helical domain of PspC (PspCN) is responsible for its binding to hFH,[24] which is composed of 20 short homologous domains, namely, CCP1−20. Binding studies of truncated proteins have localized the PspC binding of hFH to its central domains (CCP8−11)[21] then further to CCP9,[25] which exhibits nanomolar affinity to PspC. A cocrystal structure has been solved at a 1.08 Å resolution to elucidate the atomic-level details of the CCP9−PspCN interaction. The PPI interface is composed of 17 residues from PspCN and 18 residues from CCP9, which forge a constellation of polar and nonpolar interactions (Figure 1). To the end of developing PPI inhibitors that could function as novel antibacterial agents, we envisioned that a standalone CCP9 domain could effectively inhibit the PspC−hFH interaction, thereby dismantling the PspC mediated infective mechanisms. To test the hypothesis, we recombinantly

https://doi.org/10.1021/jacsau.4c00195
JACS Au 2024, 4, 2484−2491

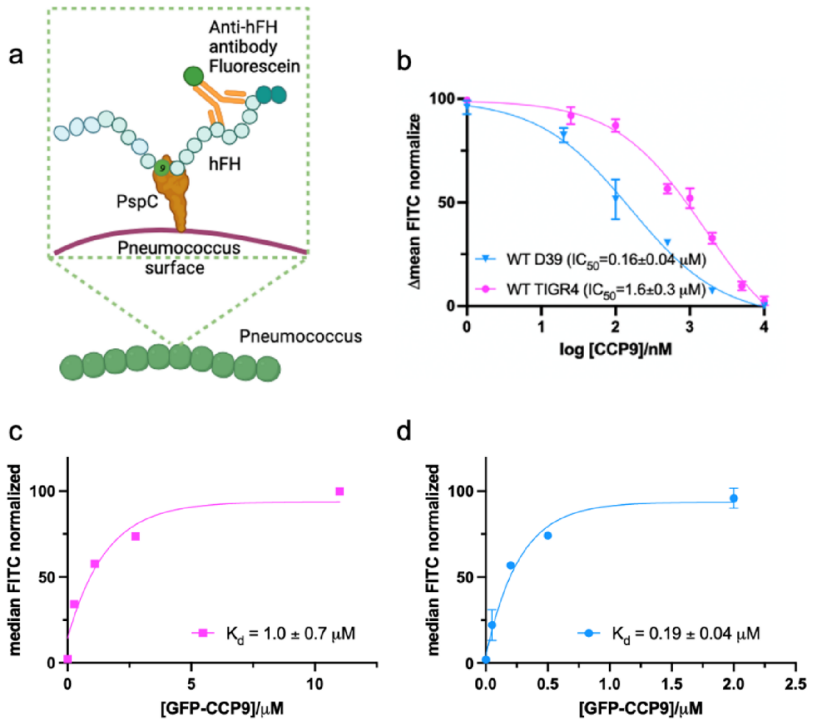


Figure 2. Recombinant CCP9 binds to PspCN and inhibits the hFH recruitment by *S. pneumoniae*. (a) Illustration of hFH recruitment assay to evaluate the PPI inhibitors. (b) Recombinant CCP9 domain inhibits the bacterial recruitment of hFH for both the D39 and TIGR4 strains. (c) Binding curve of GFP-CCP9 to TIGR4 PspCN immobilized on beads. (d) Binding curve of GFP-CCP9 to D39 PspCN immobilized on beads.

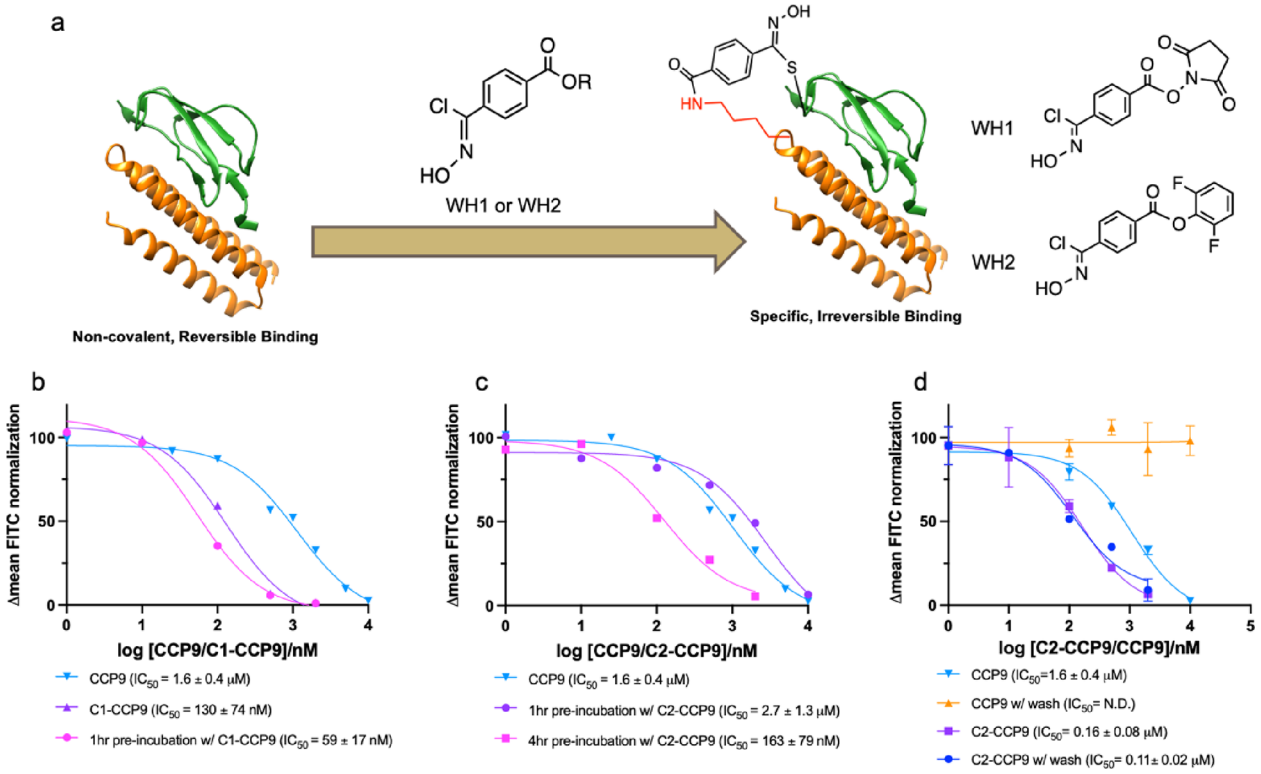


Figure 3. Covalent CCP9 affords enhanced potency and permanent inhibition. (a) CCP9 modification with WH1/2 to give C1-CCP9 and C2-CCP9, which are expected to covalently bind PspC via lysine conjugation. (b) C1-CCP9 and (c) C2-CCP9 tested in the hFH binding inhibition assay against the TIGR4 strain of *S. pneumoniae*. (d) Permanent inhibition of hFH recruitment enabled by C2-CCP9 as revealed by the inhibition assay with and without inhibitor clearance. CCP9 was studied in parallel for comparison.

expressed the CCP9 domain and characterized its inhibitory activity of the hFH binding by *S. pneumoniae* cells.

Our attempt to directly express the hFH CCP9 domain unfortunately afforded a poor yield. Instead, we expressed the

https://doi.org/10.1021/jacsau.4c00195
JACS Au 2024, 4, 2484−2491

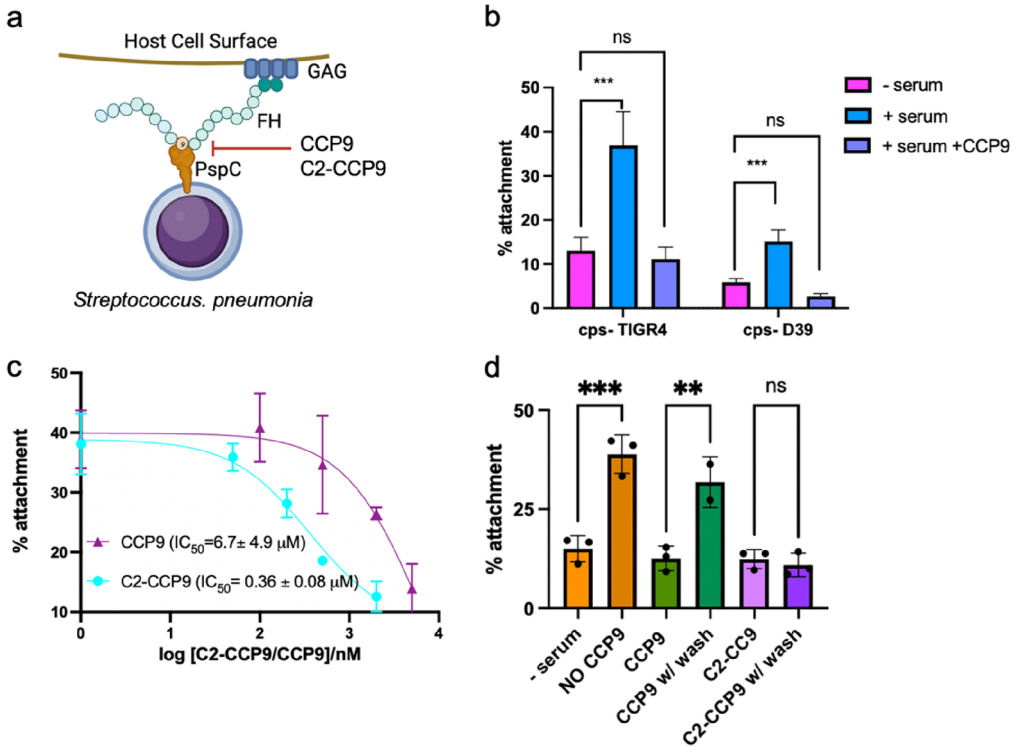


Figure 4. Effect of inhibiting the PspC−hFH interaction on bacterial adhesion to epithelial cells. (a) Illustration of bacterial recruitment of hFH facilitating pneumococcal invasion of epithelial cells, presumably via binding integrin and GAGs of host cells.[22] Multiplicity of infection (MOI) of the cell attachment assay was controlled at ∼80 for all experiments herein. (b) Percentages of the cell attachment cps− D39 and cps− TIGR4 pneumococcal cells in the presence and absence of serum and CCP9 (10 μM for cps− TIGR4 and 2 μM for cps− D39) as an inhibitor. (c) Comparative analysis of CCP9 and C2-CCP9 for inhibiting the epithelial cell attachment of TIGR4 *S. pneumoniae*. The IC_{50} of the covalent inhibitor was determined with 3 h of incubation. (d) Evaluating the inhibitory effect of CCP9 (10 μM) and C2-CCP9 (2 μM) against epithelial cell attachment with and without a washing step for inhibitor clearance. Bar graphs in panels b and d represent mean with SD ($n \geq 3$). Significance was determined by one-way ANOVA between comparison groups (**$p < 0.01$, ***$p < 0.001$).

CCP9 domain as an MBP (maltose binding protein) fusion (Figure S1). The recombinant fusion protein was incubated overnight to allow complete disulfide bond formation. A cleavage site of TEV protease, a highly sequence-specific cysteine protease from Tobacco Etch Virus (TEV), was engineered in between MBP and CCP9, which allowed facile removal of MBP to give the desired CCP9 domain (Figure S2). This protocol readily yielded CCP9 at multimilligram quantities. With the recombinant protein in hand, we analyzed its inhibition of hFH recruitment by two well-characterized *S. pneumoniae* strains, TIGR4 and D39,[26,27] which belong to the serotype 4 and serotype 2 families, respectively. Both strains originated from clinical isolates associated with severe invasive diseases and have been shown to be highly virulent in animal studies.[27,28] The hFH binding by bacterial cells was measured via a flow cytometry experiment, in which the bacterial cells were treated with 20% human serum (as a hFH source) and then the bound hFH is quantified using a fluorescently labeled anti-hFH antibody (Figure 2a). Consistent with the hFH binding, the serum treated cells exhibited greater fluorescence than the untreated controls (Figure S4). Excitingly, the addition of CCP9 inhibited the bacterial binding of hFH in a dose-dependent manner (Figure 2b). Curve fitting yielded an IC_{50} of 1.6 and 0.16 μM for the TIGR4 and D39 strains, respectively. As a random protein control, a recombinant staphylococcal sortase was tested in parallel, and no inhibitory activity was observed (Figure S4). The CCP9 elicited inhibition presumably results from the competitive binding of CCP9 to PspC against hFH in serum. To confirm this mechanism, we recombinantly expressed the N-terminal domain of PspC from both the TIGR4 and D39 strains (Figures S5−S7). To assess the CCP9 binding to these PspCN variants, we expressed a GFP-CCP9 fusion (Figure S8), which allowed the quantification of the CCP9-PspCN binding affinity using flow cytometry (Figure 2c,d). Curve fitting revealed a K_d of 1.0 and 0.19 μM for CCP9 binding to the PspCN of the TIGR4 and D39 strains, respectively. These K_d values may appear comparably higher than some previous reports. However, we would cation against simple comparison of K_d values extracted from different conditions and methods. For example, all our binding studies were conducted with 1 mg/mL BSA (bovine serum albumin) present in the medium to mimic human serum conditions. The absence of such a blocking agent may have resulted in artificially low K_d values. Finally, we note that the K_d values agree remarkably well with the IC_{50} values extracted from bacterial cell studies of hFH binding and inhibition despite the much more complex molecular composition in the latter experiment with cells immersed in serum. This near perfect agreement indicates that CCP9 binds the PspC−hFH interface with excellent specificity.

Morphing CCP9 into Covalent Inhibitors

Although CCP9 proved to be an effective PspC−hFH inhibitor, we postulated that its inhibitory potency could be significantly enhanced by installing a covalent warhead. Targeted covalent inhibitors have received much attention due to their unique mode of action.[29,30] A covalent inhibitor typically associates with its target protein through noncovalent interactions, and the binding event directs the warhead to forge a covalent bond. The covalent bond formation can afford greater potency and specificity toward the target protein.[30−35] A close examination of the TIGR4 PspCN−CCP9 complex structure revealed a solvent exposed Lys-Glu (Glu34 of CCP9 and Lys29 of PspCN) salt bridge (Figure S12), which we envisioned could be converted to a covalent interaction (Figure 3a). We produced E34C mutants of CCP9, in the forms of MBP or GFP fusion (Figures S9−S11), with the intention of using the cysteine residue as the conjugation site for a covalent warhead. The E34C mutant exhibited a marginally weakened PspC binding affinity in comparison to the wild-type CCP9 (Figure S13). We further synthesized two bifunctional molecules, namely,

https://doi.org/10.1021/jacsau.4c00195
JACS Au 2024, 4, 2484−2491

WH1 and WH2 (Figure 3a), each incorporating a chlorooxime group for fast cysteine modification[36] and an activated ester to serve as a lysine-reactive warhead (synthetic details of these small molecules can be found in the Supporting Information). There has been extensive research on lysine-reactive warheads,[37] and we have chosen the activated esters for this study due to their ease of synthesis and highly tunable reactivity. Specifically, we have synthesized a highly reactive OSu ester (WH1) and a less reactive difluorophenolic ester (WH2), which were characterized comparatively.

Treating CCP9-E34C with WH1 and WH2 afforded the corresponding covalent inhibitors, dubbed C1-CCP9 and C2-CCP9, respectively (Figures S14 and S15). With these molecules in hand, we first tested their stability in a PBS buffer and in 20% blood serum, respectively (Figure S16). C1-CCP9 exhibited a $t_{1/2}$ of 82 min in the PBS buffer and 43 min in blood serum. In contrast, C2-CCP9 exhibited much improved stability, showing no degradation for 16 h in blood serum and even 5 days in PBS buffer. Next, we assessed the capability of C1/2-CCP9 to bind the PspC protein and form a covalent conjugate (Figure S17). Under the experimental conditions, C1-CCP9, which harbors a more reactive ester (an OSu ester), rapidly conjugated with PspCN to achieve completion within 25 min. C2-CCP9 exhibited a slower conjugation kinetics in comparison, yielding a half-time of conversion of ∼3 h. Interestingly, neither C1-CCP9 nor C2-CCP9 showed conjugation with the D39 PspCN under the same experimental conditions (Figure S18). Sequence alignment indicated that the D39 PspC sequence lacks the equivalent lysine residue that is targeted by our covalent CCP9s (Figure S19). The contrasting results of the two strains highlight the superb target protein selectivity that a covalent inhibitor can afford.

Encouraged by the protein conjugation studies, we evaluated the inhibitory effect of C1/2-CCP9 against the hFH binding of TIGR4 pneumococci (Figure 3). Despite its vulnerability to hydrolysis, C1-CCP9 exhibited significantly enhanced potency in blocking the bacterium's recruitment of hFH, yielding an IC_{50} value 12 to 13 times smaller than that of CCP9 alone (Figure 3b). Incubating the bacterium with C1-CCP9 for 1 h further reduced the IC_{50} by ∼2-fold (59 vs 130 nM). Importantly, we note that C1-CCP9 appeared highly specific for inhibiting PspC as a similar inhibition curve was obtained for preincubating C1-CCP9 with bacterium versus adding C1-CCP9 directly into the bacterium−serum mixture (Figure 3b). Similarly, C2-CCP9 exhibited a significantly improved potency in blocking hFH binding, although a 4 h incubation was necessary as an 1 h incubation yielded little benefit in comparison to the noncovalent control (Figure 3c). These results are consistent with the postulated covalent mechanism of inhibition, which is further corroborated by running the same experiments but with a washing step (Figure 3d). Specifically, adding a washing step before the bacterium was mixed with the serum completely abolished CCP9's capability to inhibit hFH binding. In contrast, the inhibitory effect of C2-CCP9 was minimally affected by the washing step, again consistent with the mechanism that C2-CCP9 had been covalently linked to the PspC protein. Finally, we note that covalent variants of CCP9 afforded little benefit in inhibiting the hFH recruitment by the D39 strains (Figures S19 and 20), as expected by their lack of covalent conjugation to the D39 PspC protein.

PPI Inhibition Frustrates the Bacterial Adhesion to Epithelia

With the inhibition of PspC−hFH interaction confirmed on pneumococcal cells, we further probed the downstream consequences of inhibiting the bacterium's recruitment of hFH. As stated earlier, several reports in the literature describe that hFH recruited to pneumococcal surfaces can facilitate the bacterium's attachment to human epithelia, which is a critical step in the infective pathway of *S. pneumoniae*. We envisioned that molecular inhibition of the PspC−hFH interaction can effectively suppress the bacterium's adhesion to human epithelial cells (Figure 4a). To this end, we examined the efficiency of *S. pneumoniae* to adhere to A549 cells in the presence or absence of a CCP9 inhibitor. The A549 cell line is derived from human lung epithelia and has been previously used for pneumococcal cell adhesion studies. Under our experimental conditions, the wild-type TIGR4 strain of *S. pneumoniae* showed a small percentage of attachment to A549 cells, although the PspC deletion strain (PspC−) did result in a significantly reduced cell adhesion (Figure S21), consistent with the critical role of PspC for hFH recruitment. The effect of hFH recruitment is magnified with the capsular polysaccharide deleted strain (cps−) of TIGR4 *S. pneumoniae*. In comparison to the wild type, this cps− strain of bacterium displayed a much greater percentage (45%) of cell adhesion (Figure 4b). This observation is consistent with the literature reports on non-encapsulated *S. pneumoniae* cells.[38] The enhanced cell adhesion upon cps deletion is also consistent with an earlier paper describing cps shedding by adhering and invading bacterial cells.[39] This efficient cell adhesion requires the presence of human serum, which presumably serves as a source of hFH. Furthermore, deleting PspC in the cps− strain resulted in a dramatic reduction of cell adhesion under the same experimental conditions (Figure S21), again highlighting the critical importance of the PspC−hFH interaction in the bacterial adhesion to host cells.

With the cell attachment assay established, we tested CCP9 and C2-CCP9 for their potential to inhibit the bacterial adhesion to host cells. C2-CCP9 was chosen over C1-CCP9 as an exemplary covalent inhibitor due to its greater stability. Gratifyingly, both CCP9 and C2-CCP9 elicited dose-dependent inhibition of the bacterial attachment to the A549 cells (Figure 4c). Furthermore, at saturating concentrations, both inhibitors were able to reduce the percentage of cell attachment to that of the serum-free control (Figure 4b). Fitting the dose-dependence curve yielded an IC_{50} of 6.7 μM for CCP9 and 0.36 μM for C2-CCP9. The 20-fold greater potency of C2-CCP9 is consistent with its enhanced potency for inhibiting hFH recruitment by the bacterial cells (Figure 4c). Finally, the advantage of covalent inhibition is seen in our cell adhesion assay: the inhibitory effect of C2-CCP9 on bacterial cell adhesion was not affected by drug clearance (via a washing step before mixing the bacterium and A549 cells). In contrast, adding a washing step completely abolished the inhibitory effect of CCP9 under the same experimental conditions (Figure 4d). Finally, we note that CCP9 did efficiently inhibit the lung epithelial cell adhesion of the D39 pneumococci (Figure 4b), affording a complete inhibition at 2 μM. C2-CCP9 was not tested against the D39 pneumococci as the covalent design does not apply to the PspC of this strain of cells (Figure S18).

Effect of Blocking PspC−hFH Interaction on Complement Evasion

Considering the postulated importance of hFH in regulating the complement activities, we also probed the effect of PspC−hFH interaction inhibition on bacterial opsonization by the complement system. The complement system plays an important role in our innate immunity in terms of fending off bacterial pathogens. The complement system, upon activation, can deposit the C3b protein onto bacterial cell surfaces as an opsonin, which further recruits neutrophils and macrophages for bacterial killing and clearance. The complement system is also known to produce membrane attack complex (MAC), which can cause direct membrane lysis and cell death for an invading pathogen. However, it has been shown that this MAC mediated cell killing mechanism does not play a major role in our host defense mechanisms against *S. pneumoniae*.[40]

To quantitatively assess the complement mediated bacterial opsonization, we resorted to a fluorescently labeled anti-C3 antibody to detect C3b protein deposited onto the bacterial cell surfaces (Figure 5a). Briefly, the bacterial cells were treated with human serum and then mixed with the anti-C3 antibody. The extent of C3 deposition is recorded by flow cytometry analysis, which gives the percentage of cells with opsonization above a threshold defined by non-serum-treated cells (Figure S22). As expected, the wild-type TIGR4 and D39 cells both afforded a significant percentage of opsonized cells after 30 min treatment with human serum (Figure 5b,c). As we clearly demonstrated in Figure 2, both strains of bacteria are capable of recruiting hFH from human serum. However, blocking the hFH recruitment with CCP9 appeared to have a marginal effect

https://doi.org/10.1021/jacsau.4c00195
JACS Au 2024, 4, 2484−2491

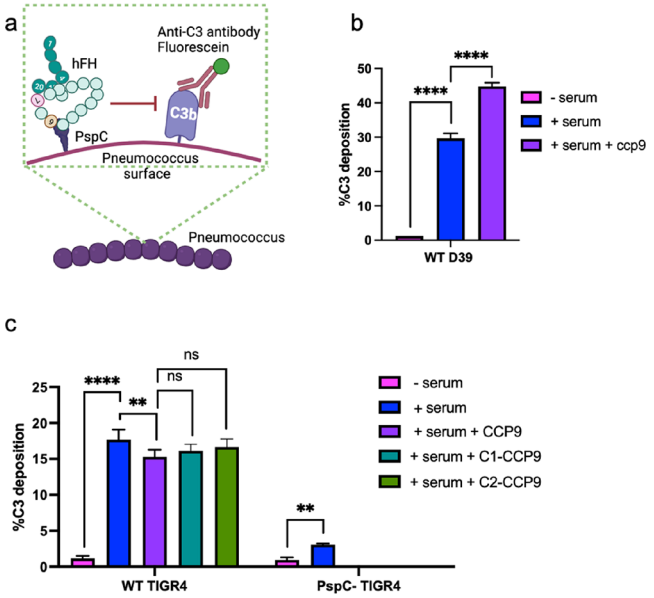


Figure 5. Effect of inhibiting the PspC−hFH interaction on complement mediated opsonization of *S. pneumoniae*. (a) Illustration of the C3 deposition assay. (b) Percentage of opsonized D39 cells upon serum treatment and in the presence and absence of CCP9 as a PspC−hFH inhibitor. (c) Percentage of opsonized TIGR4 cells upon serum treatment and in the presence of CCP9 (10 μM), C1-CCP9 (2 μM), and C2-CCP9 (2 μM). A PspC− strain of TIGR4 exhibits a lower opsonization percentage in comparison to the wild-type bacterium. Bar graphs in panels b and c represent mean with SD ($n \geq 3$). Significance was determined by one-way ANOVA between comparison groups (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

on bacterial opsonization: whereas the D39 cells exhibited a notable, but not dramatic, increase of opsonization (30 to 45%), no increase of opsonization was observed at all for the TIGR4 cells upon CCP9 treatment (Figure 5c). Instead, a slight decrease of cell opsonization was observed. Further evaluation of the covalent inhibitors yielded the same results as the unmodified CCP9 (Figure 5c). We note that all three inhibitors were used at concentrations that essentially blocked the hFH binding of the bacterium to completion, as seen in Figure 3. To better understand this surprising result, we created a PspC deletion strain of TIGR4, which is no longer capable of recruiting hFH as demonstrated by flow cytometry analysis (Figure S4). When subjected to opsonization, the PspC− strain of TIGR4 yielded a pronounced reduction of opsonization in comparison to the wild-type bacterium (blue bars, Figure 5c). This disfavored opsonization upon PspC deletion, contrary to an expected increase, is consistent with what we learned from the CCP9 inhibition studies. The greater extent of opsonization decrease caused by PspC deletion on TIGR4 strain is likely due to additional, currently unknown, functions of PspC other than hFH recruitment, which require further investigation. Nevertheless, our results presented above strongly indicate that evading the complement-mediated opsonization is not the major role of the PspC mediated hFH recruitment, contrary to the previously held beliefs.

CONCLUSIONS AND DISCUSSION

This contribution reports the first installment of our investigation of PPI inhibition as a novel strategy to develop antibacterial agents. As a case study, our work herein focused on inhibiting the PspC−hFH interaction, which has been shown to play critical roles in the pathogenesis of *S. pneumoniae*. Our results show that a standalone CCP9 domain, which is one of the 20 homologous domains of hFH, effectively inhibited the PspC mediated hFH recruitment by pneumococcal cells. Further, we show that inhibiting the PspC−hFH interaction dramatically reduced the bacterium's adhesion to human lung epithelial cells, a critical step in the pathogenic pathways of *S. pneumoniae*. On the other hand, our investigation of CCP9 in an opsonization assay suggests that, contrary to prior understandings, the bacterial recruitment of hFH does not play a major role in pneumococcal escape of the human complement mediated opsonization. This finding, while unexpected, does not detract from the value of inhibiting the PspC−hFH interaction, which we show is primarily responsible for promoting bacterial adhesion to lung epithelial cells. Finally, and importantly, we demonstrate that the inhibitory potency and specificity of CCP9 can be significantly enhanced by enlisting a covalent warhead to target a strategically chosen lysine, and the covalent CCP9 inhibitors allow potent and permanent inhibition of the hFH recruitment by the TIGR4 strain of pneumococci. We note that the covalent inhibitors, while enjoying greater potency, does elicit a narrowed coverage across different bacterial strains. For example, C1/2-CCP9 worked well on the TIGR4 strain but not on D39. However, we submit that a covalent inhibitor could also be created specifically for the D39 strain by following a similar protocol as described in this contribution.

Although our results here present a proof-of-concept demonstration, additional research is needed to assess and achieve the full potential of using PPI inhibition to treat bacterial infections. Specifically, although CCP9 conveniently functions as a PspC−hFH inhibitor, a more generalizable approach is required to develop an inhibitor scaffold for other PPIs that one wishes to inhibit for therapeutic applications. Fortunately, there has already been much work done on the front of PPI inhibition in human pathophysiology, which has resulted in both rational design and library screening to discover PPI inhibitors in general.[41,42] On a related front, it may be still nontrivial to transform a noncovalent inhibitor into successful covalent inhibitors, which requires a balance of reactivity and selectivity of warhead chemistry[43] as well as optimization of the linker that joins the warhead to the noncovalent inhibitor scaffold. This study means to provide a proof-of-concept demonstration of PPI inhibition as an antibacterial strategy; hence, we have resorted to activated esters as a lysine reactive warhead to capitalize on their ease of synthesis and tunable reactivity. Further development of these covalent inhibitors will need to take into consideration the *in vivo* stability of the covalent warheads. Again, fortunately, there has been quickly accumulating knowledge on warhead chemistry[44] as well as successful examples of covalent inhibitors in the literature.[30,37] Our covalent CCP9 inhibitors add to the earlier examples to showcase the remarkable potency and specificity achievable with covalent inhibitors. Excitingly, novel genetically encoded platforms have been recently developed to complement rational design and allow for high-throughput screening of covalent ligands and inhibitors for a protein of interest.[45,46] These synergistic advances collectively make PPI inhibition ripe for exploration in our pursuit of next-generation antibiotics, which we believe will reveal completely novel mechanisms of antibiotic action.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00195.

https://doi.org/10.1021/jacsau.4c00195
JACS Au 2024, 4, 2484−2491

Additional experimental details, methods, and characterization data (PDF)

AUTHOR INFORMATION

Corresponding Author

Jianmin Gao − *Department of Chemistry, Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, United States;* orcid.org/0000-0001-9341-1287; Email: Jianmin.Gao@bc.edu

Authors

Yuhan Lyu − *Department of Chemistry, Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, United States*

Fan Yang − *Department of Chemistry, Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, United States*

Bharathi Sundaresh − *Department of Biology, Boston College, Chestnut Hill, Massachusetts 02467, United States;* Present Address: Current address: Boston Children's Hospital, Boston, Massachusetts 02115, United States

Federico Rosconi − *Department of Biology, Boston College, Chestnut Hill, Massachusetts 02467, United States*

Tim van Opijnen − *Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, Massachusetts 02142, United States;* Present Address: Current address: Boston Children's Hospital, Boston, Massachusetts 02115, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/jacsau.4c00195

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Qiwen Su and Dr. Patrick Autissier for their help in various flow cytometry-based assays. The financial support of this work was provided by NIGMS through grants GM124231 and GM152005 (to J.G.).

REFERENCES

(1) CDC *Antibiotic Resistance Threats in the United States, 2019*; Atlanta, GA, 2019.

(2) Arkin, M. R.; Tang, Y.; Wells, J. A. Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. *Chem. Biol.* **2014**, *21* (9), 1102−14.

(3) Kahan, R.; Worm, D. J.; de Castro, G. V.; Ng, S.; Barnard, A. Modulators of protein−protein interactions as antimicrobial agents. *RSC Chem. Biol.* **2021**, *2* (2), 387−409.

(4) Prevention, C. f. D. C. a., Pneumococcal Disease. In *Epidemiology and Prevention of Vaccine-Preventable Diseases*, Hamborsky, J. K. A., Wolfe, S, Ed. Public Health Foundation: Washington D.C, 2015.

(5) Yang, Y.; Lin, J.; Harrington, A.; Cornilescu, G.; Lau, G. W.; Tal-Gan, Y. Designing cyclic competence-stimulating peptide (CSP) analogs with pan-group quorum-sensing inhibition activity in Streptococcus pneumoniae. *Proc. Natl. Acad. Sci. U.S.A.* **2020**, *117* (3), 1689−1699.

(6) Heesterbeek, D. A. C.; Angelier, M. L.; Harrison, R. A.; Rooijakkers, S. H. M. Complement and Bacterial Infections: From Molecular Mechanisms to Therapeutic Applications. *J. Innate. Immun.* **2018**, *10* (5−6), 455−464.

(7) Esparza-Gordillo, J.; Soria, J. M.; Buil, A.; Almasy, L.; Blangero, J.; Fontcuberta, J.; Rodríguez de Córdoba, S. Genetic and environmental factors influencing the human factor H plasma levels. *Immunogenetics* **2004**, *56* (2), 77−82.

(8) Moore, S. R.; Menon, S. S.; Cortes, C.; Ferreira, V. P. Hijacking Factor H for Complement Immune Evasion. *Front. Immunol.* **2021**, *12*, No. 602277.

(9) Rosenow, C.; Ryan, P.; Weiser, J. N.; Johnson, S.; Fontan, P.; Ortqvist, A.; Masure, H. R. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. *Mol. Microbiol.* **1997**, *25* (5), 819−829.

(10) Iannelli, F.; Oggioni, M. R.; Pozzi, G. Allelic variation in the highly polymorphic locus pspC of Streptococcus pneumoniae. *Gene* **2002**, *284* (1), 63−71.

(11) Janulczyk, R.; Iannelli, F.; Sjöholm, A. G.; Pozzi, G.; Björck, L. Hic, a Novel Surface Protein of Streptococcus pneumoniae That Interferes with Complement Function*. *J. Biol. Chem.* **2000**, *275* (47), 37257−37263.

(12) Quin, L. R.; Carmicle, S.; Dave, S.; Pangburn, M. K.; Evenhuis, J. P.; McDaniel, L. S. In vivo binding of complement regulator factor H by Streptococcus pneumoniae. *J. Infect. Dis.* **2005**, *192* (11), 1996−2003.

(13) Balachandran, P.; Brooks-Walter, A.; Virolainen-Julkunen, A.; Hollingshead, S. K.; Briles, D. E. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of Streptococcus pneumoniae. *Infect. Immun.* **2002**, *70* (5), 2526−34.

(14) Iannelli, F.; Chiavolini, D.; Ricci, S.; Oggioni, M. R.; Pozzi, G. Pneumococcal surface protein C contributes to sepsis caused by Streptococcus pneumoniae in mice. *Infect. Immun.* **2004**, *72* (5), 3077−80.

(15) Zhang, J.-R.; Mostov, K. E.; Lamm, M. E.; Nanno, M.; Shimida, S.-i.; Ohwaki, M.; Tuomanen, E. The Polymeric Immunoglobulin Receptor Translocates Pneumococci across Human Nasopharyngeal Epithelial Cells. *Cell* **2000**, *102* (6), 827−837.

(16) Hammerschmidt, S.; Talay, S. R.; Brandtzaeg, P.; Chhatwal, G. S. SpsA, a novel pneumococcal surface protein with specific binding to secretory Immunoglobulin A and secretory component. *Mol. Microbiol.* **1997**, *25* (6), 1113−1124.

(17) Dave, S.; Brooks-Walter, A.; Pangburn, M. K.; McDaniel, L. S. PspC, a pneumococcal surface protein, binds human factor H. *Infect. Immun.* **2001**, *69* (5), 3435−7.

(18) Kozel, T. R.; Neeleman, C.; Geelen, S. P. M.; Aerts, P. C.; Daha, M. R.; Mollnes, T. E.; Roord, J. J.; Posthuma, G.; van Dijk, H.; Fleer, A. Resistance to Both Complement Activation and Phagocytosis in Type 3 Pneumococci Is Mediated by the Binding of Complement Regulatory Protein Factor H. *Infect. Immunol.* **1999**, *67* (9), 4517−4524.

(19) Jarva, H.; Janulczyk, R.; Hellwage, J.; Zipfel, P. F.; Bjorck, L.; Meri, S. Streptococcus pneumoniae evades complement attack and opsonophagocytosis by expressing the pspC locus-encoded Hic protein that binds to short consensus repeats 8−11 of factor H. *J. Immunol.* **2002**, *168* (4), 1886−94.

(20) Quin, L. R.; Onwubiko, C.; Moore, Q. C.; Mills, M. F.; McDaniel, L. S.; Carmicle, S. Factor H binding to PspC of Streptococcus pneumoniae increases adherence to human cell lines in vitro and enhances invasion of mouse lungs in vivo. *Infect. Immun.* **2007**, *75* (8), 4082−7.

(21) Hammerschmidt, S.; Agarwal, V.; Kunert, A.; Haelbich, S.; Skerka, C.; Zipfel, P. F. The Host Immune Regulator Factor H Interacts via Two Contact Sites with the PspC Protein of Streptococcus pneumoniae and Mediates Adhesion to Host Epithelial Cells1. *J. Immunol.* **2007**, *178* (9), 5848−5858.

(22) Agarwal, V.; Asmat, T. M.; Luo, S.; Jensch, I.; Zipfel, P. F.; Hammerschmidt, S. Complement regulator Factor H mediates a two-step uptake of Streptococcus pneumoniae by human cells. *J. Biol. Chem.* **2010**, *285* (30), 23486−95.

(23) DiScipio, R. G.; Daffern, P. J.; Schraufstätter, I. U.; Sriramarao, P. Human Polymorphonuclear Leukocytes Adhere to Complement Factor H Through an Interaction That Involves $\alpha M\beta 2$ (CD11b/CD18)1. *J. Immunol.* **1998**, *160* (8), 4057−4066.

(24) Lu, L.; Ma, Y.; Zhang, J. R. Streptococcus pneumoniae recruits complement factor H through the amino terminus of CbpA. *J. Biol. Chem.* **2006**, *281* (22), 15464−74.
(25) Achila, D.; Liu, A.; Banerjee, R.; Li, Y.; Martinez-Hackert, E.; Zhang, J. R.; Yan, H. Structural determinants of host specificity of complement Factor H recruitment by Streptococcus pneumoniae. *Biochem. J.* **2015**, *465* (2), 325−35.
(26) Tettelin, H.; Nelson, K. E.; Paulsen, I. T.; Eisen, J. A.; Read, T. D.; Peterson, S.; Heidelberg, J.; DeBoy, R. T.; Haft, D. H.; Dodson, R. J.; Durkin, A. S.; Gwinn, M.; Kolonay, J. F.; Nelson, W. C.; Peterson, J. D.; Umayam, L. A.; White, O.; Salzberg, S. L.; Lewis, M. R.; Radune, D.; Holtzapple, E.; Khouri, H.; Wolf, A. M.; Utterback, T. R.; Hansen, C. L.; McDonald, L. A.; Feldblyum, T. V.; Angiuoli, S.; Dickinson, T.; Hickey, E. K.; Holt, I. E.; Loftus, B. J.; Yang, F.; Smith, H. O.; Venter, J. C.; Dougherty, B. A.; Morrison, D. A.; Hollingshead, S. K.; Fraser, C. M. Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. *Science* **2001**, *293* (5529), 498−506.
(27) Lanie, J. A.; Ng, W.-L.; Kazmierczak, K. M.; Andrzejewski, T. M.; Davidsen, T. M.; Wayne, K. J.; Tettelin, H.; Glass, J. I.; Winkler, M. E. Genome Sequence of Avery's Virulent Serotype 2 Strain D39 of Streptococcus pneumoniae and Comparison with That of Unencapsulated Laboratory Strain R6. *J. Bacteriol.* **2007**, *189* (1), 38−51.
(28) Yau, B.; Too, L. K.; Ball, H. J.; Hunt, N. H. TIGR4 strain causes more severe disease than WU2 strain in a mouse model of Streptococcus pneumoniae meningitis: a common pathogenic role for interferon-gamma. *Microbes. Infect.* **2017**, *19* (7−8), 413−421.
(29) Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A. The resurgence of covalent drugs. *Nat. Rev. Drug. Discovery* **2011**, *10* (4), 307−17.
(30) Boike, L.; Henning, N. J.; Nomura, D. K. Advances in covalent drug discovery. *Nat. Rev. Drug. Discovery* **2022**, *21* (12), 881−898.
(31) Li, Q.; Chen, Q.; Klauser, P. C.; Li, M.; Zheng, F.; Wang, N.; Li, X.; Zhang, Q.; Fu, X.; Wang, Q.; Xu, Y.; Wang, L. Developing Covalent Protein Drugs via Proximity-Enabled Reactive Therapeutics. *Cell* **2020**, *182* (1), 85−97.
(32) Klauser, P. C.; Chopra, S.; Cao, L.; Bobba, K. N.; Yu, B.; Seo, Y.; Chan, E.; Flavell, R. R.; Evans, M. J.; Wang, L. Covalent Proteins as Targeted Radionuclide Therapies Enhance Antitumor Effects. *ACS Cent. Sci.* **2023**, *9* (6), 1241−1251.
(33) Gabizon, R.; Tivon, B.; Reddi, R. N.; van den Oetelaar, M. C. M.; Amartely, H.; Cossar, P. J.; Ottmann, C.; London, N. A simple method for developing lysine targeted covalent protein reagents. *Nat. Commun.* **2023**, *14* (1), 7933.
(34) Che, J.; Jones, L. H. Covalent drugs targeting histidine - an unexploited opportunity? *RSC Med. Chem.* **2022**, *13* (10), 1121−1126.
(35) Compain, G.; Monsarrat, C.; Blagojevic, J.; Brillet, K.; Dumas, P.; Hammann, P.; Kuhn, L.; Martiel, I.; Engilberge, S.; Oliéric, V.; Wolff, P.; Burnouf, D. Y.; Wagner, J.; Guichard, G. Peptide-Based Covalent Inhibitors Bearing Mild Electrophiles to Target a Conserved His Residue of the Bacterial Sliding Clamp. *JACS Au* **2024**, *4* (2), 432−440.
(36) Chen, F.; Zheng, M.; Nobile, V.; Gao, J. Fast and Cysteine-Specific Modification of Peptides, Proteins and Bacteriophage Using Chlorooximes. *Chem. - Eur. J.* **2022**, *28* (20), No. e202200058.
(37) Pettinger, J.; Jones, K.; Cheeseman, M. D. Lysine-Targeting Covalent Inhibitors. *Angew. Chem., Int. Ed.* **2017**, *56* (48), 15200−15209.
(38) Novick, S.; Shagan, M.; Blau, K.; Lifshitz, S.; Givon-Lavi, N.; Grossman, N.; Bodner, L.; Dagan, R.; Nebenzahl, Y. M. Adhesion and invasion of Streptococcus pneumoniae to primary and secondary respiratory epithelial cells. *Mol. Med. Rep.* **2017**, *15* (1), 65−74.
(39) Hammerschmidt, S.; Wolff, S.; Hocke, A.; Rosseau, S.; Müller, E.; Rohde, M. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect. Immun.* **2005**, *73* (8), 4653−67.
(40) Jarva, H.; Jokiranta, T. S.; Würzner, R.; Meri, S. Complement resistance mechanisms of streptococci. *Mol. Immunol.* **2003**, *40* (2), 95−107.
(41) Lu, H.; Zhou, Q.; He, J.; Jiang, Z.; Peng, C.; Tong, R.; Shi, J. Recent advances in the development of protein−protein interactions modulators: mechanisms and clinical trials. *Sig. Transduct. Target. Ther.* **2020**, *5* (1), 213.
(42) Passioura, T.; Katoh, T.; Goto, Y.; Suga, H. Selection-based discovery of druglike macrocyclic peptides. *Annu. Rev. Biochem.* **2014**, *83*, 727−52.
(43) Tuley, A.; Fast, W. The Taxonomy of Covalent Inhibitors. *Biochemistry* **2018**, *57* (24), 3326−3337.
(44) Gehringer, M.; Laufer, S. A. Emerging and Re-Emerging Warheads for Targeted Covalent Inhibitors: Applications in Medicinal Chemistry and Chemical Biology. *J. Med. Chem.* **2019**, *62* (12), 5673−5724.
(45) Chen, S.; Lovell, S.; Lee, S.; Fellner, M.; Mace, P. D.; Bogyo, M. Identification of highly selective covalent inhibitors by phage display. *Nat. Biotechnol.* **2021**, *39* (4), 490−498.
(46) Zheng, M.; Chen, F.-J.; Li, K.; Reja, R. M.; Haeffner, F.; Gao, J. Lysine-Targeted Reversible Covalent Ligand Discovery for Proteins via Phage Display. *J. Am. Chem. Soc.* **2022**, *144* (34), 15885−15893.

https://doi.org/10.1021/jacsau.4c00195
JACS Au 2024, 4, 2484−2491