Synthetic peptides capable of potent multi-group *Staphylococcal* quorum sensing activation and inhibition in both cultures and biofilm communities

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ABSTRACT

The pathogen *Staphylococcus epidermidis* uses a chemical signaling process—i.e., quorum sensing (QS)—to form robust biofilms and cause human infection. Many questions remain about QS in *S. epidermidis* as it uses this intercellular communication pathway to both negatively and positively regulate virulence traits. Herein, we report synthetic multi-group agonists and antagonists of the *S. epidermidis* accessory gene regulator (*agr*) QS system capable of potent super-activation and complete inhibition, respectively. These macrocyclic peptides maintain full efficacy across the three major *agr* specificity groups, and their activity can be "mode-switched" from agonist to antagonist via subtle, residue specific structural changes. We describe the design and synthesis of these non-native peptides and demonstrate that they can appreciably decrease biofilm formation on abiotic surfaces, underscoring the potential for *agr* agonism as a route to block *S. epidermidis* virulence. Additionally, we show that both the *S. epidermidis* agonists and antagonists are active in *S. aureus*, another common pathogen with a related *agr* system, yet only as antagonists. This result not only revealed one of the most potent *agr* inhibitors known in *S. aureus*, but also highlights differences in the mechanisms of *agr* agonism and antagonism between these related bacteria. Finally, our investigations reveal unexpected inhibitory behavior for certain *S. epidermidis agr* agonists at sub-activating concentrations, an observation that can be leveraged for the design of future probes with enhanced potencies. Together, these peptides provide a powerful tool set to interrogate the role of QS in *S. epidermidis* infections and in *Staphylococcal* pathogenicity in general.

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INTRODUCTION

Many common bacteria communicate using small molecule and peptide signals to monitor their local population densities and enact beneficial group behaviors.[1-2] This process, termed quorum sensing (QS), is responsible for controlling myriad virulence traits in prevalent human, animal, and plant pathogens, including biofilm formation and toxin production, making its study particularly relevant to public health.[3-8] As antibiotic resistance poses a continually growing threat, chemical strategies that combat bacterial virulence through QS modulation without affecting growth have emerged as attractive alternate therapeutic approaches.[9-10] Over the past 20 years, our laboratory and others have developed synthetic QS inhibitors and activators for opportunistic pathogens, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and the majority of these compounds closely resemble the native QS signaling molecules used by these pathogens.[11-22] Largely as a result of this choice of chemical scaffold, these compounds are often highly sensitive to structural modification in terms of potency yet lack desired properties in many biologically relevant contexts, including (but not limited to) species selectivity, pan-species activity, or chemical stability.[19, 23-25] Looking forward, the pervasiveness of multi-species bacterial communities in nature and infections, along with the growing awareness of the relationships between these microbiomes and human health,[26-29] motivate the development of chemical modulators of QS with relevant bioactivities in both isolated cultures and complex, mixed microbial environments.[30-31]

Staphylococcus epidermidis is an opportunistic pathogen with a high propensity to form robust biofilms on abiotic surfaces, such as indwelling medical devices.[3, 32] These biofilms can enhance its resistance to antimicrobial agents and elicit strong host immune responses, which has established *S. epidermidis* as a leading cause of hospital-associated infections.[3, 33-35] *S. epidermidis* uses QS to regulate and disperse biofilm in a density-dependent manner; activation of QS has been shown to decrease biofilm, suggesting that QS agonists, rather than the far more examined QS antagonists, may make more effective “anti-virulence” tools in this pathogen.[24, 32, 36] In certain settings, however, biofilm is not *S. epidermidis’* most concerning phenotype. For example, in atopic dermatitis infections

involving *S. epidermidis*, QS activation has been linked to enhanced skin degradation, suggesting that efficacious QS antagonists could temper virulence in this setting.[37] Because *S. epidermidis* demonstrates such context-dependent QS-mediated virulence, chemical tools that can tune QS activity either up or down could significantly facilitate study of its pathogenicity.

S. epidermidis and other closely related Gram-positive bacteria use the accessory gene regulator (*agr*) QS system (Figure 1A), which includes two proteins involved in QS signal biosynthesis (AgrB and AgrD) and two proteins involved in signal transduction (AgrC and AgrA).[38-39] The pro-peptide AgrD is processed (at least in part) by the transmembrane protease AgrB to generate the autoinducing peptide (AIP) signal, which contains a five-residue thioester-bridged macrocycle and a 3-7 residue N-terminal tail.[40-41] (In certain *agr* groups of the closely related pathogen *S. aureus*, the integral membrane protease MroQ performs the final N-terminal tailoring of the AIP signal, but this mechanism has not been demonstrated in *S. epidermidis*.)[42-44] Via an unknown mechanism, the AIP is transported (and/or diffuses) out of the cell and into the local environment. AIP is generated at low basal levels at low cell densities. When a quorate-level cell density (and thus a threshold AIP concentration) is achieved, the AIP signal will bind to the extracellular sensor domain of its cognate receptor histidine kinase, AgrC, which leads to autophosphorylation of AgrC's cytoplasmic domain. AgrC then initiates an intracellular signaling cascade via phosphorylation of its partner response regulator, the transcription factor AgrA.[45] AgrA subsequently dimerizes and activates production of the agr machinery (via the P2 promoter) and the effector RNA, RNAIII (via the P3 promoter), which is believed to regulate at least certain virulence factors (including extracellular proteases).[41, 46-48] AgrA also activates the production of phenol soluble modulins (PSMs) (via designated PSM promoters), which have been implicated in the biofilm dispersal process.[3, 49-51] Although biofilm is its most prominent virulence phenotype, *S. epidermidis* produces the extracellular cysteine protease, EcpA, in a QS-dependent manner, which can exacerbate skin degradation in dermatitis (as introduced above) and potentially other infection types.[37, 52]

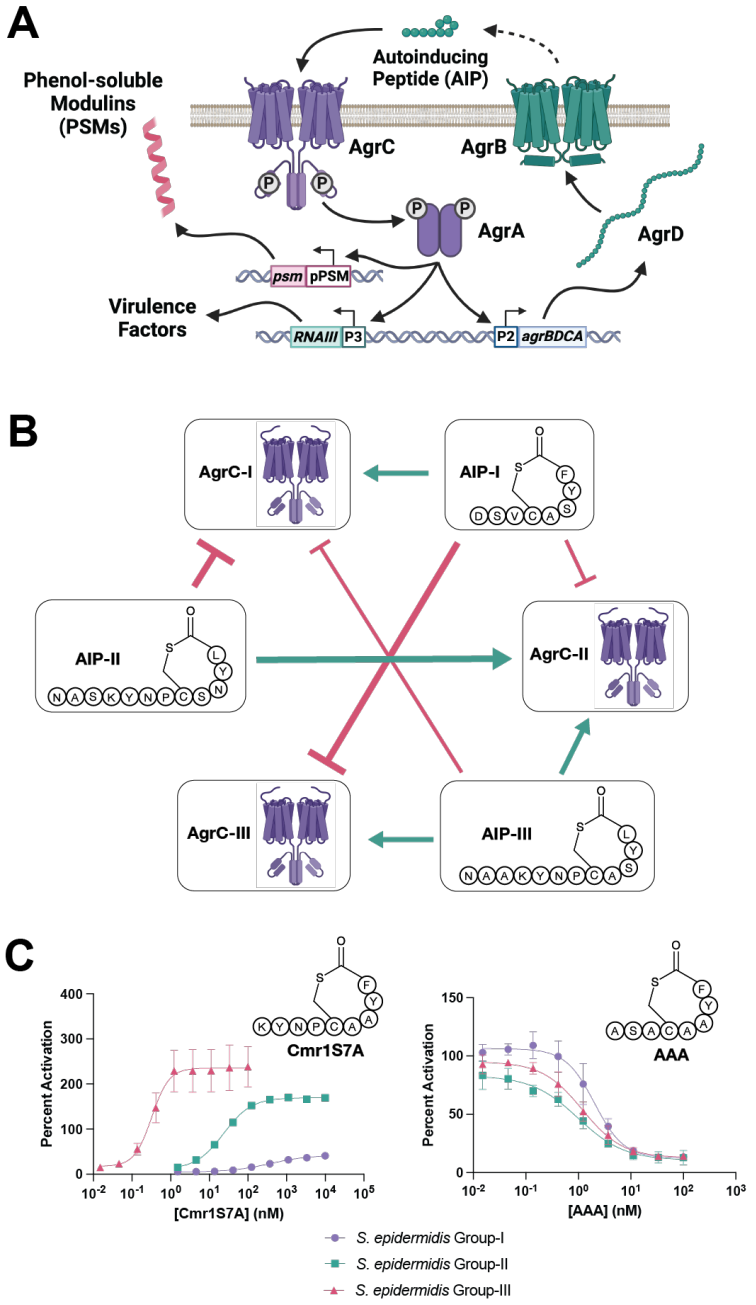

Figure 1. The *agr* QS system and AIP-receptor crosstalk in *S. epidermidis.* (A) Simplified schematic of the *agr* system. (B) The interactions between the AIPs and AgrC receptors of the three major specificity groups (I–III) of *S. epidermidis.* Pink ⊣ indicates inhibition and green ⟶ indicates activation. Line thickness indicates relative activities. (C) Previously reported lead pan-group agonist Cmr1S7A and antagonist AAA and their corresponding dose-response activity in the three specificity groups of *S. epidermidis*.

S. epidermidis' environment-specific virulence behaviors underscore the need for strategies that can attenuate *agr* activity, either up (to disperse/block biofilm) or down (to inhibit protease production), depending on the context. Inhibition or activation of *agr* in *S. epidermidis*, however, is

complicated by the coexistence of at least four divergent *agr* specificity groups (I–IV) of this pathogen, each group with its own unique AIP signal and cognate AgrC receptor.[53-54] (The recently identified *S. epidermidis* group-IV is believed to be scarce in nature, so for the purposes of this study, we focus our efforts on the better-characterized *agr* groups-I–III).[55] Interestingly, each *S. epidermidis* AIP activates its corresponding AgrC receptor, but inhibits the other, non-cognate receptors (Figure 1B).[24-25, 41] The exceptions to this are AIP-III, which also activates AgrC-II, and AIP-II, which is effectively inactive toward AgrC-III.[25, 41] Combinations of these *agr* groups have been reported in clinical isolates of *S. epidermidis*, suggesting that this cross-inhibition of QS does not necessarily preclude cohabitation.[53-54, 56-57] Consequently, for chemical *agr* probes to be most effective in physiologically relevant mixed communities of *S. epidermidis*, the probes must either universally activate or inactivate QS in all *agr* specificity groups, despite the intrinsic specificity of each AgrC receptor for its native signal. This desired activity profile is challenging to design into a molecule *a priori* and has motivated considerable research in our laboratory. We previously reported multi-group *agr* antagonists (e.g., AAA) and a partially active multi-group *agr* agonist (Cmr1S7A; Figure 1C), all of which arose from structural modifications to the native *S. epidermidis* AIPs.[24-25] However, pan-group (i.e., across the three major and historically studied specificity groups) *agr* agonists that reach endogenous levels of activation have proven elusive, perhaps because typically a single native AIP, which evolved for AgrC receptor-specific activation, and very close analogs have been used as starting scaffolds for designing QS modulators in *S. epidermidis* so far.[25]

In the current study, we report the development of universal synthetic agonists and antagonists of the *agr* QS systems in groups-I–III of *S. epidermidis*. We performed a detailed structure-activity relationship (SAR) analysis of three key domains of the known multi-group agonist, Cmr1S7A, which resulted in the discovery of the most potent and efficacious pan-group *agr* agonists in *S. epidermidis* to date. To our knowledge, these non-native macrocyclic peptides are the first compounds capable of activating the *agr* QS system above wild-type (wt) levels (in some cases, above 500%) in the three most prevalent specificity groups of this species. We demonstrate that these

compounds can significantly decrease biofilm formation *in vitro*, supporting QS agonism as a potential strategy to block the main virulence trait of *S. epidermidis*. Our investigations also revealed that certain synthetic *agr* agonists demonstrate strong antagonistic behavior at concentrations significantly lower than those at which they agonize *agr*, a curious activity trend that we were able to directly correlate to specific changes in the structure of a single residue of their exocyclic tails. In addition to these new *agr* agonists, our SAR studies revealed several new pan-group antagonists of the *S. epidermidis agr* system, some of which are the most efficacious reported to date (IC_{50} < 200 pM, >95% inhibition in cells). Lastly, we show that both our pan-group *agr* agonists and antagonists in *S. epidermidis* are only *agr* antagonists in the closely related pathogen *S. aureus*, which suggests differences in the biochemical mechanisms of *agr* agonism and antagonism between these two bacteria. This suite of highly active peptides offers exciting new utility for studying the *agr* system and combating virulence in the Staphylococci.

EXPERIMENTAL

Reagents, strains, and general methods. All standard biological and chemical reagents, amino acids, and solvents were purchased from commercial suppliers and used according to instructions. Safranin O was purchased from Ward's Science. Water (18 MΩ) was purified using an Arium Pro ultrapure water system (Sartorius). All *S. epidermidis* strains were grown in Tryptic Soy Broth (TSB NutriSelect Basic, Sigma-Aldrich) and incubated at 37 °C with shaking at 200 rpm unless otherwise noted. Cultures of *S. epidermidis* fluorescence reporter strains AH3408 (group-I), AH3623 (group-II), and AH3409 (group-III) were supplemented with 10 μg/mL of erythromycin. All *S. aureus* strains were grown in Brain Heart Infusion (BHI, Teknova) and incubated at 37 °C with shaking at 200 rpm unless otherwise noted. Cultures of *S. aureus* fluorescence reporter strains AH1677 (group-I), AH430 (group-II), AH1747 (group-III), and AH1872 (group-IV) were supplemented with 5 μg/mL of chloramphenicol. Native *S. epidermidis* AIPs and analogs previously reported by our laboratory were acquired from in-house stocks synthesized according to reported methods.[24-25] Details of all bacterial strains (Table S1) and instrumentation are included in the Supporting Information (SI).

Peptide synthesis. Linear *S. epidermidis* AIP analogs were prepared on Dawson Dbz resin via standard solid-phase synthesis, macrocyclized in solution, purified using reverse-phase high-performance liquid chromatography (RP-HPLC), and characterized using high-resolution mass spectrometry (HRMS) and analytical RP-HPLC, following previously reported methods.[25, 58] Additional experimental and characterization details of these peptides can be found in the SI (Tables S2).

Fluorescence reporter assay protocol. Agonism and antagonism assays in the *S. epidermidis* and *S. aureus* reporter strains were performed using previously established protocols with minor modifications.[24-25] Peptide stock solutions in DMSO (1 mM) were serially diluted with DMSO (either three-fold or ten-fold), and 2 µL aliquots of the diluted solutions were added to each of the wells in a black 96-well polystyrene microtiter plate (Costar). Each peptide solution was tested in technical and biological triplicate, and 2 µL of DMSO was included as a negative control. To block AgrC activation by endogenously produced AIP in *S. epidermidis* reporter strains, the synthetic multi-group antagonist AAA (identified in our previous study; Figure 1C)[24] was used for the agonism assays at a final concentration of 25 nM. A negative control of 25 nM of AAA was included on each plate to ensure full inhibition was being achieved prior to addition of agonist. Overnight cultures of *S. epidermidis* (grown for 24 h) or *S. aureus* (grown for 20 h) fluorescent reporter strains were diluted fifty-fold with fresh TSB and BHI media, respectively, and 198 µL of the diluted culture were added to each of the wells of the microtiter plate containing peptide. Plates were then incubated at 37 °C for 24 h at 1000 rpm on a Stuart SI505 microtiter plate shaker incubator, and fluorescence (Excitation 500 nm/Emission 540 nm) and OD_{600} of each well were measured using a plate reader. Data was normalized to vehicle (100%) and media (0%) controls, and then analyzed using GraphPad Prism software (v. 10.0.0) to generate sigmoidal curve fits ([compound] vs. response, four-parameters), calculate IC_{50}/EC_{50} values and corresponding 95% confidence intervals (CIs), and determine maximal percent activation and inhibition values.

Checkerboard fluorescence assays in the presence of Cmr1 and AIP-I were performed using the above protocol for *S. epidermidis* antagonism assays with slight modifications. Cmr1 stock solution in DMSO (1 mM) was serially diluted ten-fold with DMSO, and 2 µL aliquots of the diluted solutions were added from left to right across a 96-well black microtiter plate. AIP-I stock solution in DMSO (1 mM) was serially diluted three-fold with DMSO, and 2 µL aliquots of the diluted solutions were added from top to bottom across the same 96-well plate. For comparison, 4 µL aliquots of DMSO and 2 µL DMSO + 2 µL of the threefold-diluted Cmr1 were included. Overnight cultures of *S. epidermidis* fluorescent reporter strains (grown for 24 h) were diluted fifty-fold with fresh TSB medium, and 196 µL of the diluted culture were added to each of the wells of the microtiter plate containing peptide. All other growth conditions and fluorescence/OD_{600} measurements remained the same as in the above protocol. Additional details about the fluorescent reporter assay can be found in the SI.

Safranin biofilm inhibition assay protocol. Quantitative biofilm inhibition assays in *S. epidermidis* WT strain RP62A were performed using previously established protocols for crystal violet dye with significant modifications.[24, 59] Peptide stock solutions were serially diluted with DMSO (ten-fold) and 2 µL of the diluted solutions were added to each of the wells in a clear, flat-bottom 96-well polystyrene microtiter plate (Costar) that had been dried overnight in a desiccator. As a negative control, 2 µL aliquots of DMSO were included. An overnight culture of RP62A was diluted five-hundred-fold with fresh TSB medium augmented with 0.25% (w/v) glucose, and 198 µL of the diluted culture were added to each of the wells of the microtiter plate containing peptide. Bacteria were incubated at 37 °C for 24 h.

The amount of biofilm formed in the wells was quantitated using safranin dye. OD_{600} values were measured for each well prior to decanting the liquid culture. The plates were then inverted over a waste container and gently shaken to remove planktonic bacteria and culture media. The wells were washed gently with 200 µL PBS once, and inverted again over the waste container to remove liquid. The plates were then dried in an oven at 50 °C for 1 h. Following drying, 200 µL of 0.01% safranin

solution (in water) was added to each well and incubated for 25 min at RT. The wells were next washed with 200 µL of water (2x), and remaining safranin was solubilized by the addition of 250 µL of 30% acetic acid in water. The plates were then incubated with rocking (60 rpm) for 60 min at RT, after which safranin absorbance was read at 535 nm using a plate reader. Absorbance was processed by normalizing to the vehicle control (100%) and the media control (0%) and analyzed using GraphPad Prism software (v. 10.0.0). Each sample was tested in technical and biological triplicate.

Safranin biofilm dispersal assay protocol. Quantitative dispersal assays of pre-formed biofilm in *S. epidermidis* WT strain RP62A were performed as detailed above for biofilm inhibition assays with minor changes. First, an overnight culture of RP62A was diluted five-hundred-fold with fresh TSB medium augmented with 0.25% (w/v) glucose, and 200 µL of the diluted culture were added to wells of a clear, flat-bottom 96-well polystyrene microtiter plate that had been dried overnight in a desiccator. Bacteria were statically incubated at 37 °C for 6 h to pre-grow biofilm. A 24 h pre-growth incubation was also tested, and no significant differences were observed in the assay results. Following this incubation period, liquid medium containing non-adherent cells was pipetted out, with care taken to not disrupt the biofilm growing on the bottom of the plate, and 198 µL of fresh TSB medium augmented with 0.25% (w/v) glucose were added to the wells. Peptide stock solutions were serially diluted with DMSO (ten-fold), and 2 µL of the diluted solutions were added to each of the wells in the microtiter plate containing pre-grown biofilm. As a negative control, 2 µL aliquots of DMSO were included. Bacteria were incubated statically at 37 °C for 18 h. The staining and quantification procedures were identical to those in the biofilm inhibition assay protocol above.

RESULTS AND DISCUSSION

Probe design and approach for evaluation. We selected Cmr1S7A (Figure 2A), a previously identified *agr* agonist in *S. epidermidis* (see above),[25] as a starting molecule in our effort to identify highly potent and efficacious QS modulators with pan-group activity. This chimeric peptide is

comprised of the macrocycle of AIP-I and the shared abbreviated exocyclic tail of AIP-II and -III, with the serine in the macrocycle replaced with an alanine. While Cmr1S7A showed potent and efficacious *agr* activation in groups-II and -III, it was only partially activating in group-I (Figure 1C, Table 1). Thus, to improve activity in group-I, while maintaining efficacy in groups-II and -III, we initiated our investigations by making targeted modifications to three key domains of the Cmr1S7A scaffold (C-terminus, exocyclic, and N-terminus), including the incorporation of non-canonical amino acids (Figure 2), and exploring their impacts on agonistic activity.

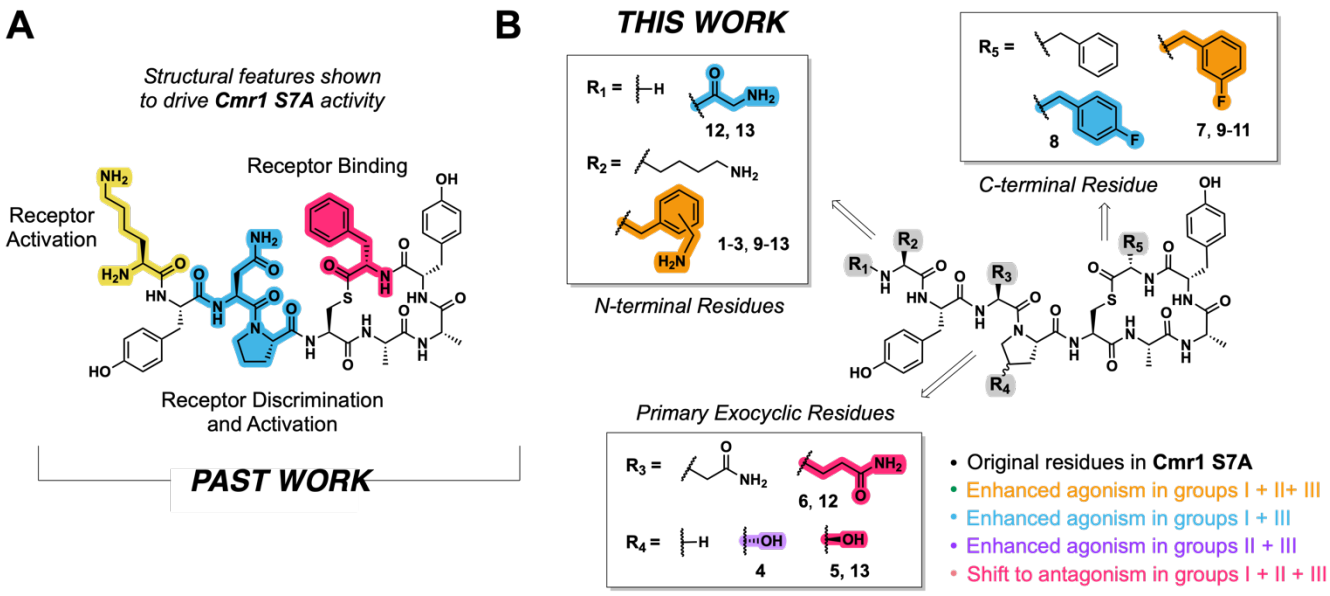

Figure 2. Summary of Cmr1S7A SAR data. (A) Previously identified structural motifs and associated activity trends in the Cmr1S7A scaffold used to guide targeted SAR analyses in the current study (see text). (B) Key modifications at the N-terminal, primary exocyclic, and C-terminal residues of Cmr1S7A that revealed motifs for improving *agr* activation and inhibition across all three groups of *S. epidermidis.* The bolded numbers below the individual modifications indicate the peptides in this study that contain those residues.

All peptide analogs in this study were generated via standard solid-phase peptide synthesis methods (using *N*-Fmoc-L-amino acids) and macrocyclized in solution. Biological activity was measured using a fluorescent transcriptional reporter assay in *S. epidermidis*. The reporter strains used in this assay produce green fluorescent protein (GFP) under the control of the *agr*-regulated P3 promoter, allowing for transcriptional modulation to be monitored via a fluorescent readout (see Experimental for synthesis and assay protocols). We note that these strains produce AIP at native

levels; thus, agonism was measured by first inhibiting AgrC with a known potent pan-group antagonist (AAA; Figure 1C). We chose 25 nM as the competing concentration of AAA because this amount has been shown to significantly inhibit quorum sensing (>85%) similarly across the three major specify groups, but is dilute enough to allow for competition with agonists.[24] This competitive agonism assay method allows for % activity measurements greater than 100% (the value for *agr* activation in the absence of exogenously added ligand). For both agonism and antagonism assays, the endogenous levels of activation were set to 100% activity to allow us to measure the ability of our agonists to stimulate activity relative to untreated cells. Over 35 peptides were iteratively synthesized and evaluated throughout the course of this study using these methods (Tables S3–S5); we focus our discussion on the most efficacious pan-group active compounds and those that drove SAR delineation and probe design here. Major SAR trends uncovered in our scans of the Cmr1S7A scaffold are summarized in Figure 2B. As these peptides resemble native AIP signals, we believe it is reasonable to interpret the result of the *agr* reporter assays to reflect effects of the peptides on the AgrC-I–III receptors.

N-terminal SAR reveal motifs that improve agonism efficacy. We began our SAR analysis by modifying the N-terminal Lys residue of Cmr1S7A, which has previously been implicated as important for AgrC receptor activation (Figure 2A).[25] This positively charged residue is conserved in the native AIPs-II and -III, but is replaced by a negatively charged aspartate in AIP-I. We hypothesized that the positively charged tail of Cmr1S7A may contribute to its diminished efficacy in AgrC-I and thus sought to make modifications at the N-terminus that could introduce activating contacts with AgrC-I without reducing efficacy in groups-II and -III. This resulted in peptides **1**–**3**, which contain 2-, 3-, and 4-(aminomethyl)phenylalanine in place of Lys at the N-terminus, respectively (Figure 2B; Table 1). Peptides **1** and **3** both showed efficacy (% activation) increases of 10-20% in group-I relative to Cmr1S7A, although potency (i.e., EC_{50}) remained effectively unchanged. We observed mixed results in group-II, as **1** was 70% less active than Cmr1S7A, but **3** surpassed 400% activation, far exceeding

the maximal activity of Cmr1S7A (170%). Notably, peptide **2** demonstrated enhanced efficacy in groups-I and -II, approximately doubling that of Cmr1S7A in both groups (Table 1). These varied activities in AgrC-I and -II suggest that the position of the aminomethyl group on this aryl side chain plays an important role in dictating the degree of receptor activation. In group-III, **1**–**3** maintained nearly the same level of activation as Cmr1S7A with analogous low-nanomolar (<100 nM) potencies, suggesting that AgrC-III is insensitive to these N-terminal modifications. The enhanced activities of **1**–**3**, particularly in group-I, made them promising scaffolds from which to continue our development of pan-group *agr* agonists in *S. epidermidis*.

Table 1. Activity of *S. epidermidis* native AIPs, previous lead modulators, and compounds 1–13 in the group I–III *agr* reporters.

		Group-I		Group-II		Group-III	
Compound	**Sequence**	**EC_{50} (nM)[a]**	**Max. Act. (%)[b]**	**EC_{50} (nM)[a]**	**Max. Act. (%)[b]**	**EC_{50} (nM)[a]**	**Max. Act. (%)[b]**
AIP-I	D-S-V-(C-A-S-Y-F)	339 *(278 - 418)*	161	--[c]	--[c]	--[c]	--[c]
AIP-II	N-A-S-K-Y-N-P-(C-S-N-Y-L)	--[c]	--[c]	>2000	917	Inactive[d]	Inactive[d]
AIP-III	N-A-A-K-Y-N-P-(C-A-S-Y-L)	--[c]	--[c]	>2000	131	871 *(624 - 1190)*	288
Cmr1	K-Y-N-P-(C-A-S-Y-F)	>2000	64.9	30.8 *(21.7 - 41.0)*	48.9	0.423 *(0.305 - 0.546)*	204
Cmr1S7A	K-Y-N-P-(C-A-A-Y-F)	378 *(290 - 523)*	40.7	23.4 *(18.2 - 29.1)*	170	0.322 *(0.0693 - 0.675)*	238
1	X_1-Y-N-P-(C-A-A-Y-F) *X_1 = 2-(aminomethyl)-Phe*	433 *(224 - 1700)*	60.4	39.4 *(13.6 - 73.8)*	98.5	35.7 *(26.6 - 44.9)*	396
2	X_1-Y-N-P-(C-A-A-Y-F) *X_1 = 3-(aminomethyl)-Phe*	93.7 *(n/c)[e]*	81.0	15.6 *(7.31 - 38.5)*	334	0.383 *(0.324 - 0.429)*	202
3	X_1-Y-N-P-(C-A-A-Y-F) *X_1 = 4-(aminomethyl)-Phe*	182 *(82.1 - 785)*	52.7	14.1 *(n/c)[e]*	442	0.142 *(n/c)[e]*	204
4	K-Y-N-X_1-(C-A-A-Y-F) *X_1 = trans-hydroxyproline*	602 *(n/c)[e]*	36.4	463 *(239 - 1370)*	544	2.93 *(2.22 - 3.85)*	378
7	K-Y-N-P-(C-A-A-Y-X_1) *X_1 = 3F-Phe*	249 *(198 - 322)*	49.6	252 *(164 - 410)*	162	0.477 *(0.385 - 0.597)*	383
8	K-Y-N-P-(C-A-A-Y-X_1) *X_1 = 4F-Phe*	1620 *(915 - 5760)*	75.3	10.8 *(5.15 - 21.7)*	78.3	0.274 *(n/c)[e]*	484

9	X_1-Y-N-P-(C-A-A-Y-X_2) *X_1 = 2-(aminomethyl)-Phe* *X_2 = 3F-Phe*	150 *(99.2 - 231)*	131	19.2 *(2.18 - 37.5)*	80.5	2.89 *(1.55 - 5.56)*	435
10	X_1-Y-N-P-(C-A-A-Y-X_2) *X_1 = 3-(aminomethyl)-Phe* *X_2 = 3F-Phe*	70.9 *(24.7 - 145)*	177	11.9 *(6.89 - 20.7)*	427	0.213 *(0.191 - 0.239)*	197
11	X_1-Y-N-P-(C-A-A-Y-X_2) *X_1 = 4-(aminomethyl)-Phe* *X_2 = 3F-Phe*	153 *(n/c)*[e]	92.5	15.2 *(n/c)*[e]	535	0.271 *(0.243 - 0.301)*	198
Compound	**Sequence**	**IC_{50} (nM)[a]**	**Min. Act. (%)[b]**	**IC_{50} (nM)[a]**	**Min. Act. (%)[b]**	**IC_{50} (nM)[a]**	**Min. Act. (%)[b]**
AIP-I	D-S-V-(C-A-S-Y-F)	--[c]	--[c]	15.7 *(8.43 - 29.3)*	19.1	3.68 *(2.65 - 5.44)*	9.64
AIP-II	N-A-S-K-Y-N-P-(C-S-N-Y-L)	7.77 *(5.90 - 11.2)*	7.54	--[c]	--[c]	Inactive[d]	Inactive[d]
AIP-III	N-A-A-K-Y-N-P-(C-A-S-Y-L)	390 *(290 - 553)*	8.99	--[c]	--[c]	--[c]	--[c]
AAA	A-S-A-(C-A-A-Y-F)	2.04 *(1.54 - 2.72)*	12.9	0.982 *(0.605 - 1.50)*	12.8	1.19 *(0.893 - 1.60)*	13.9
5	K-Y-N-X_1-(C-A-A-Y-F) *X_1 = cis-hydroxyproline*	40.4 *(31.9 - 50.3)*	3.96	0.162 *(0.113 - 0.213)*	3.14	2.93 *(2.21 - 3.74)*	5.58
6	K-Y-Q-P-(C-A-A-Y-F)	1.64 *(0.918 - 3.10)*	3.17	1.52 *(1.20 - 1.92)*	4.02	0.987 *(0.715 - 1.34)*	7.88
12	G-X_1-Y-Q-P-(C-A-A-Y-F) *X_1 = 2-(aminomethyl)-Phe*	1.21 *(1.04 - 1.43)*	3.20	0.936 *(0.691 - 1.26)*	7.45	0.560 *(0.394 - 0.857)*	7.58
13	G-X_1-Y-Q-X_2-(C-A-A-Y-F) *X_1 = 2-(aminomethyl)-Phe* *X_2 = cis-hydroxyproline*	16.5 *(14.6 - 18.5)*	4.25	4.82 *(3.01 - 8.88)*	8.68	2.26 *(1.84 - 2.79)*	7.24

[a]Agonism or antagonism assay data obtained in *S. epidermidis* group-I–III reporter strains. See Experimental Section for details. 95% confidence intervals (CI) for EC_{50} and IC_{50} values provided in parentheses under each entry. [b]100% activity corresponds to baseline activity produced by endogenous native AIP from vehicle controls in the absence of 25 nM AAA; 0% activity corresponds to media controls. See SI (Tables S3–S5) for 95% CI for max. and min. activity data. [c]Dose-response assays revealed the opposite activity (i.e., a compound does not have an EC_{50} value or Max. Act. because it is an inhibitor, and *vice-versa*). [d]Dose-response assays revealed no substantial agonism or antagonism activity over the concentration range tested. [e]Dose-response analysis did not converge over concentration range tested.

Initial exocyclic residues can be tailored to toggle between pan-group *agr* agonism and antagonism. Prior studies in our laboratory have shown that the exocyclic residues directly adjacent to the macrocycle in the *S. epidermidis* AIPs strongly impact overall peptide conformation and are critical for receptor activation (Figure 2A).[25, 60] In Cmr1S7A, the first exocyclic residue is a proline, which is native to AIPs-II and -III, but not AIP-I. We reasoned that the presence of a non-native residue at this position in Cmr1S7A may contribute to its poorer activity in group-I. Thus, we

investigated the effects of altering steric bulk and hydrophobicity at this position on pan-group activation (Figure 2B; Tables S3–S5). Proline hydroxylation led to the most significant deviation in activity profile from Cmr1S7A. Peptide **4**, which contained trans-4-hydroxyproline in place of proline, had 374% and 140% higher maximal agonistic activities in group-II and group-III relative to Cmr1S7A, respectively, while group-I efficacy remained virtually unchanged (<5% difference in activity) (Table 1). In contrast, peptide **5**, which contained cis-4-hydroxyproline in place of proline, shifted from an agonist to a potent pan-group antagonist (Table 1). This result suggests that regardless of group, AgrC activation is highly sensitive to stereochemical changes made at this residue, and that the stereochemistry of this hydroxyproline substituent effectively allows for a "mode switch" between universally activating to inhibitory interactions with AgrC. The ability to toggle between two drastically different activity profiles with two nearly identical (diastereomeric) chemical probes across three groups of *S. epidermidis* suggests interesting mechanistic and structural studies for the future.

The second exocyclic residue—asparagine—in Cmr1S7A has similarly been shown to be important for AgrC activation (Figure 2A).[25, 60] We were interested to learn the role of sidechain length on receptor activation and designed peptide **6**, which contains a glutamine instead of an asparagine at this position. This modification also resulted in a shift from pan-group activation to potent pan-group inhibition, like that observed for **4** and **5** (Figure 3, Table 1). These data reinforce the importance of the primary exocyclic residues for receptor activation and demonstrate the exquisite sensitivity of AgrC-I–III to structural changes at this position. It is currently unclear whether direct peptide-AgrC contacts or broader peptide conformational changes are driving this shift from agonism to antagonism (for either Cmr1S7A vs. **6**, or for **4** vs. **5**). While the more immediate goal of the current study was to identify pan-group *agr* agonists with enhanced potencies, examination of these exocyclic modifications to Cmr1S7A resulted in two new pan-group *agr* antagonists (**5** and **6**) with comparable potencies and superior efficacies (>95% inhibition in groups-I and -II) relative to our previous inhibitor, AAA, and thus represent upgraded tools to study QS in *S. epidermidis* and its virulence phenotypes across different settings.

C-terminal phenylalanine modifications broadly affect compound potency. We next turned to examining the endocyclic residues in Cmr1S7A to ascertain features important for *agr* activation. The native *S. epidermidis* AIPs and Cmr1S7A all contain hydrophobic residues at the C-terminus of the macrocycle that are believed to be important for receptor binding (Figure 2A).[23, 25] These residues include a conserved tyrosine at the second-to-last position, but diverge at the C-terminal residue; AIP-I and Cmr1S7A contain a phenylalanine, and AIPs-II and -III contain leucine. West et al. demonstrated that *S. epidermidis* AIP analogs containing a phenylalanine at the C-terminal position were more potent than those containing a leucine regardless of *S. epidermidis agr* group, suggesting that electronics, in addition to sterics and hydrophobicity, may play a role in this receptor binding.[25] We sought to explore this phenomenon by incorporating methyl- or fluorophenylalanine at this position, in an attempt to alter the electronics of the side chain without significantly changing its size (Figure 2B). In a preliminary screen with the AIP-I scaffold, we found that *meta*- and *para*-fluorination of the C-terminal phenylalanine improved potency in all three groups relative to AIP-I (Tables S3–S5). Given the similar size of hydrogen and fluorine, we attributed this improved potency to the altered charge density on and around the aromatic surfaces of these non-native phenylalanine sidechains.

When incorporated at the C-terminus of the Cmr1S7A scaffold, *meta*- and *para*-fluorophenylalanine-containing peptides (**7** and **8**, respectively) had mixed effects across specificity groups. In group-I, both **7** and **8** showed improved efficacy compared to Cmr1S7A, though not to the same degree as we observed when the same modifications were made to AIP-I (Table 1, Tables S3–S5). In group-II, **7** demonstrated a ten-fold decrease in potency, but maintained approximately the same efficacy, compared to Cmr1S7A. Peptide **8**, however, maintained largely the same potency as Cmr1S7A in group-II, but was far less efficacious, contrasting the effects of these substitutions in the AIP-I scaffold. Lastly, in group-III, neither *meta*- nor *para*-fluorophenylalanine incorporation substantially affected potency, but both increased the already high (>100%) maximal activity (Table 1). These data suggest that the peptide-AgrC interaction is sensitive to the electronics of the C-

terminal phenylalanine in both the AIP-I and Cmr1S7A scaffolds, and that fluorination of this residue is a useful toggle for improving group-I, maintaining group-III, and reducing group-II activity.

Second-generation analogs with improved potency and efficacy as both agonists and antagonists. Following the above initial SAR analysis, we designed a set of second-generation peptide analogs combining multiple motifs that we had observed were important for enhanced activity. The first set contained 2-, 3-, or 4-(aminomethyl)phenylalanine at the N-terminus of peptide **7** (which contains 3-fluorophenylalanine at the C-terminus), resulting in peptides **9**, **10**, and **11** (Figure 2B), respectively. These substitutions were chosen because, as shown above, they resulted in nearly universal increases in efficacy or potency relative to Cmr1S7A. Peptide **9** demonstrated similar or improved potency in all three groups compared to its parent scaffold **1**, with as much as a twelve-fold decrease in EC_{50} in group-III (Figure 3, Table 1). In addition, **9** was more efficacious in groups-I and –III, although it saw an approximately 20% decrease in maximal activity in group-II. Peptide **11** demonstrated similar potencies in all three groups compared to its parent scaffold **3**, yet was generally more efficacious, bringing group-I activity to approximately endogenous levels and maintaining well over 100% activation in groups-II and –III (Figure 3, Table 1).

The most significant finding, however, was the activity profile for peptide **10**: this analog maintained the low-nanomolar-level potencies in groups-I and –II observed in its parent scaffold **2**, but saw nearly 100% increases in maximal activity in both groups compared to **2**, and remained around 200% active in group-III (Figure 3, Table 1). To our knowledge, peptide **10** is the most potent and efficacious pan-group *agr* agonist in *S. epidermidis* identified to date. Notably, peptide **10** is the first synthetic compound to activate *agr* well over 100% in all three specificity groups, and to nearly the same maximal levels as AIP-I and -III in their cognate AgrC receptors. Moreover, **10** is significantly more potent across all three *agr* groups, with 4- to over 4000-fold lower EC_{50} values relative to each corresponding native AIP. Along with revealing this remarkably active *agr* agonist, these results for peptides **9**–**11** also indicate a degree of synergism between these two C-terminal and N-terminal

modifications on Cmr1S7A, as the level of enhancement in agonistic activities far surpassed simple additive effects.

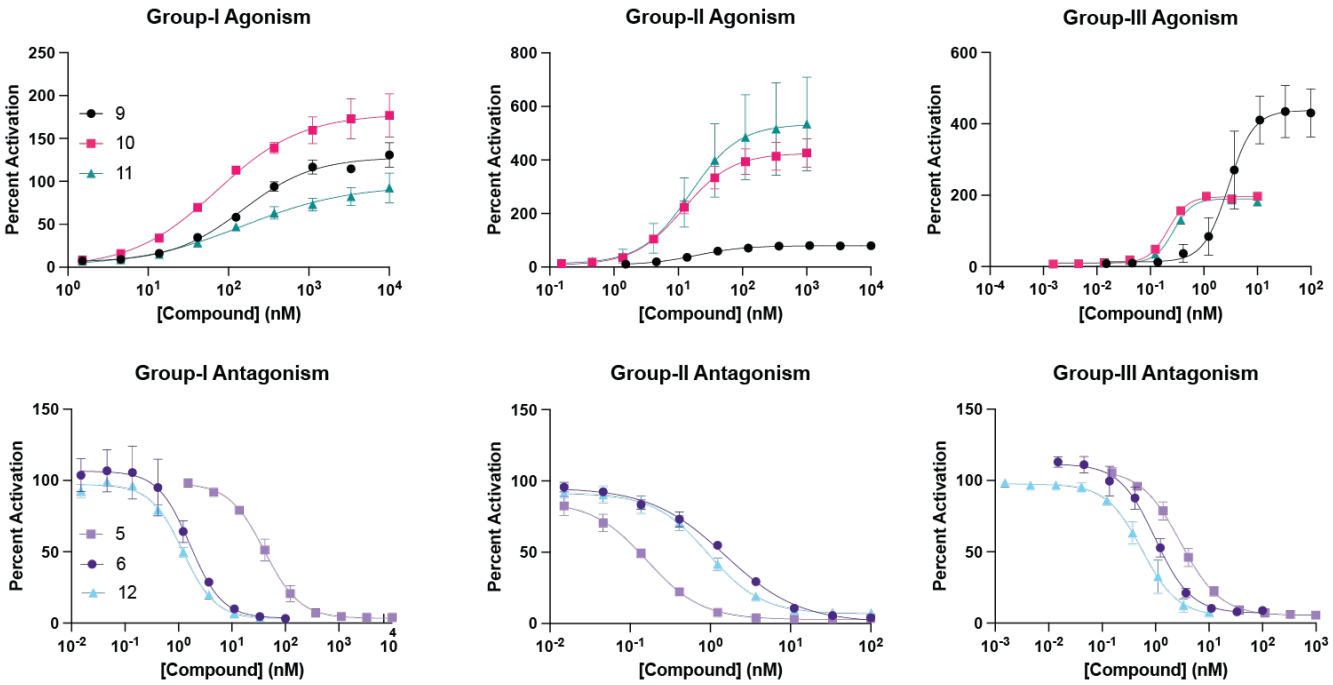

Figure 3. Selected dose-response activity curves for pan-group activators and inhibitors in *S. epidermidis*. Dose-response curves for the three lead agonists (**9**, **10**, and **11**) and the three lead antagonists (**5**, **6**, and **12**) in fluorescent reporter strains for groups-I (AH3408), -II (AH3623), and -III (AH3409). Compound legend in the left plots applies to all plots. See Experimental section for methods. All plotted data is normalized to media (0% activity) and DMSO (100% activity) controls.

We next sought to investigate the behavior of peptides containing combinations of motifs with notable agonistic or antagonistic activities identified in our initial SAR analysis. We hypothesized that motifs that engender strong antagonism (because of presumably strong competitive receptor binding), when appropriately combined with other changes that engender strong agonism, could result in overall enhancements in agonistic activity. This reasoning resulted in the design and synthesis of peptide **12** (Figure 2B), which contained the N-terminal activating residue of **1** (2-(aminomethyl)phenylalanine), the inhibiting asparagine substitution (N3Q) present in **6**, and an additional N-terminal glycine residue, which had varied effects on agonism/antagonism across specificity groups (see SI for further discussion and Tables S3–S5). Interestingly, this peptide showed

enhanced pan-group antagonism activity, with single-digit nanomolar to picomolar potencies in all three groups (Figure 3, Table 1). A similar trend is observed for peptide **13**, which combines the inhibitory cis-hydroxyproline substitution from **5** with the activating 2-(aminomethyl)phenylalanine of **1**. These results suggests that, in opposition to our hypothesis, the substitution that led to increased *agr* inhibition may force antagonistic interactions with AgrC, and additional contacts brought about by 2-(aminomethyl)phenylalanine and N-terminal Gly may simply enhance this effect. Nevertheless, peptide **12** to our knowledge represents the most efficacious pan-group inhibitor of the *S. epidermidis* *agr* system reported to date. Overall, the enhanced activities observed in these second-generation agonists (**9**–**11**) and antagonists (**12** and **13**) validated our two-stage design strategy in which structural modifications that alter either activity or potency are identified in parallel prior to being combined to further bolster overall probe activity.

Pan-group agonists effectively decrease biofilm at high concentrations in *agr* group-I. We were interested to examine the activity of the pan-group *agr* agonists identified above on *S. epidermidis* biofilm formation, the primary virulence phenotype of concern in this pathogen. Based on previous reports,[24, 32] we expected that our agonists would reduce biofilm formation in *S. epidermidis* RP62A (group-I), a known strong biofilm-forming strain,[3] through activation of the *agr* QS system. Again, this outcome is caused by *agr*'s positive regulation of PSM production (Figure 1A), as PSMs function as detergent-like peptides to disperse biofilm.[61] The *agr* system also negatively regulates the production of the autolysin AtlE, which contributes to initial surface attachment.[36, 49, 62-63] Thus, activation of *agr* should, in theory, counteract biofilm accumulation at both steps. In support of this hypothesis, we previously showed that the agonist Cmr1S7A can significantly increase the production of PSMs in *S. epidermidis* groups I–III in a prior study.[25]

We selected the new pan-group agonist **10** and Cmr1S7A for further study in biofilm assays and found that they both not only effectively inhibit biofilm formation, but also disperse preformed biofilm to varying degrees (as judged via safranin biofilm staining assays, Figures 4A and B; see

Experimental). Notably, there was a correlation between maximal activation levels seen in the *agr* reporter strains and the levels of biofilm inhibition and dispersal (Figure 4C). Peptide **10**, which had higher maximal QS activation in the reporters (177%) relative to Cmr1S7A, exhibited 43.5% biofilm inhibition and 30.8% biofilm dispersal at 10 μM (compared to a DMSO control). In turn, Cmr1S7A had lower maximal reporter activation (40.7%) relative to **10** and likewise exhibited reduced biofilm inhibition (18.2%) and dispersal (11.0%) at 10 μM. We note that 10 μM is an approximately 10-fold higher concentration than is necessary to reach maximal QS activation for **10** in the reporter strains, but was chosen for consistency between treatment conditions. Both **10** and Cmr1S7A show slightly greater anti-biofilm activity when added at t = 0 h (inhibition-style assay) than t = 6 h (dispersal-style assay). This trend suggests that, while PSMs likely play a role in dispersing biofilm at both time points, early *agr* activation in the inhibition-style assay may prevent some degree of initial surface attachment through the inhibition of AtlE. To our knowledge, these analogs are the first pan-group agonists in *S. epidermidis* shown to inhibit biofilm growth.

While examining the synthetic pan-group *agr* activators in biofilm inhibition and dispersal assays, we also tested the native *S. epidermidis* agonist, AIP-I, for comparison. Interestingly, although AIP-I reaches similar levels of activation in the group-I reporter assay relative to **10** (161% vs. 177%, respectively), this native signal exhibited moderately improved biofilm inhibition (51.0%) and dispersal (46.7%) compared to **10** (Figure 4A–C). To further characterize the effects of AIP-I and our synthetic agonists on biofilm, we generated dose-response curves for AIP-I, Cmr1S7A, and **10** in both the biofilm inhibition and dispersal assays (over 1 nM to 10 μM; Figures S2 and S3). In both assays, AIP-I demonstrated the expected concentration-dependent dose-response behavior, but Cmr1S7A and **10** yielded unexpected and variable effects on biomass at low concentrations (100 nM and lower). Treatment of *S. epidermidis* RP62A with low concentrations of Cmr1S7A (i.e., below concentrations needed to activate the *agr* system) resulted in drastically reduced biofilm formation, whereas only moderate dose-response behavior was observed with sub-activating concentrations of **10** (Figures S2 and S3). Conspicuously, these trends were only observed with certain of our Cmr1S7A-derivatives

and were not seen with our previously-reported synthetic agonists (or the native AIP-I).[24] This surprising finding in the biofilm assays led us to further investigate the activity profiles of the former derivatives in cell-based reporters, particularly at lower concentrations, to better understand their mechanisms of *agr* modulation.

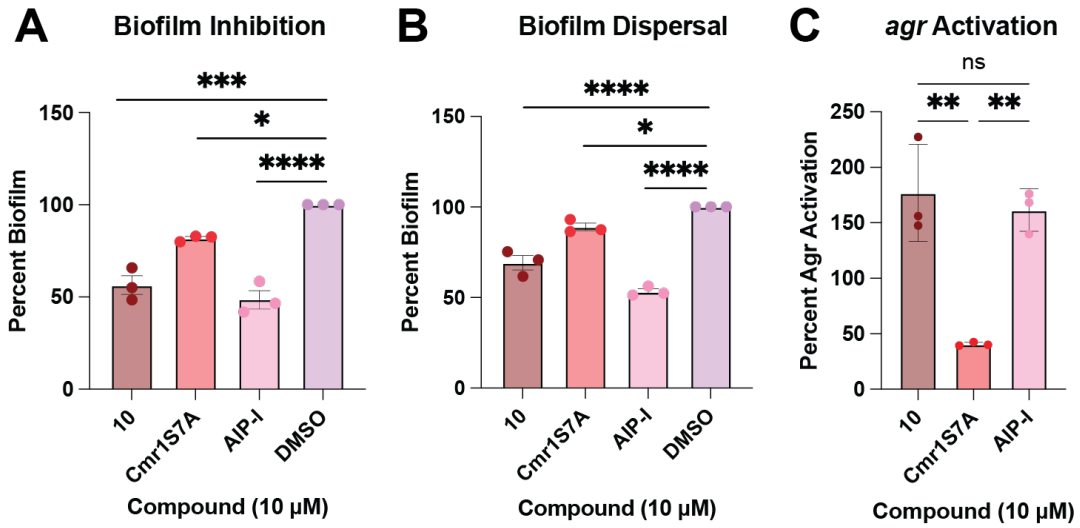

Figure 4. *S. epidermidis* agonists reduce biofilm at high concentrations. Percent biofilm observed at 10 μM of peptide **10**, Cmr1S7A, and AIP-I in (A) inhibition assays or (B) dispersal assays in group-I *S. epidermidis*. For (A) and (B), significance measurements were made using one-way ANOVA tests with respect to the DMSO control. All noted differences were deemed significant with $P < 0.05$. For comparison, *agr* activation at 10 μM for the same compounds in the group-I *S. epidermidis* reporter is shown in (C). Significance measurements for (C) were made using one-way ANOVA tests, and the differences with ** were deemed significant with $P < 0.01$. See Experimental for methods.

Non-native agonists inhibit *agr* groups-I and -III at sub-activating concentrations. We next investigated the Cmr1S7A-derived agonists in the *agr* GFP reporter assays at low concentrations and in the absence of a competing antagonist (i.e., AAA). Using this strategy, we could effectively measure *agr* activation with background endogenous AIP being produced, thus matching the conditions in the biofilm inhibition/dispersal assays. We began by testing **10** in the group-I *agr* reporter, and we observed the expected partial agonism profile at high concentrations (10 μM). However, we also saw complete *agr* inhibition at approximately 10 pM. At sub-10 pM concentrations, the activity returned to vehicle-level activation, generating a “U-shaped” curve (Figure 5A). This result is consistent with **10** behaving as an *agr* antagonist at sub-activating concentrations in the group-I

reporter strain. We then conducted the same experiment with Cmr1 (Table 1), a previously identified group-I agonist and the parent compound of Cmr1S7A,[25] and observed the same U-shaped agonism profile as **10** (Figure 5B). This result showed that agr inhibition at sub-activating concentrations is not unique to **10**, but may instead represent a common activity profile across certain non-native agonists.

The unusual activity profiles of **10** and Cmr1 led us to consider different mechanistic hypotheses for AgrC agonism and antagonism at varying concentrations. A possible heterodimeric model of AgrC:ligand interactions attracted our interest, shown schematically in Figure 5B. AgrC is activated by its native AIP as a homodimer, forming a two protein:two ligand complex.[45] At high concentrations (10 μM) of our synthetic agonists, we see high *agr* activation levels, presumably because the agonist is saturating the two AgrC binding sites. At sub-activating concentrations, however, we reasoned that one AgrC monomer may bind to synthetic agonist and the other to the natively produced AIP (being generated at native levels in these reporter strains). We propose that in this heterodimeric state with at least **10** and Cmr1, AgrC adopts an inactive conformation, resulting in *agr* inhibition. Notably, the concentration at which this inhibition is observed appears to correlate with compound potency (i.e., more potent agonists inhibit the *agr* system at lower concentrations). Finally, at very low concentrations of added synthetic agonist, the native AIP presumably dominates AgrC binding, the AgrC:AIP homodimer prevails, and we see a return to vehicle levels of activity. This hypothesis matches the “U shape” in these curves, starting with agonism by native AIP and ending with agonism by non-native AIP analog.

To test this heterodimer antagonism hypothesis, we conducted checkerboard reporter assays in group-I *S. epidermidis* in which we dosed in varying amounts of AIP-I to a ten-fold dilution series of the agonist Cmr1. These checkerboard data showed that as the amount of AIP-I increases, the concentration at which we observe *agr* inhibition shifts (i.e., the low part of the “U” shape) toward higher concentrations of Cmr1, indicating that more Cmr1 is needed to reach the inhibitory ratio (Figure 5C). As the concentration of AIP-I increases, the basal activity also increases, an expected result given that AIP-I has significantly greater maximum activity (161%) than Cmr1 (64.9%) in group-I

S. epidermidis. These findings support our heterodimer binding model, as they indicate that there is some amount of competition between the native AIP and Cmr1 that leads to *agr* inhibition at sub-activating concentrations. Clearly, additional biochemical studies, ideally *in vitro* with purified AgrC, are required to further probe this mechanistic model. The concentration dependence of this shift in activity in the presence of competing ligand, however, lends support to the hypothesis and motivate such additional inquiry.

In an effort to determine the prevalence of this U-shaped activity profile for *agr* agonists, we screened all of the agonists reported in this study, as well as select previously reported compounds (see SI for full data set),[25] using group-I–III *agr* reporter assays in the absence of AAA, and noticed several trends across compounds and *S. epidermidis* specificity groups. First, we observed that group-I agonists containing a proline residue in the first exocyclic position also inhibited the group-I *agr* system at sub-activating concentrations, but this behavior was not seen in analogs with a valine residue (which is native to AIP-I) in this same position. This activity profile is clearly demonstrated when comparing the activities of Cmr1 and Cmr2, two previously reported agonists in group-I that only differ in their first exocyclic residue (valine in Cmr2, which is native to AIP-I, and proline in Cmr1, which is not native to AIP-I). As shown in Figure 5D, we observe the characteristic U-shaped dose-response curve in this assay for Cmr1, but for Cmr2 we see the expected dose-response curve of a super-agonist (i.e., activity starts at 100% and increases above 100% sigmoidally). This trend was consistent across many (but not all) of our AIP analogs screened in group-I with non-native residues in the first exocyclic position, including trans-hydroxyproline, homoproline, α-aminobutyric acid, and 4-fluoroproline (see SI for dose-response curves).

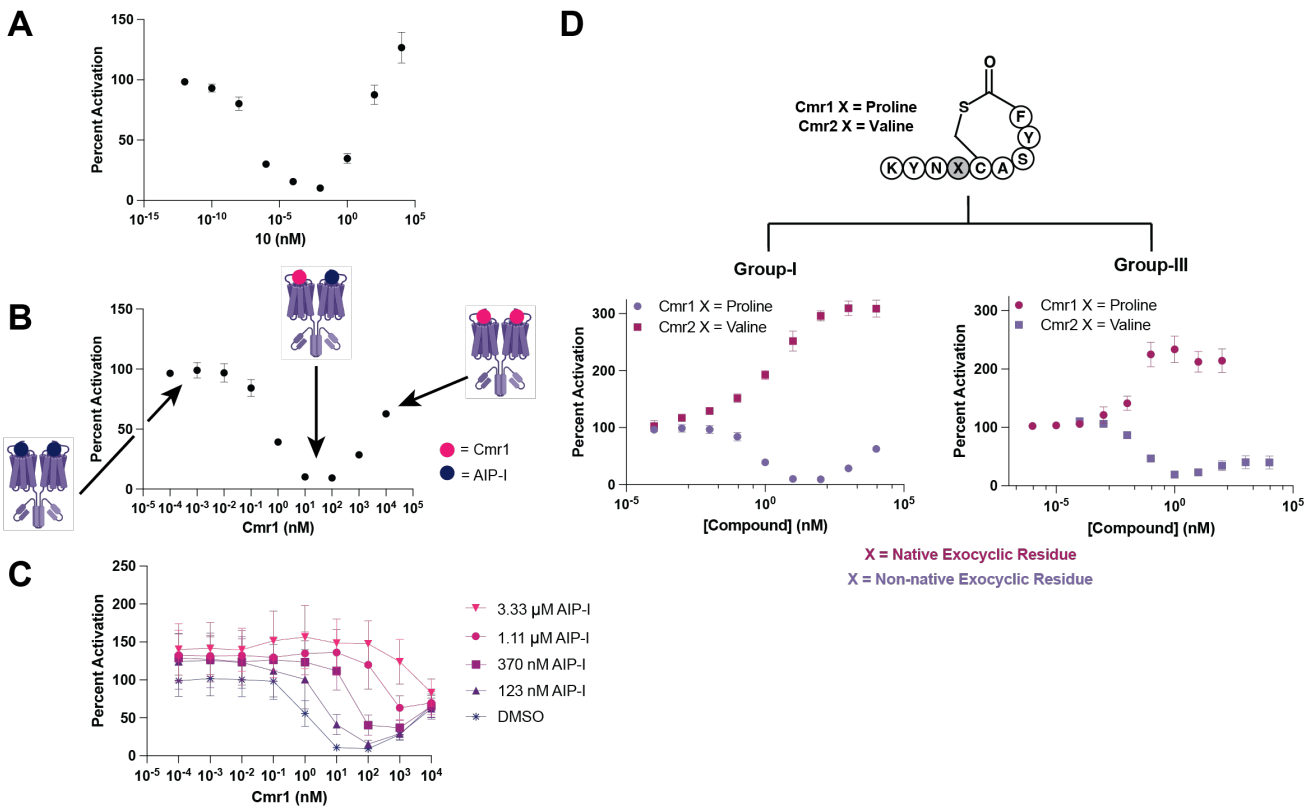

Figure 5. Inhibition of *agr* activity by agonists at sub-activating concentrations. (A) Dose-response curve for peptide **10** in the absence of competing inhibitor (AAA), demonstrating the unexpected "U-shaped" behavior. (B) Schematic detailing the proposed heterodimeric AgrC binding model, using the dose-response curve for Cmr1. (C) Checkerboard reporter assay where dilutions of AIP-I are added to a dose-response of Cmr1. As more AIP-I is added, the inhibition shifts to the right, suggesting more Cmr1 is needed to compete for AgrC binding. (D) Comparison of compounds Cmr1 and Cmr2 in *S. epidermidis* groups-I and -III. Inhibition is observed when the non-native exocyclic residue (indicated in purple) is present (Cmr1 in group-I and Cmr2 in group-III). Inhibition is not observed when the native exocyclic residue (indicated in pink) is present (Cmr2 in group-I and Cmr1 in group-III).

We also observed this U-shaped activity trend, albeit less pronounced, in group-III *S. epidermidis*. AIP-III contains a proline in this first exocyclic position, meaning that for group-III (and opposite to group-I), Cmr1 contains the native residue and Cmr2 contains a non-native residue at the corresponding position. As with group-I, we observed inhibition at low concentrations of agonists containing a non-native exocyclic residue (Cmr2), but not with those containing the native residue (Cmr1) (Figure 5D). Again, this behavior was observed across a wide range of compounds with non-native residues in this first exocyclic position (see SI for dose-response curves). Interestingly, this behavior was not seen in our reporter strain for *agr* group-II for any of the compounds tested, containing the identical reporter plasmid (AH3623). It is currently unclear if this lack of behavior in

group-II is due to a difference in our reporter strain, or something inherent to the *S. epidermidis* group-II *agr* system.

Pan-active *S. epidermidis agr* modulators also potently inhibit *agr* in all four groups of *S. aureus*. As the final part of this study, we were curious whether the new *agr* agonists and antagonists identified herein were also active in other Staphylococci, and we selected the ubiquitous pathogen *S. aureus* for study. In contrast to *S. epidermidis* virulence, in which the primary phenotype under control of *agr* is biofilm formation, *S. aureus agr* positively regulates a plethora of virulence factors and toxins, including several hemolysins, enterotoxins, and (in group-III) toxic shock syndrome toxin-1.[5] Previous work has shown that *S. aureus agr* inhibition is an effective means of reducing this toxin production,[19, 64] underscoring the potential of QS inhibition for anti-virulence applications.[65-66] In addition, *S. epidermidis* and *S. aureus* are frequently found together in the skin microbiome[4, 67] and have been reported to coexist in mixed biofilms at least *in vitro*.[4, 68-70] Thus, having chemical probes with activity across both species would be valuable tools for broad virulence control. Like *S. epidermidis, S. aureus* has evolved into four *agr* specificity groups, so we also were interested to gauge our *S. epidermidis* modulators across each *S. aureus* group.

We tested our most potent *S. epidermidis* pan-group modulators in *S. aureus agr* reporter strains (groups-I–IV) analogous to those in *S. epidermidis* (see Experimental). Across the board, we found that all the *S. epidermidis agr* modulators tested, whether agonists or antagonists in *S. epidermidis,* were potent and efficacious *antagonists* in all four groups of *S. aureus* (Tables S6–S9). None of these compounds displayed appreciable agonism in *S. aureus*. This result yielded two divergent and unprecedented sets of QS modulators: (1) a set that pan-activate *agr* in *S. epidermidis*, but also pan-inhibit *agr* in *S. aureus* (i.e., Cmr1S7A, **2**, **9**, **10**, and **11**), and (2) a set than pan-inhibit *agr* across both species (i.e., AAA, **5**, **6**, and **12**). These opposing activity profiles are readily apparent in the heat map shown in Figure 6 (orange vs. blue). Notably, peptide **10**, the most potent pan-group agonist in *S. epidermidis*, is an extremely potent and efficacious antagonist in *S. aureus*, fully

inhibiting *agr* with IC_{50} values in the low-nanomolar to picomolar range across all four groups of *S. aureus* (Tables S6–S9). This level of potency is comparable to that observed with our previously reported pan-group inhibitor in *S. aureus,* AIP-III D4A,[19] which places it among the most potent reported inhibitor of QS in any bacterial species reported to date. Collectively, this set of AIP analogs represent powerful new tools for studying *agr* signaling and potentially modulating virulence in both of these *Staphylococcal* species simultaneously.

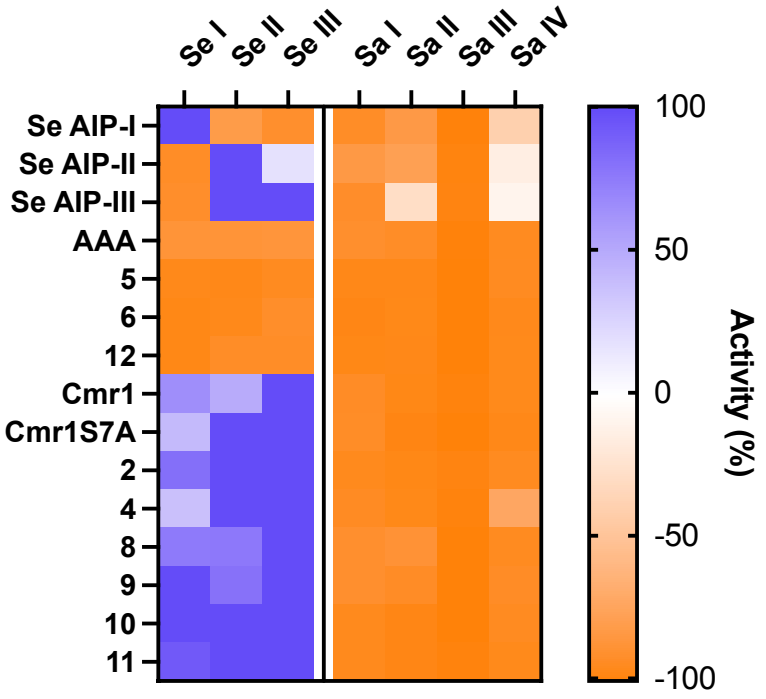

Figure 6. Heat map of the activity of native and synthetic *agr* modulators in *S. epidermidis* and *S. aureus*. Activity data for the *S. epidermidis* AIPs, lead pan-group agonists, and lead pan-group antagonists at 10 μM in *S. epidermidis* (Se) groups-I–III and *S. aureus* (Sa) groups-I–IV. See Experimental for details of strains. Activity is scaled to 100% activation (blue) or 100% inhibition (orange, shown as -100%). Note, many of the agonists activate *S. epidermidis* over 100% (see text and Table 1).

SUMMARY AND CONCLUSIONS

Coagulase-negative Staphylococci, such as *S. epidermidis*, cause a significant percentage of human infections, many of which are strongly connected to the biofilm-forming capacity of these pathogens.[67] In *S. epidermidis*, biofilm formation is regulated, in part, by the *agr* QS system, as *agr* activation can lead to the dispersal of the biofilm matrix. Universal *agr* activation with chemical probes has been

proposed and explored as a means of combating biofilm formation,[25] but is complicated by the existence of multiple *agr* types with strict specificity for their cognate AIPs. Although biofilm formation is the primary mechanism of pathogenicity in *S. epidermidis,* another virulence factor of concern is the protease, EcpA, which has been shown to be detrimental in several human skin diseases.[37, 71] Unlike biofilm formation, EcpA production is positively regulated by *agr*.[4, 41] Thus, the development of potent pan-group *agr* inhibitors is also relevant for *S. epidermidis* virulence control. Having a suite of chemical probes with potent and efficacious pan-group activities in both the activating and inhibitory directions would facilitate investigations into the diverse pathogenic behavior of *S. epidermidis* in a variety of complex environments and contexts. Identifying such a compound suite was the broad goal of the current study.

We began by systematically altering of the N-terminal, exocyclic, and C-terminal domains of a previously identified peptide, Cmr1S7A, with promising albeit limited pan-group agonist activity, and evaluating the resulting peptides in cell-based reporter assays. These studies revealed key SARs dictating both *agr* agonism and antagonism. Incorporation of 2-, 3-, and 4-(aminomethyl)phenylalanine in place of lysine at the N-terminus of Cmr1S7A (**1**, **2**, and **3**, respectively) generally maintained or improved efficacy in all specificity groups. Interestingly, replacing the fourth position proline with trans- and cis-hydroxyproline (**4** and **5**, respectively) resulted in an almost “on-off” mode switch from pan-group activation (by **4**) to pan-group inhibition (by **5**). In addition, fluorination of the C-terminal phenylalanine of Cmr1S7A affected efficacy and potency in different manners across specificity groups (e.g., **8** had improved efficacy in groups-I and -III but diminished efficacy in group-II), suggesting that the peptide C-terminus may interact with AgrC-I–III in distinct ways. Strategic combinations of various activating and inhibiting motifs within the Cmr1S7A scaffold resulted in, to our knowledge, the most potent pan-group *agr* agonists and antagonists in *S. epidermidis* to date. For example, the agonist **10** activated over 100% in all three groups and reached levels of activation comparable to those of native AIPs with their cognate receptors. In turn, the antagonist **12** inhibited *agr* almost completely (to >90%) in all three *agr* groups. The resulting suite of chemical probes

provide powerful tools to toggle between targeting the two key phenotypes in *S. epidermidis*—biofilm formation and EcpA protease production—that are inversely regulated by *agr*. This feature will be particularly useful to investigate mixed bacterial communities in which intra- and interspecies *agr* cross-inhibition could be prevalent. In support of such future research, we show that agonist **10** can significantly inhibit *S. epidermidis* biofilm formation and disperse preformed biofilm, underscoring its utility as a tool for studying QS-mediated virulence phenotypes.

Another significant finding of this study was that certain synthetic *agr* agonists demonstrated inhibitory activities at sub-activating concentrations in *S. epidermidis*, an activity trend that could be directly linked to specific structural changes in the exocyclic region of the peptide scaffold. Their "U-shaped" activity profiles in cell-based *agr* assays led us to propose a possible model of AgrC inhibition for both AgrC-I and AgrC-III in which each protein forms heterodimers bound to native AIP and non-native agonist ligands. This competition between native AIP and our compounds has important implications for the activity of agonists *in vivo*, and the observed lack of this behavior in AgrC-II suggests interesting differences between these receptors across the *S. epidermidis* specificity groups.

Beyond their strong activity profiles in *S. epidermidis,* the pan-group *agr* modulators reported herein also were active across all four specificity groups of *S. aureus.* Interestingly, both the agonists and the antagonists behaved as highly potent pan-group *agr* antagonists in *S. aureus*. This result was unexpected and suggests that the mechanism of AgrC activation and inhibition between these two species differs, at least for this set of peptide ligands. As a notable example, our lead *S. epidermidis* pan-group agonist, **10**, has IC_{50} values in the low-nanomolar to picomolar range for all four *S. aureus agr* groups, placing this compound, to our knowledge, among the most potent *agr* inhibitors known. This level of pan-species inhibition almost certainly cannot be achieved by treatment with combinations of native AIPs, which have variable activities across different specificity groups and species (shown clearly in the heat map in Figure 6), thus underscoring the potential utility of these synthetic compounds to modulate QS within mixed communities. For example, these pan-species QS modulators could allow us to switch between targeting different aspects of *S. epidermidis* virulence

through either *agr* activation or inhibition, while simultaneously inhibiting *S. aureus* toxin production via *agr* inhibition. Given the native AIP-AgrC receptor specificity that pervades *agr*-type QS systems, the discovery of multispecies-active compounds with the ability to block virulence phenotypes, such as the compounds introduced herein and recently-reported *agr* modulators in *Listeria monocytogenes,*[72] represent a meaningful advance for studying and attenuating QS in complex, disease-relevant contexts.

ASSOCIATED CONTENT

Additional experimental details, MS and HPLC data for analogs, full characterization of compound activity and dose-response curves in both *S. epidermidis* and *S. aureus* reporter strains, and additional SAR discussion.

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E.L.E. and T.D.V. contributed equally to this work. E.L.E., T.D.V., and H.E.B. conceived this project. E.L.E., T.D.V., and B.N.P. performed all experimental work. A.R.H. developed and contributed bacterial reporter strains. E.L.E., T.D.V., B.N.P., and H.E.B. analyzed all experimental results. E.L.E., T.D.V., and H.E.B. wrote the manuscript. All authors edited the manuscript and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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