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Inhibition of Virulence as Treatment Strategies for Staphylococcus aureus Infection

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INHIBITION OF VIRULENCE AS TREATMENT STRATEGIES FOR *STAPHYLOCOCCUS AUREUS* INFECTION

BY

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DISSERTATION

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DEDICATION

To everyone who kept me hearty and hale through a decade of college.

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ABSTRACT

Staphylococcus aureus is a global health threat due to its ability to cause significant morbidity and mortality and the exceptional capacity to acquire antibiotic resistance. One method to combat antibiotic resistance, proposed by the NIAID, is anti-virulence strategies focused on bacterial disarmament. *S. aureus* regulates over 200 virulence factors with the *accessory gene regulator* (*agr*) operon, which encodes a quorum sensing (QS) system, and many of the *agr*-regulated virulence factors are required for invasive infection. Therefore, we hypothesized that bacterial disarmament through the inhibition of *agr* quorum sensing will both complement immune function and mitigate the severity of *S. aureus* infection. First, in **Chapter 2**, we report the development and demonstrate the efficacy of the first active vaccine targeting the secreted signal of the *agr* system. The use of stable peptide mimotopes displayed with high valency on a virus-like particle proved successful in limiting *agr*-dependent pathogenesis *in vivo*, a significant advance in the field. Second, in **Chapter 3**, we utilize a novel natural product inhibitor of AgrA, ω-hydroxyemodin, to demonstrate that bacterial disarmament can both limit *S. aureus* pathogenesis and support host innate defense resulting in increased bacterial clearance.

This is the first small molecule *S. aureus* QS inhibitor to demonstrate *in vivo* efficacy and a definitive mechanism of action. Both of the approaches demonstrate that inhibitors of QS would be efficacious as treatments to limit skin infections. Understanding the role of *agr* in other disease states such as pneumonia and bacteremia would enhance the clinical utility. Likewise, it will be essential to determine if QS inhibitors can function as adjunctive therapy to extend the clinical lifetime of current antibiotics. Inhibition of QS alone is not going to solve the antibiotic resistance crisis, but it is another tool that can be used to fight resistance.

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CHAPTER 1: Introduction and Background

*S*taphylococcus aureus, which roughly translates to 'bunch of grapes' for the

characteristic purple cocci clusters following Gram stain and 'golden' for the color

of colonies on solid media, was first described over a hundred years ago (reviewed in

(1)). In the pre-antibiotic era, *S. aureus* was attributed mortality rates exceeding 80% (2).

The advent of penicillin in the 1940s dawned the 'Golden Age' of antibiotics, but within

two years resistance developed and to date less than 5% of *S. aureus* are sensitive to

penicillin (3). The introduction of methicillin in 1961, which was designed to target

penicillin-resistant strains, was met only a few years later with resistant strains termed

methicillin-resistant *S. aureus* or MRSA (3, 4). Resistance to a wide range of antibiotics

has continued to develop following introduction to the clinic, including to the gold

standard of care vancomycin (4). In 2013, the Centers for Disease Control and Prevention

(CDC) released an antibiotic threats document that named MRSA a 'Serious Threat' level

organism (5). In this document the CDC attributed over 11,000 deaths to MRSA in 2011,

and it is important to note this number does not address deaths due to methicillin-

sensitive (MSSA) infections (5). The continual rise of resistance, in all bacteria, coupled

with the dearth of novel pharmaceuticals, is a crisis that has been approached at national

levels with initiatives for antibiotic discovery, such as the British "Antibiotic Action" and

the American "10 x '20" programs (6, 7). However, non-traditional methods for

addressing the resistance threat are gaining traction due to the seemingly inevitable

development of resistance anticipated for even future antibacterial agents (8, 9). These

non-traditional methods include harnessing and enhancing host immune responses and

inhibition of bacterial virulence (8, 9). Herein, two approaches that fall into these non-

traditional methods for combating *S. aureus* infection will be described following a review of the relevant literature.

Disease severity and risk factors

S. aureus is a human commensal, colonizing the anterior nares of ~20% of people persistently and 60% transiently (10). Importantly, this commensal has the capacity to become a significant pathogen, and nasal carriage increases the risk of autoinfection (11). Skin and skin structure infections (SSSIs) represent the major burden of infection caused by *S. aureus*, accounting for about 90% of all infections (12). In a study of SSSIs presenting to emergency departments in the United States, 76% were caused by *S. aureus* and 59% of those infections were due to MRSA strains (13). Additionally, the SENTRY Antimicrobial Surveillance Program found *S. aureus* to be the leading etiological agent of SSSIs in North America, Latin America, and Europe (14, 15). SSSIs can range from simple furuncles to life-threatening systemic infections such as necrotizing fasciitis (14, 16). Finally, recent or concurrent SSSI is a risk factor for developing blood stream infection (17). Although SSSI represent the majority, other disease states are also associated with this pathogen, such as endocarditis, bone and joint infections, pneumonia, device related infections, and bacteremia (12). Even with current therapeutics and best practices, mortality rates for bacteremia are estimated at 10-40% (3, 18).

Populations susceptible to *S. aureus* infections can be grouped into either genetic or non-genetic risk factors. Genetic predispositions include neutrophil disorders such as: Job's syndrome where mutations in signal transducer and activator of transcription 3 (STAT3), a transcription factor important for immune signaling, lead to hyper-IgE and

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dysfunctional T_H17 responses (19); Chediak-Higashi syndrome where mutations in the lysosomal trafficking regulator (LYST), leads to lysosomal transport errors (20); and chronic granulomatous disease which is due to a defect in the enzyme NADPH oxidase, resulting in a lack of oxidative killing in phagosomes (21). Other genetic predispositions include defects in toll-like receptor 2 (TLR2), a bacterial pattern recognition receptor (22), and complement deficiencies (23). Non-genetic risk factors include: hospitalization, contact sports, imprisonment, military service, diabetes, intravenous access (illicit and licit), surgical intervention, immunosuppression (chemical and infections) and old age (3, 14, 24–27). Additionally, although MRSA was previously thought of primarily as a nosocomial, or healthcare associated infection (HA-MRSA), in the 1990s the epidemiology shifted to include infections associated with onset in the community (CA-MRSA) in otherwise healthy persons (reviewed in (28)). Interestingly, CA-MRSA are paradoxically more susceptible to antibiotics but more virulent than HA-MRSA (28). Not only is MRSA resistant to a wider range of antibacterial agents compared to MSSA, it is also associated with increased mortality, length of hospital stay, increased healthcare cost, and overall increased economic burden (29–32).

Current and Proposed Therapy

Although *S. aureus* is becoming increasingly resistant to antibiotics, some efficacious antibiotics are still available and their use depends on the severity infection. Recommended treatment of simple furuncles and abscesses is incision and drainage with concomitant culture and sensitivity testing (16). SSSIs of increased complexity requires the use of empiric and then defined antibiotic treatment following susceptibility testing

(16). MSSA can be treated with nafcillin, oxacillin, dicloxacillin, cefazolin, and cephalexin while vancomycin, dalbavancin, oritavancin, linezolid, daptomycin, ceftaroline, clindamycin, doxycycline, and trimethoprim-sulfamethoxazole are also effective against MRSA (16). Although there are still clinically useful therapeutics available for the treatment of MRSA, many are administered by intravenous route only, have significant adverse effects, and are cost prohibitive (33). Additionally, the continual increase in minimal inhibitory concentration (MIC) values and emergence of vancomycin-intermediate (VISA) and vancomycin-resistant strains (VRSA) will require novel treatments (33, 34). Although there are a few antibiotics in development for the treatment of *S. aureus* infections (33, 35), antibiotic development as a whole is declining, resistance is rising, and clinical needs are not wholly met (8, 35). To address this rise in resistance, the CDC outlined four stratagems to prevent resistance in their 2013 Drug Resistance Threat Report (5): (**a**) prevention of infections, which precludes the use of antibiotics, (**b**) tracking of infections, to assess risk factors and put control programs in place, (**c**) antibiotic stewardship, to control and educate about the proper use of antibiotics, and (**d**) developing novel antibiotics and tests to track resistance. In addition to the CDC recommendations, the National Institute of Allergy and Infectious Diseases (NIAID) has proposed the following non-traditional approaches to combat antimicrobial resistance (9):

- Systems Biology and Antibacterial Resistance

- Harnessing the Immune System to Combat Bacterial Infections

- Disarm, But Leave Unharmed: Exploring Anti-Virulence Strategies

- Synthetic Microbiota: An Ecobiological Approach

- Less is Better: Diagnostics to Guide Use of Narrow-Spectrum Therapeutics

- Exploiting Natural Predators: the Specificity of Phage Therapy

- Teaching Old Drugs New Tricks: Extending the Clinical Utility of Antibacterial Drugs

The body of work reported in this dissertation focuses on two not mutually exclusive strategies, 'anti-virulence' and 'harnessing the immune system,' that can be used to combat *S. aureus* infections.

S. aureus virulence regulation

Bacterial virulence is the capacity of an organism to cause disease, so inhibition of virulence, as proposed by NIAID, seeks to prevent the pathogenesis of infection. The expression of virulence factors is energetically expensive for an individual bacterium, as such many bacteria have evolved mechanisms that allow the 'switching' from avirulent to virulent phenotypes (36, 37). One such mechanism is quorum sensing (QS), which is a bacterial density-dependent cell to cell communication system (reviewed in (36)). In brief, QS functions through a secreted messenger (autoinducer, AI) that acts as both an autocrine and paracrine signal (36). At low cell density the AI is not at sufficient concentration to activate the regulatory QS circuit. As bacterial density increases, the AI concentration hits a critical concentration, positively regulating the QS circuit (feed-forward) while also activating the QS regulon (36). Many Gram positive and Gram negative organisms use QS to coordinate virulence gene expression (36); therefore, inhibition of QS represents an excellent target for anti-virulence strategies as requested by the NIAID.

S. aureus utilizes a two-component QS system consisting of a peptide AI binding to a membrane bound receptor histidine-kinase, which in turn activates a cytoplasmic response regulator (36, 38). The *accessory gene regulator* (*agr*) originally identified in 1986 (39), is a Staphylococcal QS operon controlling the switch between colonizing (adherent) and invasive (virulent) phenotypes. (reviewed in (38)). As shown in **Figure 1.A**, the *agr* locus contains two divergent promoters P2 and P3, which encode the four gene QS operon, *agrBDCA*, and the effector molecule RNAIII, respectively. Briefly, AgrB is membrane endopeptidase that cleaves and cyclizes the propeptide AgrD, and transports the mature autoinducing peptide (AIP) into the extracellular environment. The mature thiolactone cyclized AIP can then bind the cognate receptor histidine-kinase AgrC, resulting in activation (phosphorylation). Active AgrC phosphorylates the response regulator AgrA, which can then bind and initiate transcription at the P2 and P3 promoters as well as the *phenol soluble modulin* α and β (*psmα*, *psmβ*) promoters. P2 transcription results in the feed-forward activation in the *agrBDCA* operon, resulting in population synchrony (36). Transcription of P3 results in the effector molecule, RNAIII, which is the master regulator of virulence. RNAIII post-transcriptionally regulates the *agr* regulon by binding to mRNAs, masking or unmasking Shine-Dalgarno sequences, and thus preventing or enabling translation, respectively.

Evolutionary variation has resulted in four *agr* alleles (*agr*-I, -II, -III, and -IV) among *S. aureus*. Strains representing the four alleles differ in the composition of the AIP molecule (AIP1-4), both in amino acid composition and length (**Figure 1.B**). Due to the differences in the AIP messenger, both the C-terminus of AgrB and N-terminus of AgrC vary to account for processing and recognition, respectively, of the cognate AIP. The

response regulator, AgrA, is conserved across all four *agr* types. Interestingly, due to the variation in AIP, cross-strain interference occurs whereby each strain's AIP can inhibit the non-cognate AgrC. The exception is between *agr*-I and *agr*-IV, presumably because the AIPs only differ in a single amino acid. Not surprisingly, because the *agr* system is conserved across Staphylococci, *in vitro* inter-genus and intra-species *agr* interference has been reported (40–43).
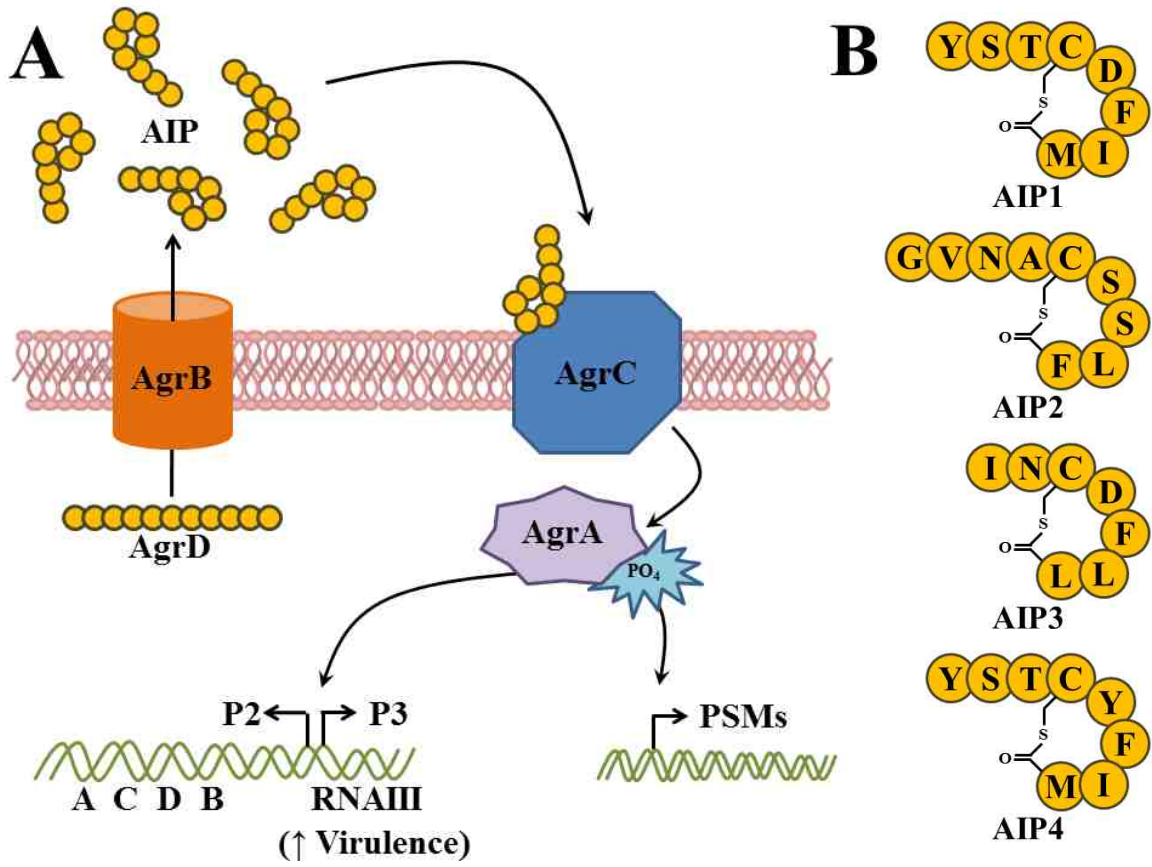
Figure 1.1 - Schematic of the *S. aureus* accessory gene regulator quorum-sensing system. (A) The *agr* P2 promoter drives the expression of the four genes of the operon *agrBDCA*. AgrD is a propeptide that is cyclized to form autoinducing peptide (AIP) and secreted via AgrB. AIPs from the four *agr* alleles vary in length from seven to nine amino acids, but all contain a five-membered thiolactone ring. Secreted AIP binds to its cognate receptor AgrC, activating its histidine kinase function leading to the phosphorylation of AgrA. AgrA binds to the divergent promoters P2 and P3 as well as the promoters for the transcription of the phenol-soluble modulin (PSM) toxins. P2 drives a positive-feedback loop resulting in the upregulation of the *agr* operon, whereas P3 drives the transcription of the effector molecule RNAIII. RNAIII leads to the upregulation of virulence factors that contribute to invasive infection. (B) Sequences of the AIP1-4 molecules of the respective *agr* types I-IV.

The *agr* system regulates expression of more than 200 virulence genes (44). In general, *agr* activation results in the upregulation of secreted proteins that aid in tissue invasion, while down regulating membrane proteins that are essential for adherence, colonization, and biofilm formation (38, 45). Virulence factors positively regulated by *agr* in an RNAIII-dependent manner include: (**a**) membrane-active toxins, exemplified by the heptameric pore forming α-hemolysin (Hla) (46), but also including γ-, β-, and δ-hemolysin and the two-component leukocidins (D/E, S/F, and G/H), (**b**) tissue degrading proteases and lipase, (**c**) the ROS scavenging pigment staphyloxanthin (which also lends the characteristic gold colony color) and (**d**) numerous superantigens which can cause sepsis and adaptive immune cell death (38). Virulence factors negatively regulated by *agr* include: (**a**) fibronectin binding proteins A and B, (**b**) the immunoglobulin binding protein A, and (**c**) coagulase (38). In addition to the RNAIII regulated proteins, AgrA directly activates the transcription of the phenol soluble modulins encoded by *psmα* and *psmβ* leading to the translation of six small amphipathic proteins involved in host cell chemotaxis and membrane damage (PSMα1-4 and PSMβ1-2) (47). Together, these and other virulence factors contribute to invasive infection through a variety of functions. Many of the virulence factor effects are concentration dependent, including direct killing of immune cells, prevention or activation of chemotaxis, prevention of phagocytosis, intracellular survival and lysosomal escape after phagocytosis, and dysregulation of immune signaling (48–51).

The rising antibiotic resistance of *S. aureus* coupled with the large burden of disease requires novel approaches to combating this pathogen. Due to the pleiotropic effects on virulence, QS inhibition of *S. aureus* has been suggested as a target for clinical

intervention (36, 52–54). There are several lines of support for this stratagem. First, infection with *agr* deletion mutants drastically attenuates the severity of multiple disease states (44, 55, 56). Second, innate immunity has evolved responses that inhibit QS such as ROS inactivation of the AIP molecule (57), binding and subsequent sequestration of AIP by lipoproteins (58, 59), and QS inhibition by hemoglobin (60). Third, because inhibitors of quorum sensing do not have direct bactericidal activity they have been posited not to drive resistance. In support of this hypothesis, it was recently demonstrated that *S. aureus* resistance to a classical antibiotic developed readily *in vitro* and *in vivo* but *agr* remained susceptible to small molecule inhibition (55). Therefore, inhibition of *S. aureus* QS may be a clinically viable option for prevention and/or treatment of *S. aureus* infection.

Progress towards a *S. aureus* vaccine

Vaccination is a cornerstone of modern medicine that has resulted in the eradication of smallpox, the drastic reduction of polio, and immense reduction of death due to multiple bacterial and viral infections. Importantly, vaccines directed at virulence factors such as *Haemophilus influenzae* B (HiB) capsule (61), and the secreted bacterial diphtheria, tetanus and pertussis toxins (DTaP) have been highly successful (62). It would seem straightforward, therefore, that since *S. aureus* can be encapsulated and produces toxins, a similar vaccination strategy would be effective. However, to date, vaccine approaches which are effective against other pathogens have failed against *S. aureus*.

The failure of conventional vaccine approaches to protect against, *S. aureus* infection may be due to its status as a common commensal. *S. aureus* colonization

happens very early in life leading to a large number of antibodies against many Staphylococcal antigens in sera of adults (63). Sadly, these natural antibodies do not protect from infection and recurrent infections are common even with increased anamnestic response post-infection (63–65). Additionally, patients with B cell or antibody deficiencies are not at increased risk for infection (26), calling into question the potential efficacy of B-cell targeted vaccination for *S. aureus* infection. Conversely, while colonized patients are more likely to be auto-infected, they are also less likely to succumb to infection (11, 66), and the number of *S. aureus* virulence factors dedicated to humoral evasion suggest that antibody responses are important (63). Interest in a Staphylococcal vaccine is not a modern medical phenomenon. In 1930, then the pre-antibiotic era, Weise reported some clinical success using purified toxin (now known to be Hla) as an active vaccination for recurrent furunculosis (67). However, now almost a century later, there is still no licensed *S. aureus* vaccine despite a large volume of basic science and clinical vaccine trials.

To illustrate the significant amount of work already completed clinically, **Table 1.1** is a compilation of the completed and active efficacy trials (phase II and III), accessible on clinicaltrials.gov, to treat or prevent *S. aureus* disease. Most of the studies have been completed as per clinicaltrials.gov (see NTC# identifiers), but no results are posted or available in the primary literature. The majority of trials have been formulated with one or two surface-associated antigens. These antigens include *S. aureus* capsule 5 and 8, the cell wall-associated lipotechoic acid (LTA) and poly-N-acetyl glucosamine (PNAG), and the binding factors: clumping factor A (ClfA), iron-regulated surface determinant B (IsdB), and serine-aspartate repeat G (SdrG). The goal of antibodies

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against these targets, generated actively through immunization or passively by injection, has been opsonization of *S. aureus* leading to complement or phagocyte-mediated killing. Additionally, there are several trials addressing the efficacy of antibodies directed at preventing the action of secreted toxins such as Hla and Panton-Valentine leukocidin. Of the seven completed studies with published results (**Table 1.1**), five showed no protection compared to placebo (68–73), with one actually showing increased mortality in vaccinated patients upon infection (72). Of the remaining two completed studies, one demonstrated a decrease in length of hospital stay but no reduction in bacteremic mortality (74). The other study demonstrated protection of hemodialysis patients from bacteremia up to ~40 weeks, but there was no significant protection at the pre-identified endpoint of 52 weeks (75).

Table 1.1 – Completed active and passive vaccines targeting *S. aureus*

Identifier	Target(s)[a]	Phase	Patients/Disease	Result(s)	Author[b]
Passive					
Altastaph	CP5, CP8	II	Adults with bacteremia (Std. Rx + pAb or placebo)	No mortality difference vs. placebo Significantly reduced LOS	(74)
		II	Preventing nosocomial infection in neonates (pAb vs. placebo)	No difference vs. placebo	(68)
Aurexis (Tefibazumab)	ClfA	II	Adults with bacteremia (mAb vs. placebo)	No difference vs. placebo	(69)
Aurograb	ABC transporter	II	Outcome of infected patients on vancomycin (Fab vs. placebo)	Completed, no results published	NCT00217841
KBSA301	Hla	II	Determine PK/PD, outcome of current pneumonia patients (mAb vs. placebo)	Recruiting as of April 2012	NCT01589185
SAR279356	PNAG	II	Determine PK/PD, prevention of infection of ventilator patients (mAb vs. placebo)	Terminated, no results posted	NCT01389700
514G3	Not disclosed	II	Determine PK, outcome of current bacteremia patients (Proprietary Ab vs. placebo vs. Std. Rx)	Starting April 2015	NCT02357966
Pagimaximab	LTA	II / III	Preventing nosocomial infection in neonates (mAb vs. placebo)	No difference vs. placebo	(70, 73)
Veronate	ClfA, SdrG	III	Preventing nosocomial infection in neonates (pAb vs. placebo)	No difference vs. placebo	(71)

[a]Commas indicate combined antigens. [b]If no peer-reviewed results are published the clinicaltrials.gov identifier is listed.

Abbreviations: ABC Transporter - ATP-binding cassette transporter; ClfA - clumping factor A; CP5, CP8 - capsule type 5, 8; Fab - fragment antigen-binding; Hla - alpha-hemolysin; LOS - length of stay; LTA - lipotechoic acid; mAb - monoclonal antibody; PD - pharmacodynamics; PK - pharmacokinetics ; pAb - polyclonal antibody; PNAG - poly-N-acetyl glucosamine; SdrG - serine-aspartate repeat G; Std. Rx - Standard Treatment

Table 1.1 – Continued.

Identifier	Target(s)[a]	Phase	Patients/Disease[b]	Result(s)	Author[c]
Active					
SA3Ag; SA4Ag	CP5-DT, CP8-DT, ClfA; +MntC	II	Immunogenicity	Completed, no results published	NCT01643941
SA4Ag	CP5-DT, CP8-DT, ClfA, MntC	II	Preventing postoperative infection after spinal surgery	Starting June 2015	NCT02388165
Toxoids' USUHS	Hla	II	Immunogenicity	Completed, no results published	NCT01011335
	LukS-PV	II	Immunogenicity	Completed, no results published	NCT01011335
	Hla, LukS-PV	II	Immunogenicity	Completed, no results published	NCT01011335
StaphVax	CP5-exoA, CP8-exoA	III	Preventing bacteremia in hemodialysis patients	Protection to about 40 weeks (Booster study demonstrated increased Ab levels)	(75, 76)
		III	Immunogenicity in cardiovascular surgery patients	Completed, no results published	NCT00211913
		III	Immunogenicity in orthopedic implant patients	Completed, no results published	NCT00211926
V710	IsdB	III	Preventing infection in surgical patients	Terminated. No difference vs. placebo, but vaccinated had elevated mortality risk	(72)

[a]Hyphenation represents conjugation of antigens, while commas indicate combined antigens. [b]All vaccinations are compared to placebo controls. [c]If no peer-reviewed results are published the clinicaltrials.gov identifier is listed.

Abbreviations: Ab - antibody; ClfA - clumping factor A; CP5, CP8 - capsule type 5, 8; DT - diphtheria toxin; exoA - exotoxin A; Hla - alpha-hemolysin; IsdB - iron-regulated surface determinant B; LukS-PV - Panton-Valentine leukocidin subunit S; MntC - manganese transport protein C; USUHS - Uniformed Services University of the Health Sciences

There have been numerous reviews addressing the potential reasons for *S. aureus* clinical vaccine failures (reviewed most recently in (77, 78)). Some of the potential reasons for failure include, but are not limited to: (**a**) inappropriate choice of clinical trial populations and endpoints (79–81); (**b**) widespread *S. aureus* colonization, which may suggest immune tolerance, and the numerous and redundant immune circumvention mechanisms utilized by this pathogen (49, 66, 82, 83); (**c**) animal models, especially mice, have proven to be poor predictors of vaccine efficacy in humans, and because human correlates of protection are not well known, developing models is difficult (84, 85); (**d**) the variability of *S. aureus* antigen expression, due both to strain and growth-phase/QS control, suggests that single antigens may not be effective (77, 78, 86); and (**e**) cell-mediated immunity, especially T_H17/IL-17 responses as opposed to opsonizing antibodies, may be required for human protection (77, 78, 86). As it becomes more apparent that cellular immunity is an important protective correlate (87), vaccines which address this need should be in the forefront.

More recent clinical trials have begun to address some of the aforementioned vaccine issues, including the use of multiple-antigens and vaccines that drive cell-mediated T_H17/IL-17 responses. For example, Pfizer is developing three- and four-antigen vaccines (phase II complete, **Table 1.1**), GSK is pursuing a four antigen vaccine comprised of CP5-TT, CP8-TT, Hla, and ClfA (phase I complete, NCT01160172), and Vaccine Research International has completed a phase I trial of a trivalent vaccine containing collagen binding protein (Can), extracellular adherence protein (Eap) and Clf (http://www.vri.org.uk). Additionally, there are two completed phase I trials of NDV-3, which is based on Candidal agglutinin-like sequence 3 protein (Als3) that shares

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homology with Staphylococcal adhesins (88), and in mice protection is mediated by

T_H17/IL-17 responses (88–90).

To demonstrate the potential for inhibiting *S. aureus* QS as a vaccination strategy, **Table 1.2** is a list of vaccines tested *in vivo* that contain at least one *agr*-regulated antigen. **Table 1.2** was generated by searching the PubMed (www-ncbi-nlm-nih.gov) database with the two searches 'aureus and vacc*' and 'aureus and immunother*' from 2005 through 2015, which resulted in 935 publications (* utilizes lemmatization [*e.g.* – vacc* would search for vaccine, vaccination, and vaccinology, *etc.*]). These results were then filtered for vaccines utilizing at least one *agr*-regulated virulence factor, and if literature on the same vaccine formulation was reported more than once, the seminal paper was identified. Due to conflicting reports, vaccines involving the RNAIII-activating protein (RAP) or related proteins were not included (91, 92). Of note, besides the intended targets, this search yielded three additional interest areas with significant literature: (**a**) epidemiology and economic analyses to identify at risk populations and the benefits of vaccination, (**b**) literature reviews addressing the failures, potential reasons for failure and the urgent clinical need for an *S. aureus* vaccine, and (**c**) a large volume of literature looking for efficacious zoonotic vaccines, mostly for bovine mastitis. Since the table was designed to demonstrate the large amount of *agr*-regulated antigens that are protective *in vivo*, it does not list antigen modification (e.g. inactivating mutations), conjugation to carriers, or adjuvants, even though all are important in vaccine formulation and ultimate efficacy.

In addition to identifying novel targets with protective efficacy, many of the studies listed in **Table 1.2** seek to address, in animals, the shortcomings identified in

human trials. First, 12 of the 25 studies (48%) contain multiple antigens, addressing the variability of antigen expression due to disease, growth-phase, and strain. Many combinations of *agr-* and non-*agr*-regulated antigens have been conjugated or formulated together with *in vivo* success (93–95). One study even combined two antigens oppositely regulated by *agr*, which may also drive protection at different *agr* activation states (94). Second, multiple investigators have assessed vaccination with small linear epitopes, domains, or discontiguous epitopes of *S. aureus* virulence factors that are protective (96–98). Additionally, Pozzi *et al.* demonstrated that conjugating protein antigens to non-protein antigens can overcome antibody interference following vaccination with multiple antigens (94). These studies are essential to understanding the antigen requirements for the formulation of successful multivalent vaccines. Third, many of the vaccines assessed efficacy in multiple disease models (94–96, 99–103), immune deficient states (99, 104), and in animals other than mice (93, 105–107), although mouse models are predominant. These are all an effort to identify protective correlates for different human disease states and host-susceptibilities. Fourth, it has been suggested that anti-toxin antibodies may be less desirable than opsonizing antibodies that lead to *S. aureus* killing, because toxins are usually expressed after initiation of infection (79), although their use has also been championed (108). The large number of successful anti-toxin strategies assessed in animals (**Table 1.2**), especially by Spaulding *et al.* who demonstrated that a large cocktail of toxin antigens alone is protective(105), suggests that anti-toxin approaches are effective. Even more encouragingly, several of the toxin only approaches actually showed reductions in CFUs *in vivo* (96, 101, 102, 105, 109), which is secondary to their neutralizing activity. This is an example of the not mutually exclusive strategy outlined

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by the NIAID, where 'anti-virulence' leads to 'harnessing the immune system.'

Ultimately, the two human clinical anti-toxin vaccine trials actively being pursued (**Table 1.1**) should elucidate the contribution of toxin components to a successful clinical vaccine. Fourth, Bagnoli *et al.* and Yeaman *et al.* are assessing antigens that drive T_H17/IL-17 responses (95, 110), which furthers the understanding of cell-mediated immunity in *S. aureus* vaccination. Fifth, two studies have demonstrated that antibody responses to the immunoglobulin-binding protein A (SpA) are protective in animal models (99, 111), suggesting that antibodies raised to immunoevasive antigens may be effective. Lastly, and most relevant to the work herein, Park *et al.* demonstrated the efficacy of targeting the *agr* quorum sensing operon to globally downregulate virulence in multiple disease models of *S. aureus* infection (103).

Table 1.2 – Animal model tested *S. aureus* vaccines containing at least one *agr*-regulated antigen (2005-2015).

Year	Target(s)[a] and *agr* [(+/-)b]	Strategy	Model	Major *in vivo* Positive Results	Author
2015	Csa1A, EsxA⁺, EsxB, FhuD2, Hla⁺	Active	Ms bacteremia; SSSI; peritonitis, pneumonia	↑Survival and ↓CFU; ↓CFU and abscesses; ↑survival; ↑survival	(95)
2015	Hla⁺	Active, Passive Rb	Ms SSSI	↓Dermonecrosis and bacterial luminescence	(98)
2015	SpA⁻	Passive Ms	Ms SSSI, bacteremia	↑Survival; ↑Survival	(99)
2014	AIP4⁺	Active	Ms SSSI	↓Abscess, ulcer, IL-1β and Hla	(112)
2014	Als3⁻	Active	Ms SSSI	↓Dermonecrosis, CFU and bacterial luminescence	(110)
2014	ClfA⁻, FnBPB⁻, SdrD⁻, SpA⁻	Active	Luekopenic Ms bacteremia	↑Survival and ↓CFU	(104)
2014	Combinations of: TSST-1⁺, SEB⁺, SEC⁺, SEl-X, Hla⁺, Hlb⁺, Hld⁺	Active, passive Rb	Rb pneumonia	↑Survival and ↓CFU	(105)
2014	CP5⁺-Hla⁺	Active, passive Rb	Ms bacteremia, pneumonia	↑Survival and ↓CFU, weight loss; ↑Survival and ↓CFU	(100)
2014	CP5⁺-TT or CP8⁺-TT, Hla⁺	Active	Rt osteomyelitis	↓CFU and osteomyelitis pathology	(107)
2014	EpiP⁺	Active	Ms peritonitis	↑Survival	(113)
2014	FnBPA⁻	Active	Ms bacteremia	↑Survival and ↓CFU	(114)

[a]Hyphenation of target antigens indicates conjugation, while commas indicate combined antigens. [b]Superscript +/- denotes positive or negative *agr* regulation, respectively.

Abbreviations: AIP4 - autoinducing peptide 4; Als3 - agglutinin-like sequence 3 protein; CFU - colony forming unit; ClfA - clumping factor A; CP5, CP8 - capsule type 5, 8; Csa1A - conserved staphylococcal antigen 1A; EpiP - epidermin leader peptide processing serine protease; EsxA, EsXB - ess extracellular A, B; FhuD2 - ferrix hydroxamate-binding lipoprotein; FnBP A, B - fibronectin binding protein A, B ; Hla, Hlb, Hld - alpha-, beta-, gamma-hemolysin; Ms - mouse; Rb - rabbit; Rt - rat; SdrD - serine-aspartate repeat D; SEB, SEC - staphylococcal enterotoxin B, C; SEl-X - staphylococcal enterotoxin-like X; SSSI- skin and skin structure infection; TSST-1 - toxic shock syndrome toxin-1; TT - tetanus toxin

Table 1.2 – Continued.

Year	Target(s)[a] and $agr^{(+/-)[b]}$	Strategy	Model	Major *in vivo* Positive Results	Author
2013	Hla$^+$	Active	Ms SSSI (ear), bacteremia, implant	↓Dermonecrosis and ↓CFU; ↓CFU; no protection	(101)
2013	IsdB$^+$-Hla$^+$	Active	Ms bacteremia	↑Survival and ↓CFU	(115)
2013	LukS$^+$	Active, passive	Ms bacteremia	↑Survival and ↓CFU in multiple organs	(109)
	LukF$^+$	Active	Ms bacteremia	↑Survival and ↓CFU in multiple organs	
	LukF$^+$, LukS$^+$	Active	Ms bacteremia	↑Survival (best) and ↓CFU in multiple organs	
2013	SEB$^+$	Active, passive Ms	Ms bacteremia. SSSI	↑Survival; ↓Abscess, dermonecrosis and CFU	(102)
2012	ClfA$^-$-PNAG	Passive Gt	Ms bacteremia	↓CFU	(93)
2012	Coa$^{-/+}$	Active, passive Rb	Ms bacteremia	↑Survival	(97)
	vWbp	Active, passive Rb	Ms bacteremia	↑Survival	
	Coa$^{-/+}$, vWbp	Active	Ms bacteremia	↑Survival	
2012	Glc-Hla$^+$	Passive Rb	Ms pneumonia; SSSI	↑Survival and ↓CFU; ↓CFU	(94)
	CP5$^+$-ClfB$^-$	Passive Rb	Ms SSSI; colonization	↓CFU; ↓CFU	
	CP8$^+$-IsdB$^+$	Passive Rb	Ms SSSI	↓CFU	
	Glc-Hla$^+$, CP5$^+$-ClfB$^-$, CP8$^+$-IsdB$^+$	Passive Rb	Ms SSSI	↓CFU	
2012	Hla$^+$	Active, passive Rb	Ms bacteremia, pneumonia	↑Survival and ↓CFU, ↑Survival and ↓CFU	(96)

[a]Hyphenation of target antigens indicates conjugation, while commas indicate combined antigens. [b]Superscript +/- denotes positive or negative *agr* regulation, respectively.

Abbreviations: CFU - colony forming unit; ClfA, B - clumping factor A, B; Coa - coagulase; CP5, CP8 - capsule type 5, 8; Glc - PNAG fragment; Gt - goat; Hla - alpha-hemolysin; IsdB - iron-regulated surface determinant B; LukS, LukF - Panton-Valentine leukocidin subunits; Ms - mouse; PNAG - poly-N-acetyl glucosamine; Rb - rabbit; SEB - staphylococcal enterotoxin B; SSSI- skin and skin structure infection; vWbp - von Willebrand factor binding protein

Table 1.2 – Continued.

Year	Target(s)[a] and *agr* [(+/-)b]	Strategy	Model	Major *in vivo* Positive Results	Author
2011	Hlb[+], CAMP	Active Hlb, Passive CAMP	Ms SSSI	↓Dermonecrosis	(116)
2010	Hla[+]	Active, passive Rb	Ms SSSI	↓Abscess, dermonecrosis	(117)
2010	Spa[-]	Active, passive Rb	Ms bacteremia	↓Abscesses and CFU	(111)
2007	AIP4[+]	Passive Ms	Ms SSSI, bacteremia	↓Abscess, dermonecrosis; ↑Survival	(103)
2006	IsdA[+], IsdB[+], SdrD[+], SdrE[+]	Active	Ms bacteremia	↑Survival	(118)
2006	IsdB[+]	Active	Ms bacteremia, Rh titers	↑Survival; ↑Titer, long lasting	(106)

[a]Hyphenation of target antigens indicates conjugation, while commas indicate combined antigens. [b]Superscript +/- denotes positive or negative *agr* regulation, respectively.

Abbreviations: AIP4 - autoinducing peptide 4; CAMP - Christie-Atkins-Munch-Peterson factor; CFU - colony forming unit; Hla, Hlb - alpha-, beta-hemolysin; IsdA, B - iron-regulated surface determinant A, B; Ms - mouse; Rb - rabbit; Rh - rhesus; SdrD, E - serine-aspartate repeat D, E; SpA - Staphylococcal protein A; SSSI- skin and skin structure infection

The strategy of inhibiting virulence via vaccination against a quorum sensing target is astute because antibody blocking QS could downregulate many virulence factors. This is especially significant when considering that many of the human clinical trial antigens are *agr*-regulated and protection has been shown in animal models for single or multiple antigens regulated by *agr* (**Table 1.1** and **Table 1.2**). Although AgrB and AgrC are extracellular, and thus accessible to antibodies, they are difficult targets as AgrB and AgrC are membrane-bound and targeted vaccination may not generate functional antibodies that block AIP secretion or binding, respectively. Similarly, the secreted AIP macrocycle ranges from seven to nine amino acids and is labile *in vivo* due to the five-membered thiolactone ring. The small size and lability of the active (cyclic) molecule, prevents the direct use of AIP as a vaccine antigen. However, in 2007, Park *et al.* pioneered QS vaccination by haptenizing a lactone AIP4-mimic to a protein carrier to generate monoclonal antibody (mAb) AP4-24H11 (103). Passive transfer of the mAb AP4-24H11 down-regulated *agr*-dependent virulence, protecting mice from SSSI and bacteremia (103). Herein, **Chapter 2** describes an active vaccination approach that builds upon the protective anti-AIP mAb developed by Park *et al.* (112).

Small molecule inhibitors of quorum sensing

In addition to vaccine approaches to interfere with *S. aureus* QS, small molecule inhibitors of QS are being actively investigated (reviewed in (37, 119)). In contrast to active vaccine approaches, small molecules and antibodies can both be administered therapeutically. However, small molecules have several beneficial attributes not found in therapeutic antibodies. Monoclonal antibodies are expensive to manufacture and,

although they are not rapidly cleared, large doses are required for efficacy (120). Antibodies require secreted or extracellular targets because of membrane exclusion, whereas small molecules can target both extracellular and intracellular targets due to their size and, often, membrane permeability (121). Finally, chemical optimization following lead compound identification has been highly successful, especially if the binding site has been identified (122). These benefits suggest that small molecule inhibitors of QS would also be useful in the fight against *S. aureus*.

Natural products, compounds produced by any organism, are a wellspring of bioactive small molecules as evidenced by their substantial contributions to anti-cancer and anti-infective therapeutics (123). Impressively, 77% of antibacterial therapeutics in the past 30 years are natural products or derived from natural product pharmacophores (123). Encouragingly, natural products have several beneficial attributes not found in chemical libraries or combinatorial chemistry approaches. Natural products are a massively diverse and vast set of structures unmatched by combinatorial and synthetic chemistry libraries (124, 125). Additionally, natural products often successfully break Lipinski's rules for 'drug-like' molecules (126) and often contain many chemical moieties avoided by medicinal chemists (*e.g.* -quinones, polyhydroxyls) (127, 128). This is likely due to the co-evolution of structures with biological targets, a sort of "biological validation," to quote Lahlou (129, 130). Finally, multiple bioactive natural products may synergize naturally, and their utility as multi-component drugs is beginning to be recognized (122, 129, 130). In light of these benefits, it is not surprising that most of the QS inhibitors identified to date are natural products.

The first studied natural product small molecule inhibitors of QS were the cross interfering AIPs, which are competitive inhibitors of AgrC (40–43). Further work in the field identified truncations of AIPs, mutated AIPs and peptides from other microbes (solonamide A and B) that are known to inhibit QS (37, 119). However, the lability of the thiolactone and amino acid side chains (57), the relatively short half-life (131), the hypermutability of AgrC (132), and the requirement of a specific inhibitor per *agr* type severely limit their utility clinically. In a similar matter, the peptide inhibitors of SpsB, which is involved in AIP processing, are not as labile as the thiolactone AIPs but presumably suffer from the same short half-life due to their peptidic nature (133). Even more importantly, the most potent SpsB inhibitory peptide is also antibacterial and carries the threat of resistance development, which is contrary to the goals of QS inhibition (133). As described above, the RIP peptide, which is part of the RAP system, will not be described due to continuing debate about its relevance (91, 92).

Interestingly another bacterial QS signal, the Gram negative *P. aeruginosa* homoserine lactone (HSL) inhibits *S. aureus* QS (134, 135) and its synthetic analogues have shown efficacy in a mouse arthritis model. The HSLs are thought to act either via inhibition of a membrane sensor (*e.g.* - sarA), allosteric inhibition of AgrC dimerization, or inhibition of AgrC interaction with AgrA (134, 135). However, these molecules have inherent antibacterial activity at concentrations above the putative *agr* inhibitory concentrations, which makes separating QS effects due to direct *agr* versus growth inhibition difficult (134, 135). Additionally, membrane binding and insertion of the HSL is required for *S. aureus* QS-inhibitory activity (although a receptor may be involved), and this potential rote membrane perturbation may contribute to the mechanism of action

(134, 135). Similarly, a quinolone from *P. aeruginosa* can inhibit *agr* signaling, although it drives formation of small-colony variants, enhances antibiotic resistance, and is antibacterial (37, 132).

Other microbial products that inhibit *S. aureus* QS include the dipeptides of *Lactobacillus reuteri*, proline-phenylalanine and proline-tyrosine, and these molecules inhibit *agr* signaling potentially though competitive binding of AgrC (136). Ambuic acid, isolated from an unidentified fungus, inhibited *agr* activation and hemolysin production, and may act by inhibition of AIP production (137). High-throughput screening of actinomycete cultures has also identified potential *agr* inhibitors (138). Additionally, indole and derivatives, produced by enteric bacteria, have been shown to inhibit staphyloxanthin and Hla production, reduced *agr*-regulated transcription, and protected against *S. aureus* in a *Caenorhabditis elegans* virulence model (139). Furthermore, polyhydroxyanthraquinones isolated from *Penicillium restrictum* isolated from a milk thistle (*Silybum marianum*), demonstrated potent inhibition of *agr*-I QS as measured by luminescent *agr* reporter and reduction in δ-toxin production (140). Herein, **Chapter 3** characterizes the most potent of the polyhydroxyanthraquinones, ω-hydroxyemodin, which is efficacious in a mouse model of SSSI and functions by binding to AgrA inhibiting activation of cognate promoters (141).

Many plant derived natural products have also been identified that inhibit *agr* promoter activity and/or *agr*-regulated transcripts or toxins, but their mechanisms of action have not been identified. These include: (**a**) licochalcone A and E from licorice root (*Glycyrrhiza inflata*) (142, 143); (**b**) thymol from thyme (*Thymus vulgaris*) (144); (**c**) luteolin derived from many plants (145); (**c**) α-cyperone isolated from a Chinese herb

(*Cyperus rotundus*)(146); (**d**) ethanolic extracts of Italian plants (*Ballota nigra*, *Castanea sativa*, and *Sambucus ebulus*) (147); (**e**) extracts of the goldenseal (*Hydeastis canadensis*) (148); (**f**) salicylic acid, the active metabolite of aspirin from willow bark (*Salix alba*) (149); and (**g**) puerarin, found in many plants (150). It should be noted that compounds **a-c** have antibacterial effects at high concentrations, but *agr* inhibition is evidenced at sub-inhibitory concentrations. There are additional reports of plant-derived structures, but their antibacterial activities make determination of true *agr*-dependent effects difficult (151, 152).

Although natural products have several beneficial attributes, chemical libraries have also proven effective at identifying bioactive molecules (123). In this regard, several traditional chemical library screens have resulted in compounds active against *S. aureus* QS. The National Cancer Institute's small molecule library was virtually screened against the AgrA phosphorylation site and identified multiple aryl compounds, including diflunisal, which inhibited Hla production, AgrA DNA-binding activity, and transcription of *agr*-regulated genes (153). Virtual screening of the lead optimized Maybridge Ro3 fragment library for AgrA binding compounds identified three aryl compounds that dose dependently inhibited AgrA DNA-binding activity (154). Furthermore, a screen of the NIH Molecular Libraries Small Molecule Repository identified several compounds that inhibited *agr* activation (155) and ultimately led to the identification of savirin (55). Savirin was shown to be largely specific to *agr* inhibition, showed efficacy in a mouse model of SSSI and did not induce resistance *in vivo* (55). Additionally, surface plasmon resonance was recently used to demonstrate direct binding of savirin to AgrA, providing a mechanism of action for *agr* inhibition (Pamela Hall, unpublished data).

Concluding remarks and hypotheses

The prevalence and range of diseases caused by *S. aureus*, together with the crisis of antibiotic resistance, suggest that a multi-pronged strategy, including both prophylactic (vaccine) and therapeutic approaches, will be required to limit human disease. Encompassing both approaches, we hypothesized that bacterial disarmament through the inhibition of *agr* quorum sensing will both complement immune function and mitigate the severity of *S. aureus* infection. As outlined below and in **Figure 1.2**, the studies presented in **Chapter 2** and **Chapter 3** test this hypothesis using both an active vaccine and a natural product approach to QS inhibition.

In **Chapter 2**, we utilize a previously described mAb, AP4-24H11, which binds and neutralizes AIP4 (103), to develop an active *S. aureus* vaccine. Specifically, we hypothesized that (**a**) AP4-24H11 could be used to identify a mimotope of AIP4 from a random library of phage displayed peptides, and (**b**) that this mimotope could in turn be used in a virus-like particle (VLP) based vaccine to generate protection against subsequent infection by an *S. aureus agr*-IV isolate.

In **Chapter 3**, we seek to validate ω-hydroxyemodin, the most potent of several natural product polyhydroxyanthraquinones identified by Figueroa *et al.* (140), as a universal *S. aureus agr* inhibitor. We hypothesized that ω-hydroxyemodin would inhibit QS in all four *agr* types, and would reduce disease severity in an *agr*-dependent model of *S. aureus* SSSI by inhibiting production of *agr*-regulated virulence factors while concomitantly bolstering the immune system. In addition, since the power of combinatorial chemistry is greatly enhanced by target and, ultimately, binding site

identification (122), we sought to determine the mechanism of action by which ω-

hydroxyemodin inhibits *S. aureus agr*.
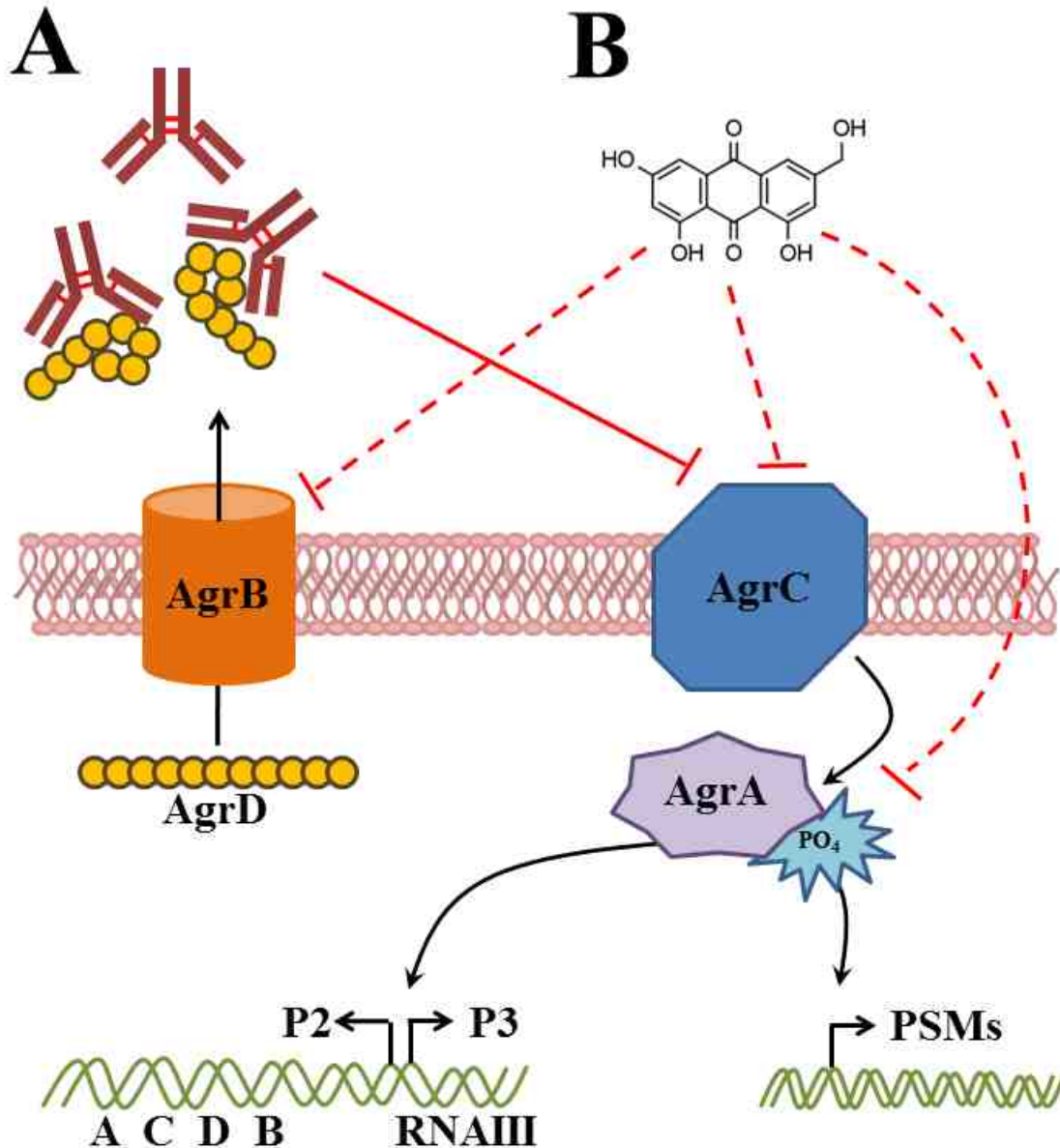
Figure 1.2 – Model for inhibition of the *S. aureus accessory gene regulator* via vaccination or small molecule inhibition. The *agr* operon is depicted with antibodies (A) or small molecules (B) inhibiting the function of the operon. (A) Antibody binding of the secreted AIP molecule sequesters the signal from reaching the AgrC receptor (solid line), thus preventing *agr* activation. (B) ω-Hydroxyemodin is depicted with potential sites for *agr* inhibition (dashed lines). Inhibition of AgrB processing of AIP prevents mature AIP from being secreted, halting *agr* signaling. Inhibition of AgrC by AIP competition, preventing dimerization, or preventing interaction with AgrA ceases *agr* signaling. Finally, inhibition of AgrA binding to target promoter DNA prevents transcription of the regulatory RNAIII, stopping signaling.

CHAPTER 2: Development of a Mimotope Vaccine Targeting the *Staphylococcus aureus* Quorum Sensing Pathway

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1.

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Abstract

A major hurdle in vaccine development is the difficulty in identifying relevant target epitopes and then presenting them to the immune system in a context that mimics their native conformation. We have engineered novel virus-like-particle (VLP) technology that is able to display complex libraries of random peptide sequences on a surface-exposed loop in the coat protein without disruption of protein folding or VLP assembly. This technology allows us to use the same VLP particle for both affinity selection and immunization, integrating the power of epitope discovery and epitope mimicry of

traditional phage display with the high immunogenicity of VLPs. Previously, we showed

that using affinity selection with our VLP platform identifies linear epitopes of

monoclonal antibodies and subsequent immunization generates the proper antibody

response. To test if our technology could identify immunologic mimotopes, we used

affinity selection on a monoclonal antibody (AP4-24H11) that recognizes the

Staphylococcus aureus autoinducing peptide 4 (AIP4). AIP4 is a secreted eight amino

acid, cyclized peptide produced from the *S. aureus* accessory gene regulator (agrIV)

quorum-sensing operon. The agr system coordinates density dependent changes in gene

expression, leading to the upregulation of a host of virulence factors, and passive transfer

of AP4-24H11 protects against *S. aureus* agrIV-dependent pathogenicity. In this report,

we identified a set of peptides displayed on VLPs that bound with high specificity to

AP4-24H11. Importantly, similar to passive transfer with AP4-24H11, immunization

with a subset of these VLPs protected against pathogenicity in a mouse model of S.

aureus dermonecrosis. These data are proof of principle that by performing affinity

selection on neutralizing antibodies, our VLP technology can identify peptide mimics of

non-linear epitopes and that these mimotope based VLP vaccines provide protection

against pathogens in relevant animal models.

Introduction

The small particulate nature and multivalent structure of virus-like particles cause them to

provoke strong immune responses and make them effective scaffolds for displaying

heterologous antigens in a highly immunogenic format. Peptide-based vaccines are

typically poorly immunogenic, however, peptides displayed on the surface of VLPs elicit

high-titer and long-lasting antibody responses (156–160). Although VLPs can be utilized to increase the immunogenicity of peptides, identifying relevant target epitopes and then presenting them to the immune system in a highly immunogenic context that mimics their native conformation, has largely been an unpredictable process of trial-and-error. The most widely used method for epitope identification is through affinity selection using peptide libraries displayed on a filamentous phage. This technology has identified the epitopes of many monoclonal antibodies (mAbs), and is a powerful technique for mapping linear epitopes and discovering peptide mimics of conformational and non-peptide epitopes. Nevertheless, peptides displayed on a filamentous phage are typically poorly immunogenic due to the low valency display of peptides on the phage surface. Thus, epitopes identified by phage display must be produced synthetically, linked to a carrier, and displayed in a structural context unrelated to the selected phage. Often, in this new conformation the peptides have vastly decreased affinity for the selecting molecule and frequently lose the ability to induce antibodies that mimic the selecting antibody.

VLP technology has not previously been adapted for use in epitope identification because recombinant VLPs are not well-suited for the construction of diverse peptide libraries. Insertion of heterologous peptides into viral structural proteins often result in protein folding and VLP assembly defects (161–163). To overcome these limitations, we engineered a version of the bacteriophage MS2 coat protein whose folding and assembly is highly tolerant of short peptide insertions (162). This system has allowed us to generate large, complex libraries of VLPs displaying random peptide sequences. Because VLPs encapsidate the mRNA that encodes coat protein and its peptide (162, 164), VLPs with specific binding characteristics can be affinity selected and then the nucleic acid encoding

the selected peptide can be recovered by RT-PCR. Most importantly, the same VLP can be used for both affinity selection and immunization. Thus, this system integrates the power of epitope/mimotope discovery of traditional phage display with the high immunogenicity of VLPs. We recently showed the utility of this VLP technology to identify linear epitopes and to elicit the proper antibody response by performing affinity selection using a set of well-characterized mAbs (165).

In this study we used this VLP vaccine discovery platform to identify immunogenic mimics of a quorum-sensing peptide from the Gram positive pathogen *Staphylococcus aureus*. *S. aureus* is the leading cause of skin and soft tissue infections (SSSI) presenting to emergency departments in the United States (13). The *S. aureus* accessory gene regulator (*agr*) quorum-sensing system coordinates a density dependent switch in gene expression that includes upregulation of virulence factors critical for invasive SSSI (56, 103, 131, 166). The *agr* system signals through the use of a secreted thiolactone-cyclized autoinducing peptide (AIP) which, upon binding to its cognate surface receptor AgrC, initiates a regulatory cascade leading to changes in transcription of more than 200 genes (44, 167). Among the upregulated genes are those encoding secreted virulence factors essential for invasive skin infection, including upregulation of the pore-forming toxin alpha-hemolysin (Hla). Infection with *agr* or *hla* deletion mutants, loss of the Hla receptor ADAM10, or neutralization of Hla significantly attenuates virulence in mouse models of SSSI (44, 56, 117, 168–170). Furthermore, we and others have shown that host innate effectors which disrupt agr-signaling also provide defense against *S. aureus* infection (57–60, 171). These results suggest that a VLP-based epitope identification approach to vaccine development targeted towards disruption of *agr*

signaling would be efficacious against *S. aureus SSSI*.

Among *S. aureus* strains there are four *agr* alleles (*agr*I to *agr*IV) and strains from a given allele secrete a unique thiolactone cyclized AIP (AIPI to AIPIV) ranging from seven to nine amino acids in length. Due to their size, these peptides are inherently non-immunogenic. To overcome this, Park et al. described the production of a monoclonal antibody (AP4-24H11) against a synthetic AIP4 hapten that binds with nM affinity to AIP4 and that largely did not bind to other AIP family members, including AIP1 (µM affinity), which differs by a single amino acid (103, 172). The crystal structure of AP4-24H11 bound to AIP4 reveals that the antibody recognizes the characteristic AIP thiolactone ring conformation, but does not interact with the N-terminal, linear region (173). Importantly, passive transfer of AP4-24H11 protected against *S. aureus* pathogenicity in a mouse model of dermonecrosis and against a lethal intraperitoneal *S. aureus* challenge. The protection afforded by AP4-24H11 administration occurred without affecting normal bacterial growth, confirming that the AP4-24H11 mechanism of action was specific to inhibiting *S. aureus* virulence. Therefore, this work provided proof of principle that antibodies targeting AIP could be efficacious against *S. aureus* SSSIs (103).

We aimed to develop an active vaccine to provide protection against *S. aureus agr*-mediated pathogenesis. Traditionally, subunit vaccines utilize whole proteins, domains or epitopes conjugated to a carrier. We initially produced Qß VLPs with many copies of chemically conjugated AIP1 peptide, but they failed to elicit a protective response in the dermonecrosis mouse model (unpublished data). This failure may have resulted from potential instability of the native AIP molecule (103). For example, the

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conformational restraint imposed by the AIP thiolactone bond is necessary for binding AgrC and induction of *agr*-signaling, as linearization of the AIPs results in loss of function. Furthermore, oxidation of the C-terminal methionine of AIP1 or AIP4 by host-generated reactive oxygen species is sufficient to inactivate the peptides. Thus, if the VLP-linked AIPs became linearized or oxidized during the conjugation or vaccination process, they would no longer be presented to the immune system as an authentic antigenic target. Therefore, we pursued the novel approach reported here using our affinity selection technology and the previously reported AP4-24H11 mAb targeting *S. aureus* AIP4.

Our approach was to identify peptides that immunologically mimic AIP4. Starting with random sequence peptide libraries on MS2 VLPs, we conducted biopanning on the AP4-24H11 mAb and identified 8 different VLPs displaying peptides that specifically bound the antibody. Vaccination with two of these VLPs elicited an immune response that protected in a *S. aureus* mouse model of dermonecrosis. These data demonstrate the feasibility of our VLP technology to identify immunologic peptide mimics of conformational epitopes and the potential to develop efficacious vaccines against otherwise non-immunogenic, conformationally constrained peptides such as those regulating *S. aureus agr*-dependent virulence. To our knowledge, this is the first report of an efficacious active vaccine targeting the secreted autoinducing peptides of the *S. aureus agr* quorum-sensing system.

Materials and Methods

Plasmid construction and random peptide libraries

The plasmids pDSP62 and pDSP62(am) were previously described (165). Briefly, pDSP62 expresses the single chain dimer of the MS2 coat protein under the control of the inducible T7 promoter. VLPs produced using pDSP62 contain 90 copies of the displayed peptide per VLP. pDSP62(am) is the same construct except it contains an amber stop codon at the junction of the two coat protein monomers in the single chain dimer. The pDSP62(am) vector produces VLPs that display peptides at low valency (~3 copies of the peptide per VLP) when expressed in a *E. coli* strain containing pNMsupA a plasmid that expresses an alanine-inserting, amber suppressing tRNA under the control of the lac promoter. The suppressor mediates occasional read through of the stop codon, so that pDSP62(am) produces a mixture of wild-type coat protein and the peptide-displaying single-chain dimer, which then coassemble into a mosaic VLP.

We have previously constructed random peptide plasmid libraries for use in our VLP affinity selection protocol that display peptides in the downstream AB loop of the MS2 single chain dimer coat protein (165). Briefly, oligonucleotides were synthesized with 6, 7, 8 or 10 NNS codons, where N represents an equimolar mixture of all four nucleotides and S is an equal mixture of C and G. NNS codons encode all 20 amino acids and only a single stop codon. Using the Kunkel method, we produced plasmid libraries of at least 10^{10} individual transformants for each peptide library. All plasmids in this study were isolated using Qiagen Qiafilter or minipreps kits (Qiagen, Valencia CA).

VLP production and purification

Plasmid libraries from affinity selection or single plasmids containing defined sequences that bound to AP4-24H11 were electroporated into the *E. coli*, T7 expression strain

C41(DE3) (Lucigen, Middleton WI) and grown to mid-log phase. Coat protein expression was induced by the addition of IPTG (1 mM, Sigma-Aldrich, St. Louis MO) for three hours and bacteria were collected by centrifugation and the pellet was stored at –20°C overnight. Bacteria were lysed in SCB buffer (50 mM Tris, pH 7.5, 100 mM NaCl) by addition of 10 µg/ml of lysozyme, sonicated and purified from bacterial debris by centrifugation. The supernatant was treated with 10 units/mL of DNaseI (Sigma-Aldrich, St. Louis MO) and the VLPs were purified away from contaminating bacterial proteins by size exclusion chromatography using sepharose CL-4B resin (Sigma-Aldrich, St. Louis MO). Fractions that contained VLPs were combined and precipitated by the addition of ammonium sulfate at 50% saturation. Precipitated VLPs were collected by centrifugation, solubilized in SCB buffer and dialyzed in SCB overnight (Slide-a-lyzer cassettes 20,000 MWCO, Millipore, Billerica MA). Purified VLPs were quantitated by Bradford assay (Biorad, Hercules CA) and analyzed by agarose gel electrophoresis and by SDS gel electrophoresis.

Affinity selections

VLP affinity selection was performed on the neutralizing AIP4 monoclonal antibody AP4-24H11 (a generous gift from Gunnar Kaufmann and Kim Janda, Scripps Research Institute). VLP affinity selections were performed as previously described (165). Briefly, for the initial round of selection, we coated Nunc MaxiSorp ELISA plates (eBiosciences, San Diego, CA) with 250 ng of AP4-24H11 in PBS overnight at 4°C. After washing, wells were blocked with 0.5% nonfat dry milk in PBS and the four VLP-peptide libraries (2.5 µg each of VLPs displaying 6, 7, 8 and 10 mers) were applied to the blocked wells

for 2 hours (10 µg total VLP/well). After extensive washing (PBS), bound VLPs were eluted with 0.1 M glycine, pH 2.7 and immediately neutralized by the addition of 1/10 volume of 1 M Tris, pH 9. To make enriched VLP libraries for subsequent rounds of affinity selection, RNA from the eluted VLP were reverse transcribed and the RT products (containing the downstream coat protein and AB loop peptide) were amplified by PCR, digested with Bam HI and Sal I, and ligated into the pDSP62(am) vector. Ligation products were electroporated into the *E. coli* 10 G bacterial strain (Lucigen, Middleton WI), with a one-hour outgrowth and then immediately placed into 100 mL of LB media containing 60 µg/mL of kanamycin. After overnight growth, plasmids were isolated (Qiafilter Midi kit, Valencia CA) and used for VLP production for the next round of affinity selection. All plasmid libraries constructed after affinity selection contained at least 10^6 individual transformants. Two additional rounds of affinity selection were performed with low valency peptide display (~3 peptides/VLP); one using 250 ng of mAb per well and the final round used 50 ng of mAb per well.

Identification and characterization of VLPs

After the final round of affinity selection, plasmid libraries enriched for VLPs displaying peptides that bound to the AIP4 mAb were isolated as described above. 1 pg of each library was electroporated into the C41(DE3) *E. coli* strain to ensure single transformants and bacteria were plated on agar plates containing kanamycin. The next day, single colonies were grown in 1 mL LB to an A_{600} of 0.6 and induced for VLP production with the addition of IPTG for 3 hours. Before induction, a 100 µL aliquot was removed for subsequent plasmid isolation. VLPs were isolated by sonication in SCB buffer and

genomic DNA digested by incubation with 10 units of DNaseI. Crude VLP preps were

assayed for binding to AP4-24H11 or an unconjugated control mouse IgG (Jackson

ImmunoResearch Laboratories, West Grove PA) by ELISA. VLPs that bound to AP4-

24H11 at least 5-fold higher then IgG control were further analyzed.

Plasmids were isolated from bacteria that produced VLPs that specifically bound

to AP4-24H11, but not control antibody. Nucleotide sequences encoding the various

peptides were determined (Eurofins Genomics, Huntsville AL) and plasmids encoding

unique peptide sequences were electroporated into C41(DE3) for large scale VLP

isolation as described above.

SDS PAGE and agarose gel electrophoresis was used to assess the purity and

characterize the isolated VLPs. We ran 2 µg of total protein on a 10% NuPAGE SDS gel

(Life Technologies, Grand Island NY) and stained the gel for total protein using

Coomassie Blue (BioRad, Hercules CA). Since VLPs encapsulate their RNA we could

also characterize VLP selectant particles by electrophoresis on an agarose gel. 10 µg of

VLPs were run on a 1% TBE agarose gel containing ethidium bromide.

ELISA

ELISA was used to assess relative binding of VLP selectants to AP4-24H11. Briefly,

wells were coated with 250 ng of purified VLPs in PBS and incubated overnight at 4°C.

Wells were washed 3 times with PBS and blocked for 1 hour using 3% BSA. Different

concentrations of AP4-24H11 in 3% BSA were applied to each well, and incubated at

room temperature for 1 hour. Unbound antibody was removed by washing with PBS.

Goat anti-Mouse IgG HRP conjugated antibody (Jackson ImmunoResearch Laboratories,

West Grove PA) was diluted 1:5000 in 3% BSA and incubated for 1 hour at room temperature. ABTS solution (EMD Millipore, Billerica MA) was used to detect bound HRP antibody and color change was measured by absorbance at 405 nm (Opsys Plate Reader, Thermo Scientific, Waltham MA).

For competition ELISAs, plates were prepared as above. AP4-24H11 (100 ng/well) was mixed with different concentration of the cyclical, bioactive AIP4 peptide (10 µM–0.1 µM) for 10 minutes prior to incubation with VLPs. Secondary antibody and detection was the same as above. As control peptides, we used a linear form of AIP4 or a cyclical peptide from the L2 protein of human papillomavirus 16.

Immunization and skin infection model

Animal studies were carried out in accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and U.S. federal law. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico Health Sciences Center. Four to six week old female Balb/c mice (Harlan Laboratories, South Easton MA) were immunized with 10 µg of VLPs in PBS (50 µL total) without the addition of adjuvant by intramuscular injection into the caudal thigh muscle. The initial immunization was followed with 2 boosts each 2 weeks apart. The initial experiment used 3 mice for each VLP and subsequent experiments used 5 mice per vaccine candidate. As negative controls, 2 groups of mice were immunized with either PBS alone or a control VLP (displays no peptide).

The dermonecrosis model of mouse skin infection was previously described and performed with minor modifications (174). Briefly, one week after the final vaccine

boost, mice were anesthetized with isoflurane and inoculated subcutaneously with 1×10^8 CFU of early-exponential phase *S. aureus agr*IV (AH1872–MN TG; generously provided by Dr. Alex Horswill, University of Iowa (175)). Animals were monitored for weight loss and lesion formation for three days post-infection. Lesion formation was assessed by measuring the maximal width and length of the abscess and necrotic ulcer with calipers. Area of the abscess was determined using the equation $A = (\pi/2)\times L\times W$, while the necrotic ulcer area was determined using the equation $A = L\times W$ (117). On day three post-infection animals were euthanized by CO_2 asphyxiation and abscesses (2.25 cm^2 area) were collected. The tissue was homogenized and serially diluted for CFU enumeration.

Cytokine analysis of abscess tissues

Abscess homogenates were stored at –80°C until cytokine analysis. Homogenates were rapidly defrosted at 37°C and clarified by centrifugation at $12,500\times g$ for 10 minutes. Cytokine concentrations in clarified supernatants were determined using a custom designed multiplex assay performed as per manufacturer's recommendations (EMD Millipore, Billerica, MA). The assay was read on a Bio-Plex 200 instrument and data analyzed using the Bio-Plex Manager Software (Bio-Rad, Hercules, CA).

Alpha-hemolysin Western blot

Frozen homogenates were thawed and clarified as described above. Briefly, clarified tissue homogenate was separated by SDS-PAGE on a 16% Tris-glycine gel (Life Technologies, Grand Island, NY) before transfer to a polyvinylidene fluoride membrane.

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After blocking using TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) with

5% non-fat dry milk, immunodetection was performed using an anti-Staphylococcus

alpha hemolysin antibody (Abcam, Cambridge, MA). Immunoreactive band intensity was

determined using a FluorChem R System and AlphaView software (ProteinSimple, Santa

Clara, CA). Relative intensity is the ratio of measured intensity divided by the total

protein concentration based on absorbance at 280 nm.

Statistical analysis

Statistical significance was determined using GraphPad Prism v.5.04. The two-tailed

Student's *t*-test was used for analysis of *in vitro* data, and *in vivo* data were analyzed by

the Mann-Whitney U test for non-parametrics. Results were considered significantly

different at $p < 0.05$.

Results

In order to identify mimotopes of the AIP4 mAb AP4-24H11 epitope, we performed

affinity selection on AP4-24H11 using our random sequence peptide libraries displayed

on MS2 VLPs. The basic methodology is found in (**Figure 2.**). We used a mixture of four

libraries, each displaying 6-, 7-, 8- or 10-amino acid inserts, with each library containing

more than 10^{10} transformants (162, 165). We find that many antibodies have strong

preferences for peptide sequences of specific lengths, and using a mixture increases the

probability of finding optimal binding peptides. Three iterative rounds of affinity

selection were performed, each at increasing stringency; in round 2 we increased the

stringency by reducing the display valency from 90 to about 3 peptides per particle. In

round 3 stringency was further increased by reducing the amount of antibody 5-fold to 50 ng. Reaction of the selectant population with AP4-24H11 was monitored by ELISA after each round. By the end of round 3 binding was elevated more than 200-fold (data not shown).
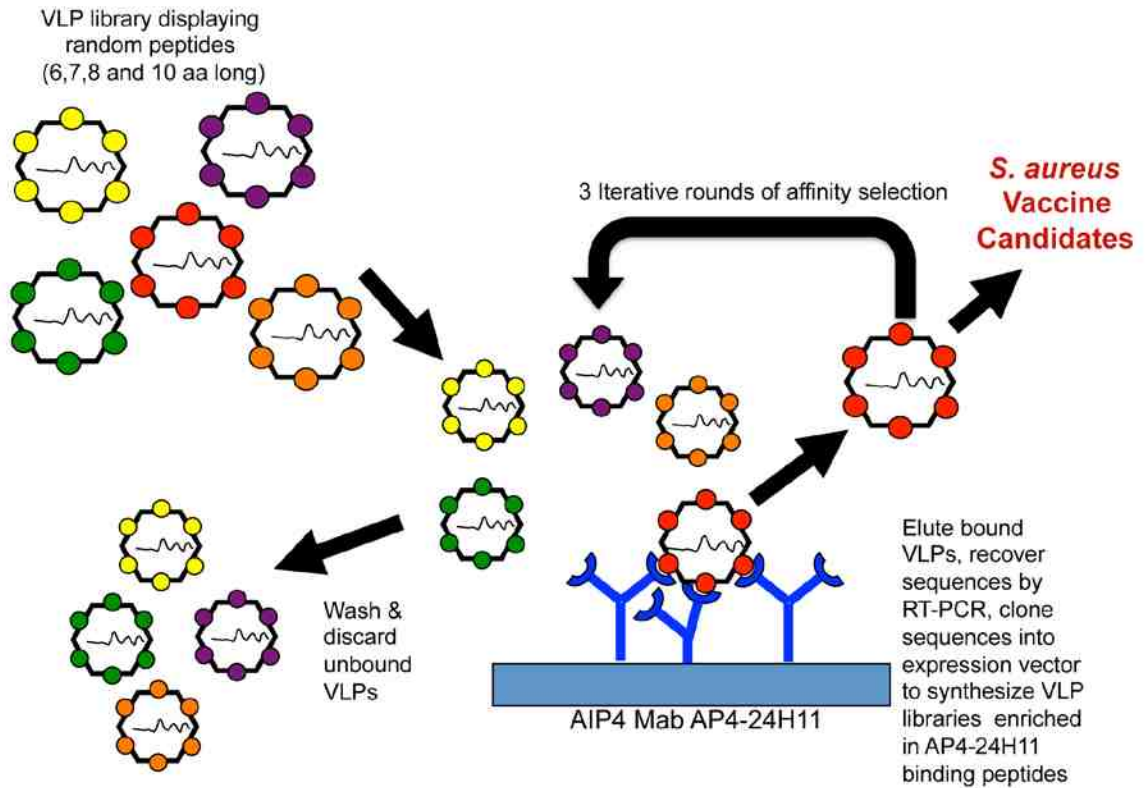
Figure 2.1 - VLP affinity selection to identify mimotopes of mAb AP4-24H11. Wells of an ELISA plate were coated with the mAb AP4-24H11 and were incubated with VLP libraries displaying random peptides. RNA sequences from bound VLPs were recovered by RT-PCR and re-cloned into VLP expression constructs and VLP libraries enriched for peptides binding to AP4-24H11 were produced. Three rounds of biopanning were used and clones of the resulting VLPs were sequence for peptide identification and subsequent functional analysis.

Ten cloned, round 3 selectants were subjected to sequence analysis, which identified 8 different peptide sequences (**Figure 2.B**). The peptide sequence SGIMPH was found in 3/10 selectants, while the other seven clones had unique sequences. When performing affinity selections on antibodies with linear epitopes, families of related sequences are often encountered. However, there was little primary sequence homology amongst the different peptides, with the exception of peptide 2 and peptide 3. Furthermore, none showed sequence identity to AIP4 (**Figure 2.A**) suggesting that these peptides somehow structurally mimic AIP4, or that they bind an antibody paratope distinct from that occupied by AIP4.

The AP4-24H11 selectant VLPs were expressed in *E. coli* and purified by procedures we have described elsewhere (162). Their elution behavior from the gel filtration matrix Sepharose CL-4B shows that each assembles into a particle the size expected of the VLP (not shown). **Figure 2.C** shows the electrophoretic behavior of each VLP in agarose. Since only intact particles contain RNA, their staining with ethidium bromide verifies their intactness. Differences in electromobility are due mostly to charge differences imparted by the presence of the peptides on the VLP surface. To assess the purity of the VLP preparation prior to binding assays and immunizations, we analyzed protein content by SDS-PAGE followed by Coommassie blue staining. VLPs (single chain dimer ~28 kD) were effectively purified away from contaminating bacterial protein (**Figure 2.D**).
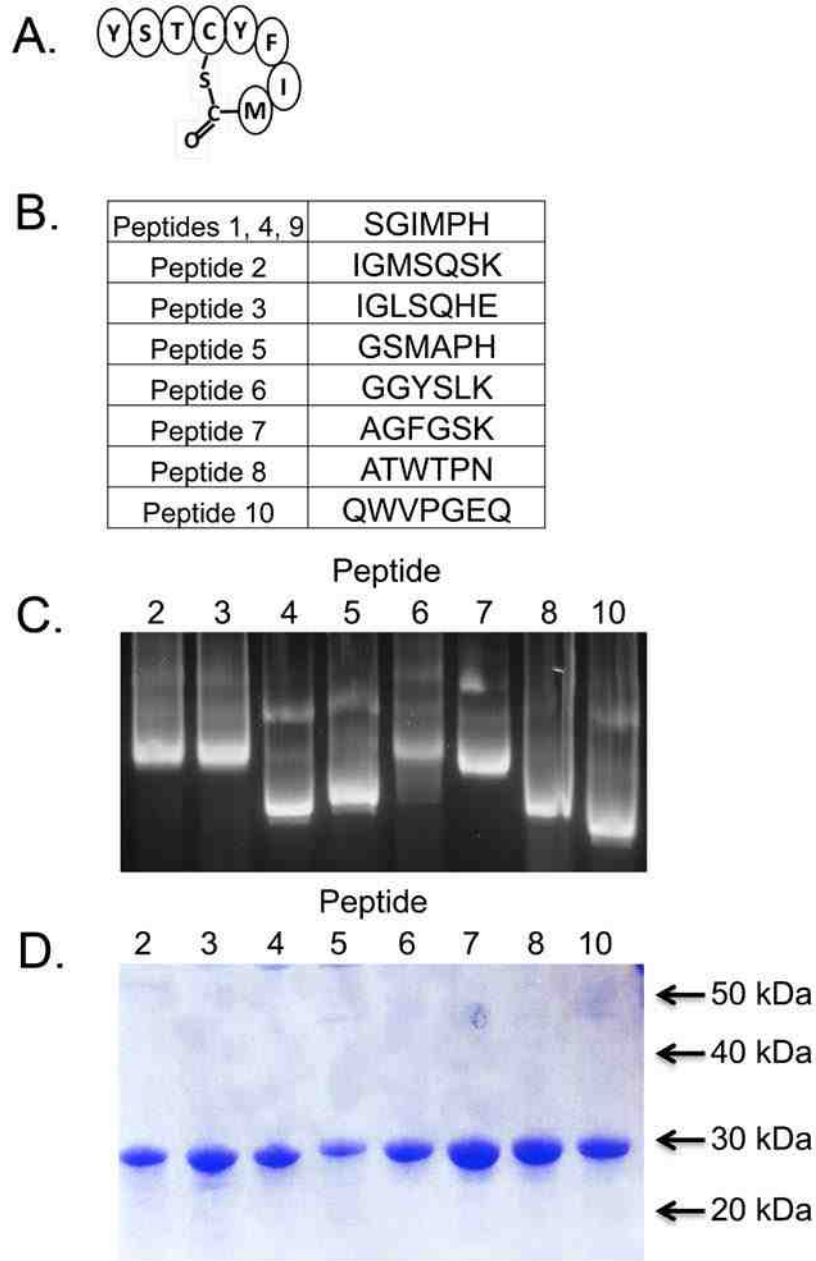
Figure 2.2 - Identification and purification of affinity selected VLP displayed peptides. (A) The sequence and structure of the AIP4 peptide. (B) 10 VLP clones were sequenced after three rounds of VLP affinity selection. Peptide inserts are shown and demonstrate little primary sequence homology to the native AIP4 peptide. (C) Agarose gel analysis of purified VLPs. RNA staining is indicative of intact VLPs (which encapsulate their RNAs) and differences in VLP mobility are mostly due to differences in the charges of the peptides displayed on the VLP surface. (D) SDS-PAGE. Two µg of purified VLP protein were run of a 10% SDS gel and protein was detected by staining the gel using Coommassie Blue.

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A direct ELISA was used to confirm that the affinity selected VLPs bound specifically to AP4-24H11. VLPs were used as the coating antigen (250 ng/well) and various amounts (0.1 to 500 ng) of AP4-24H11 were added to each well. AP4-24H11 bound to all of the VLPs displaying the selected peptides, whereas little to no binding of AP4-24H11 was observed with a control VLP (**Figure 2.A**). The selected VLPs demonstrated a range of binding to AP4-24H11 with a ~3.5-fold difference between the strongest binder (VLP displaying peptide 5) and the lowest (VLP displaying peptide 8). As an additional control, VLP coated wells were incubated with a mouse IgG control antibody using the same dilutions as used with the AIP4 mAb. Little or no binding was detected with the control antibody for any VLP samples (data not shown).

To ensure that selecting VLPs are binding to the antigen-binding site of AP4-24H11 rather than the Fc region, we investigated the ability of bioactive AIP4 peptide to compete with VLPs for antibody binding. VLPs were the coating antigen, and prior to the addition of AP4-24H11 (100 ng/well) various amounts of bioactive AIP4 peptide (10 µM–0.1 µM) were incubated with the mAb. The peptide/antibody mixture was added to the VLP coated wells and incubated for 1 hour. Similar to the results shown in **Figure 2.A**, there was a range of peptide concentrations required to inhibit antibody binding (**Figure 2.B**). Importantly, all VLPs were competed off the antibody by bioactive AIP4, suggesting that the selected VLPs are interacting with the antigen binding site of AP4-24H11.These data demonstrate that affinity selection can identify a population of VLPs displaying peptides that bind specifically to the mAb AP4-24H11.
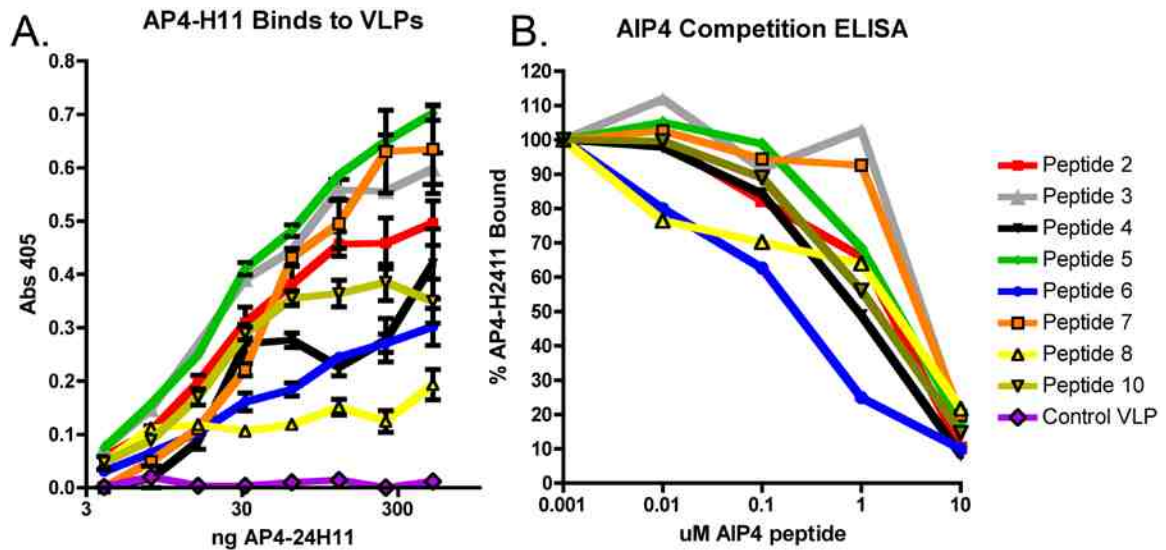
Figure 2.3 - Affinity selected VLPs bind to and occupy the antigen binding site of mAb AP4-24H11. (A) ELISA was used to assess specific binding of VLP selectants to AP4-24H11. Wells were coated with the indicted VLPs and different concentrations of AP4-24H11 were applied. Error bars represent standard error of the mean. (B) For competition ELISAs, wells were coated with the indicated VLPs and AP4-24H11 was mixed with different concentration of the cyclical, bioactive AIP4 peptide prior to incubation with VLPs. Secondary antibody and detection was the same as above. As a control peptide, we used a linear form of AIP4. Results are representative of an experiment performed twice.

Next, we tested whether any of the selected VLPs could serve as an immunologic mimic of AIP4. Secreted virulence factors regulated by *agr*, such as Hla, mediate dermonecrosis, suggesting that vaccination with VLPs presenting immunologic mimics of the AP4-24H11 epitope would elicit protection against *agr*IV-mediated dermonecrosis. To test this, we vaccinated groups of 3 mice with VLPs presenting AP4-24H11-selected peptides or a VLP control, and challenged the mice by subcutaneous injection *with S. aureus agr*IV isolate AH1872 (58) (**Figure 2.A**). We observed the mice for three days post-infection as we typically see maximum ulcer development by this time point followed by resolution over approximately the next seven days (55, 176). Compared to VLP control vaccinated mice, mice vaccinated with peptide 4 VLPs showed significantly reduced abscess area on days 1 and 3 post-infection (**Figure 2.B**). In addition, mice vaccinated with either peptide 2 or peptide 4 VLPs showed a trend toward reduced dermonecrosis (ulcer area) on days 2 and 3 post-infection, although this did not reach statistical significance (**Figure 2.C**). Vaccination with VLPs displaying peptide 3 and peptides 5–10 were included in pilot testing but no protection was observed, therefore these VLPs were not included in further studies (data not shown).
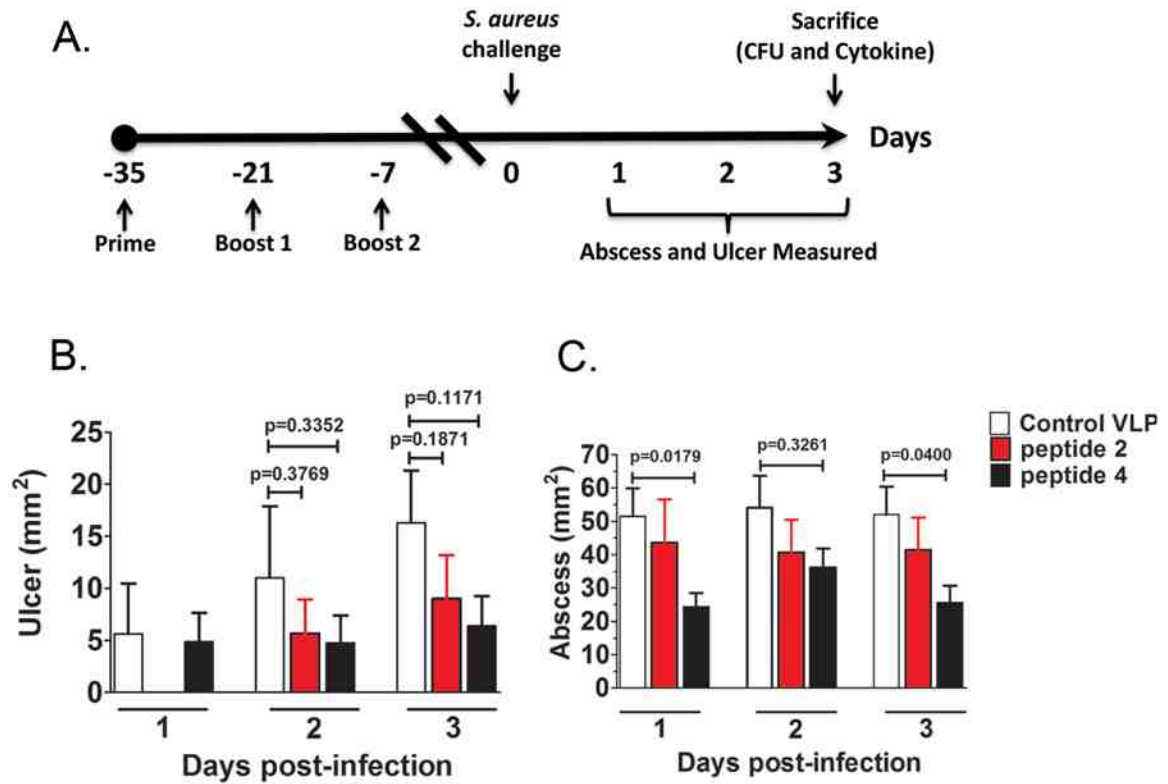
Figure 2.4 - Vaccination with VLP mimotopes of AIP4 is efficacious in a mouse model of *S. aureus* SSSI. (A) Mice were vaccinated three times at two week intervals with 10 µg of VLP. One week after the final vaccination mice were inoculated subcutaneously with 1×10^8 CFU of *S. aureus agr*-IV isolate AH1872, and abscess (B) and ulcer (C) areas were measured over the course of 72 hours. Data are shown as the mean ± SEM of at least two independent experiments totaling 8–10 mice per group.

Based on the reduced ulcer area in mice vaccinated with peptide 2 or 4 VLPs, we asked whether vaccination with a combination of peptide 2 and peptide 4 VLPs (peptide 2/4-VLPs) would result in a significant reduction in dermonecrosis following *S. aureus* challenge. To address this, mice were vaccinated with a mixture (1:1) of VLPs displaying peptide 2 and 4, control VLPs or PBS alone, and then challenged by subcutaneous injection with *S. aureus* AH1872. Whereas reductions in ulcer area in mice immunized with either peptide 2- or peptide 4-VLPs alone failed to reach statistical significance, mice immunized with combined peptide 2/4-VLPs had significantly reduced dermonecrosis compared to control vaccinated mice on day three post-infection (**Figure 2.A**). Decreased ulcer area in peptide 2/4-VLP vaccinated mice was not due to a reduction in bacterial burden at the site of infection (**Figure 2.B**) suggesting the decreased dermonecrosis resulted from inhibition of *agr* signaling. In support of this view, decreased dermonecrosis in the peptide 2/4-VLP vaccinated mice was associated with a significant decrease in local IL-1β levels, but not decreases in IL-6 or keratinocyte-derived chemokine (KC), compared to control vaccinated mice (**Figure 2.C**). Such a decrease in local IL-1β levels is consistent with reduced *agr*-mediated expression of Hla, which causes pore-formation in host cells leading to NLRP3 inflammasome activation and IL-1β production (46, 177–182). To demonstrate that reduced IL-1β at the site of infection in peptide 2/4-VLP vaccinated mice was associated with decreased *agr*-mediated virulence factor expression, we measured Hla in tissue homogenate by Western blot analysis. As expected, peptide 2/4-VLP vaccinated mice had significantly less Hla at the site of infection compared to controls (**Figure 2.D**). Together, these data suggest that peptides 2 and 4 identified by VLP affinity selection can

serve as immunologic mimics of the *S. aureus* AIP4 mAb AP4-24H11 epitope and

provide protection against *agr*-mediated dermonecrosis.
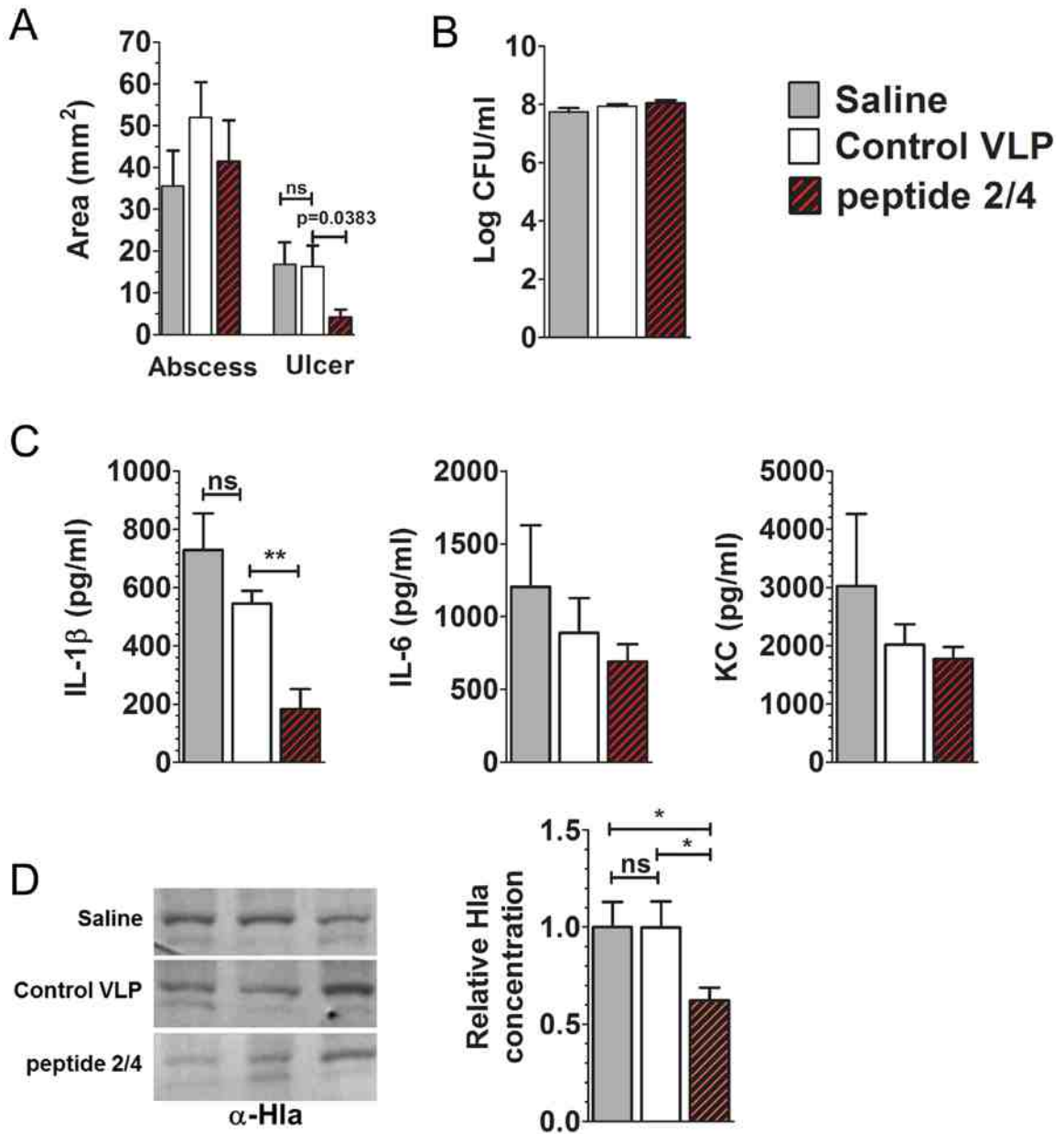
Figure 2.5 - A combination vaccine of two VLP mimotopes limits pathogenesis in a mouse model of *S. aureus* dermonecrosis. Mice were vaccinated with 10 µg of a 1:1 suspension of peptide 2 and peptide 4 and inoculated with 1×10^8 CFU of AH1872 as described previously. At the apex of infection (day 3) (A) abscess and ulcer area were measured and (B) bacterial burden at the site of infection was determined, and (C) local cytokine and chemokine levels were determined. (D) Western blot showing relative HLA levels in tissue homogenate of vaccinated and challenged mice. Quantification based on Western blot band intensity relative to total protein concentration. Data are shown as the mean ± SEM of 6–10 mice per group. ns, not significant; *, p<0.05; **, p<0.01.

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Discussion

Recent technological advances have resulted in the isolation and characterization of a host of broadly neutralizing monoclonal antibodies having prophylactic and therapeutic effects against a variety of pathogens. We have developed a novel vaccine technology that takes advantage of these newly identified antibodies that allows for epitope discovery and mimicry on a highly immunogenic platform. We recently reported the use of this VLP selection platform to identify epitopes for several previously characterized mAbs that recognize linear epitopes (165). In this paper, we extend these observations by identifying peptide mimics of the conformational epitope from the *S. aureus* AIP4 mAb AP4-24H11. Of critical importance is that compared to controls, co-immunization with two of the selected VLP candidates limited agr-signaling and pathogenesis during S. aureus SSSI as indicated by (1) decreased expression of the agr-regulated virulence factor Hla, (2) reduced local levels of the inflammatory cytokine IL-1β and (3) reduced dermonecrosis. Furthermore, although immunization did not impact bacterial burden at the time point evaluated, we and others have shown that, along with preventing or limiting dermonecrosis, disruption of *agr*-signaling or neutralization of Hla leads to increased bacterial clearance during resolution of infection (55, 168, 183). This suggests the potential for vaccination with VLP-based AIP mimotopes to not only limit *agr*-dependent pathogenesis, but also to eventually contribute to host-mediated bacterial clearance. Importantly, these data provide proof-of-principle that our VLP technology provides a background upon which to develop efficacious vaccines against otherwise non-immunogenic, conformationally constrained epitopes. Antibodies are by nature polyspecific. In the universe of all possible short peptide sequences, an antibody may be

capable of binding a number of them. Therefore, it is possible that only some affinity-selected peptides will bind the antigen-combining site through interactions mimicking those of the authentic antigen. It is well known, for example, that M13 phage display frequently finds so-called functional mimics, peptides that bind the antibody at paratopes distinct from the antigen itself. When utilized as immunogens, functional mimics fail to elicit antibodies with the desired specificity against the original antigen. We suspect functional mimics are especially readily encountered with antibodies like AP4-24H11 whose binding sites have not been optimized for binding to a simple linear peptide epitope. Immunogenic mimics, on the other hand, form molecular contacts with the selecting antibody similar to those that engage the antigen itself, and are therefore more likely to provoke antibodies that bind the antigen. Even in these cases, without detailed structural analysis it may be impossible to discern any obvious structural similarity between the original epitope and its affinity-selected immunogenic mimic. Of the 10 peptides we characterized here, 8 are apparently in the functional mimic category; they bind the antibody but fail to elicit antibodies with specificity for AIP4. However, two peptides elicited antibodies that served as immunogenic mimics as determined by their ability to provoke an immune response that protected against *S. aureus*-mediated dermonecrosis.

To date, no anti-*Staphylococcus aureus* vaccine has succeeded in Phase III clinical trials (78). Such vaccines have primarily relied on immunization with *S. aureus* surface protein antigens, suggesting that strategies aimed at inducing opsonophagocytic antibodies are not sufficient to prevent disease by this pathogen. We are not alone in recognizing the possible utility of vaccines that target secreted virulence factors (108,

184). For example, neutralization of the secreted virulence factor Hla using active vaccination or passive transfer of neutralizing antibodies has proven efficacious in several *S. aureus* infection models (96, 117, 170, 181, 182, 185, 186) and a vaccine targeting recombinant Hla was part of a recent clinical trial (NCT01011335). The previous example is one of several strategies based on inhibition of a single secreted virulence factor; however, immune-based approaches to inhibit *S. aureus* virulence factor expression on a global level have been limited (reviewed in (184)).

Park et al. recently demonstrated *in vivo* protection via passive administration of a mAb, directed against a synthetic AIP4 hapten, which prevents global virulence factor expression by inhibiting *agr* quorum sensing (103). However, the availability of both prophylactic vaccines and therapeutic mAbs targeting *S. aureus agr*-regulated virulence would significantly increase the translational spectrum of this anti-staphylococcal virulence approach. Herein, we expanded on the work of Park et al. using MS2 VLP libraries and affinity selection to develop a vaccine strategy for *agr*-inhibition. Using this technique against the mAb AP4-24H11, we identified candidate vaccines with *in vivo* efficacy in a mouse model of *S. aureus* SSSI.

CHAPTER 3: ω-Hydroxyemodin Limits *Staphylococcus aureus* Quorum Sensing-Mediated Pathogenesis and Inflammation

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Abstract

Antibiotic-resistant pathogens are a global health threat. Small molecules that inhibit

bacterial virulence have been suggested as alternatives or adjuncts to conventional

antibiotics, as they may limit pathogenesis and increase bacterial susceptibility to host

killing. *Staphylococcus aureus* is a major cause of invasive skin and soft tissue infections

(SSSIs) in both the hospital and community settings, and it is also becoming increasingly

antibiotic resistant. Quorum sensing (QS) mediated by the accessory gene regulator (*agr*)

controls virulence factor production essential for causing SSSIs. We recently identified

ω-hydroxyemodin (OHM), a polyhydroxyanthraquinone isolated from solid-phase

cultures of *Penicillium restrictum*, as a suppressor of QS and a compound sought for the

further characterization of the mechanism of action. At concentrations that are nontoxic

to eukaryotic cells and subinhibitory to bacterial growth, OHM prevented *agr* signaling

by all four *S. aureus agr* alleles. OHM inhibited QS by direct binding to AgrA, the

response regulator encoded by the *agr* operon, preventing the interaction of AgrA with

the *agr* P2 promoter. Importantly, OHM was efficacious in a mouse model of *S. aureus*

SSSI. Decreased dermonecrosis with OHM treatment was associated with enhanced

bacterial clearance and reductions in inflammatory cytokine transcription and expression

at the site of infection. Furthermore, OHM treatment enhanced the immune cell killing of

S. aureus in vitro in an *agr*-dependent manner. These data suggest that bacterial

disarmament through the suppression of *S. aureus* QS may bolster the host innate

immune response and limit inflammation.

Introduction

Due to the widespread and seemingly inevitable development of bacterial resistance to
antibiotics shortly after their introduction, there is a great need for alternatives or adjuncts
to classical antimicrobials (8, 187, 188). Along with ongoing efforts to identify novel
antibacterial targets, interventions that are not directly bactericidal may prove efficacious.
These include approaches aimed at modifying or augmenting the host response, as well as
approaches that inhibit bacterial virulence mechanisms and thus limit pathogenesis (8,
187, 188). Many pathogenic bacteria coordinate the expression of virulence factors
important for invasive infection and pathogenesis through a density-dependent
communication system called quorum sensing (QS) (36, 52). Therefore, approaches
aimed at disrupting QS hold promise to limit pathogenesis in the host and/or serve as
adjuncts to extend the utility of existing antibiotics (36, 53, 54, 189–191).

Skin and soft tissue infections (SSSIs) represent the majority of infections caused
by *Staphylococcus aureus* (3, 12, 13), and many of the virulence factors contributing to
SSSIs are globally regulated by the accessory gene regulator (*agr*) (**Figure 1.A**) (38, 44,
167). The *agr* system utilizes a small secreted autoinducing peptide (AIP) to activate a
receptor histidine kinase, AgrC, in the bacterial cell membrane. AgrC phosphorylates the
transcription factor AgrA, which in turn activates transcription at the P2 and P3
promoters of the operon. P3 activation drives the production of the effector of the operon,
RNAIII, which regulates the expression of >200 virulence genes that contribute to
invasive infection (44). *S. aureus* isolates have one of four *agr* alleles (*agr*-I to *agr*-IV),
each encoding factors that secrete a unique AIP (AIP1 to AIP4, respectively; **Figure 1.B**)
that is detected by a cognate AgrC histidine kinase; isolates from each allele can cause

human disease (192, 193). Importantly, we and others have shown that the disruption of *agr* signaling by mutagenesis, monoclonal antibodies, or host factors limits *S. aureus* infection and reduces pathogenesis (44, 56–58, 60, 103, 194), demonstrating that *agr* QS is a robust target for combating invasive *S. aureus* infection.

Recently, we reported on a synthetic small molecule inhibitor of *S. aureus* QS called savirin, providing proof of principle that small molecule-mediated inhibition of QS can be efficacious against *S. aureus in vivo* (55). Natural products also represent a wealth of bioactive compounds, as approximately 65% of the antibacterials introduced in the last 30 years are natural products or compounds designed based on a lead natural-product pharmacophore (123). Therefore, we extended our search for inhibitors of QS to natural products and in doing so identified a series of polyhydroxyanthraquinones isolated from cultures of the fungus *Penicillium restrictum* that inhibited *S. aureus* QS (140). Among these, ω-hydroxyemodin (OHM) (**Figure 3.**) demonstrated the most potent activity for inhibiting *agr*-I signaling. Therefore, we sought to characterize OHM as an inhibitor of *agr* signaling and to evaluate its efficacy *in vivo*.
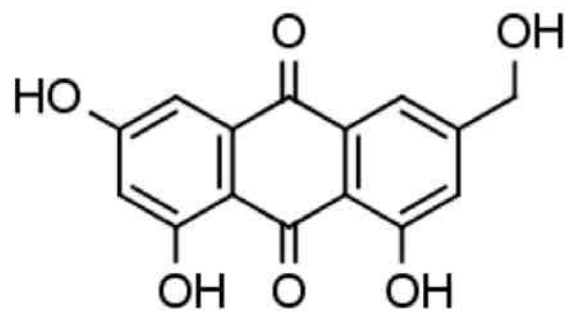
Figure 3.1 - Structure of ω-hydroxyemodin (OHM).
Molecular mass, 286.24 Da.

Approaches aimed at augmenting the host response and those aimed at inhibiting bacterial virulence mechanisms provide alternatives to conventional antibiotic therapy. However, these two approaches are not mutually exclusive, and because many *S. aureus* virulence factors antagonize the host innate immune response, we proposed that inhibiting bacterial virulence would itself augment host defense. Specifically, we postulated that small molecule-mediated disruption of *S. aureus* QS-dependent virulence would not only limit pathogenesis but would also reduce inflammation and result in enhanced bacterial clearance. Here, we report that OHM inhibits *S. aureus* QS by isolates from all four *agr* alleles at concentrations that are noncytotoxic to *S. aureus* or eukaryotic cells. Mechanistically, OHM inhibits *agr* activation by binding directly to AgrA and blocking binding to *agr* promoter DNA. Importantly, as predicted, OHM limits tissue damage and inflammation and promotes bacterial clearance in a mouse model of *S. aureus* SSSI. In addition, OHM promotes the killing of *agr*$^+$, but not *agr*-negative, *S. aureus* by both mouse macrophages and human polymorphonuclear leukocytes (PMNs), and it limits neutrophil lysis caused by *agr*-regulated *S. aureus*-secreted virulence factors. This is the first report of a polyhydroxyanthraquinone with *in vivo* efficacy against *S. aureus* QS-dependent virulence. In addition, these data demonstrate that antivirulence approaches can limit disease by disarming the bacteria while concurrently bolstering host innate defense.

Materials and Methods

Ethics statements

The animal work in this study was carried out in strict accordance with the

recommendations in the Guide for the Care and Use of Laboratory Animals (195), the

Animal Welfare Act, and U.S. federal law. The protocol was approved by the

Institutional Animal Care and Use Committee of the University of New Mexico Health

Sciences Center. The polymorphonuclear cells were isolated from whole blood samples

from consenting healthy human volunteers, according to the protocol (no. 11-005)

approved by the Human Research Protections Office of the University of New Mexico

institutional review board.

Bacterial strains and growth conditions

Methicillin-resistant *S. aureus* (MRSA) strain USA300 LAC (*agr*-I) was provided as a

generous gift from Frank DeLeo (Rocky Mountain National Laboratories, National

Institutes of Health, Hamilton, MT). *S. aureus* strains AH1677 (*agr*-I), AH430 (*agr*-II),

AH1747 (*agr*-III), and AH1872 (*agr*-IV) expressing yellow fluorescent protein (YFP)

under the control of the *agr*::P3 promoter were previously described (175).

Staphylococcus epidermidis strain AH3408 (*agr*-I) expressing superfolder green

fluorescent protein (sGFP) under the control of the *agr*::P3 promoter was also previously

described (41). *S. aureus* strains AH3469 (AgrC wild type [WT]) and AH3470 (AgrC

R238H) are described below. Unless otherwise noted, the bacteria were cultured at 37°C

and 220 rpm, with at least a 5:1 air-to-culture ratio in trypticase soy broth (TSB) (Becton,

Dickinson and Company, Sparks, MD). Early exponential-phase bacteria were prepared

as described previously (196). The frozen stocks were maintained at −80°C in TSB

supplemented with 10% glycerol. The bacteria were enumerated by serial dilution and

plating onto trypticase soy agar containing 5% sheep blood (Becton, Dickinson and Company), followed by overnight incubation at 37°C. The limit of detection was 2-$\log_{10}$ CFU.

agr::P3 promoter activation assays

Overnight cultures of *S. aureus agr*::P3 reporter strains were grown in TSB supplemented with 10 μg/mL chloramphenicol (Cam). The cultures were diluted 1:250 into fresh TSB with Cam, and 100-μl aliquots were transferred to 96-well microtiter plates (Costar 3603; Corning, Tewksbury, MA) prefilled with 100 μL of medium and a 2-fold serial dilution series (200 to 0.1 μM) of OHM. OHM was purified from solid-phase cultures of *P. restrictum*, as described previously (140), and was >95% pure, as measured by ultrahigh-performance liquid chromatography (UPLC) (**Figure 3.**).
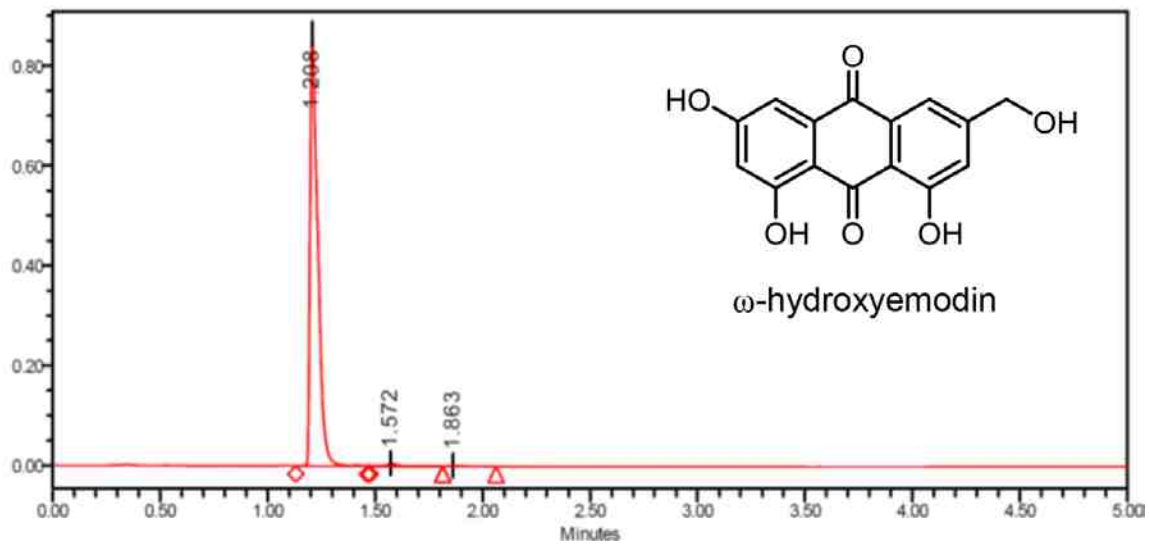
Figure 3.2 - The purity of OHM was evaluated via a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA). UPLC utilized a Waters BEH C18 column (1.7 μm; 2.1×50 mm) and a CH_3CN-H_2O gradient that increased linearly from 20 to 100% CH_3CN over 4.5 min. The chromatogram was monitored at 254 nm.

After mixing in the microtiter plate, the effective dilution was 1:500, and the final OHM concentration ranged from 100 to 0.05 μM, with a final dimethyl sulfoxide (DMSO) concentration of 0.1% (vol/vol) in all wells. Four dilution series were prepared for each reporter; in addition, 4 mock DMSO dilution series were included for each reporter strain. The microtiter plates were incubated at 37°C with shaking (1,000 rpm) in a Stuart SI505 incubator (Bibby Scientific, Burlington, NJ) with a humidified chamber. Fluorescence (top reading of 493 nm excitation, 535 nm emission, and gain of 60) and optical density at 600 nm (OD_{600}) readings were recorded at 30-min increments using a Tecan Systems (San Jose, CA) Infinite M200 plate reader.

S. epidermidis AH3408 (*agr*-I::P3-sGFP) was cultured overnight in TSB supplemented with 10 μg/mL erythromycin (Erm). To collect exogenous *S. epidermidis* AIP1 peptide, the spent medium was centrifuged at $3,000 \times g$, passed through a 0.2-μm HT Tuffryn membrane (Pall, Port Washington, NY), and stored at −20°C until use. An overnight culture of AH3408 was diluted 1:200 into 500 μL of TSB (broth) or TSB with 10% spent medium containing 5 μg/mL OHM or DMSO (vehicle). The cultures were incubated for 24 h at 37°C, centrifuged, and resuspended in 10% formalin fixative for 1 min. The cultures were washed twice by centrifugation and resuspended in phosphate-buffered saline (PBS). The mean channel fluorescence (MCF) of sGFP was analyzed using an Accuri C6 flow cytometry system (BD Biosciences, San Jose, CA). The data were normalized to the broth cultures containing no exogenous AIP1.

Quantitative PCR

For transcriptional quantification of mouse mRNA, 2.25-cm^2 sections of skin including and surrounding the abscess were excised, minced, and stored in RNA*later* (Qiagen, Valencia, CA) at −20°C until use. mRNA was purified using the RNeasy kits (Qiagen), and cDNA was generated using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using an ABI 7900HT real-time PCR system with TaqMan Gene expression master mix, according to the manufacturer's directions (Applied Biosystems). Predesigned primer and probe sets (Integrated DNA Technologies, Coralville, IA) were used for the quantitation of mouse *il-6*, *il-1β*, *tnfα*, *nlrp3*, and *hprt*. The data are represented as the fold increase relative to *hprt* compared to that in uninfected tissue.

For the quantification of *S. aureus* gene transcription, 500-μL cultures at 2×10^7 CFU/mL of LAC and/or LAC∆*agr* were grown in TSB at 37°C, with aeration, for the indicated times with 50 nM exogenous AIP1 (Biopeptide Co., Inc., San Diego, CA) and treatments (vehicle versus OHM), as indicated in the appropriate figure legends. The bacteria were stored at −20°C in RNAprotect cell reagent, according to the manufacturer's recommendations (Qiagen), until the RNA was purified as previously described (55). cDNA generation and quantitative PCR (qPCR) were performed as described above for eukaryotic qPCR. The primer and probe sets for the quantification of the *S. aureus* genes are listed in **Table 3.**.

Table 3.1 – Oligonucleotides used for *S. aureus* qPCR.

Focus	Gene	Oligo	Sequence (5'-3')
Control	16S	F	TGA TCC TGG CTC AGG ATG A
		R	TT CGC TCG ACT TGC ATG TA
		Probe	CGC TGG CGG CGT GCC TA
Virulence	*hla*	F	ACA ATT TTA GAG AGC CCA ACT GAT
		R	TCC CCA ATT TTG ATT CAC CAT
		Probe	AAA AAG TAG GCT GGA AAG TGA TAT TTA ACA
	*psm*α	F	TAT CAA AAG CTT AAT CGA ACA ATT C
		R	CCC CTT CAA ATA AGA TGT TCA TAT C
		Probe	AAA GAC CTC CTT TGT TTG TTA TGA AAT CTT ATT TAC CAG
	RNAIII	F	AAT TAG CAA GTG AGT AAC ATT GCT AGT
		R	GAT GTT GTT TAC GAT AGC TTA CAT GC
		Probe	AGT TAG TTT CCT TGG ACT CAG TGC TAT GTA TTT TTC TT
	saeR	F	TGC CAA AAC ACA AGA ACA TGA TAC
		R	CTT GGA CTA AAT GGT TTT TTG ACA TAG T
		Probe	TTT ACG CCT TAA CTT TAG GTG CAG AT
	set7	F	ACG GAA AAA CCA GTT CAT GC
		R	GCT TAT CTT TGC CAA TTA AAG CA
		Probe	CAG GTT ATA TCA GTT TCA TTC AAC CA
	spa	F	GAT GGT AAC GGA GTA CAT GTC GTT
		R	TTG CTG GTT GCT TCT TAT CAA CA
		Probe	ACA TTG CAA AAG CAA ACG GCA CTA CTG C
Stress response	*asp23*	F	GTT AAC CAC CTT TCA TGT CTA AGA TAC
		R	AAA TTA ACT TTC TCT GAT GAA GTT GTT GA
		Probe	CTT CAC GTG CAG CGA TAC CAG CAA TTT
	crtM	F	GTT TGA AAC GGA CGC TGA ATT A
		R	ACC AAG TCT TCT TGC GAC AT
		Probe	TGG TGT TGC TGG TAC AGT AGG
	clpB	F	TGG TGT GCG TAT TCA AGA TAG AG
		R	GCA CAT GCT TGG TCA ACT AAA T
		Probe	ATC AGA CAA TTC AGC GGC AGC AAC
Metabolism	*atpG*	F	GCC ACT GAT AAT GCA ACT GAA C
		R	GCG AAA CCA CCA ACA ATT TC
		Probe	AGA GCG AGA CAA GCA GAA ATT ACG CA
	murQ	F	CTG GTG GAC AAC ATG CTA TGA
		R	CAC TCG CGG CAA TTC CTA TAA
		Probe	ATG GCT GTA GAA GGT GCG AAA GAT
	sdhA	F	GCA TTA ATG GTG CGG TCA ATA C
		R	CTG CCT CTG TCA TCG CTT TA
		Probe	ATG GTG GCG ATT CCT TGC AAA CC
Efflux/ antibiotic resistance	*norA*	F	CGA GAG TGA TTG GTG GTA TGA G
		R	TCG CTG ACA TGT AGC CAA AG
		Probe	TGC TGG TAT GGT AAT GCC TGG TGT
	mdrA (SAUSA300_2299)	F	AGT CCT GCA TCT GGA CAA ATT A
		R	CTT TCG TTT CGC CAT CTT GAC
		Probe	ACA AGG TGA CAA ACT CGA TAA AGG TGA CA
	NaMDR (SAUSA300_0335)	F	TGG GAT TAT GTG AAG GTG TTG TA
		R	ACG CCG ATA GAC ATG ATA ACT G
		Probe	ACG TCT TTC ATA CGG CCT TTA TTT GCC

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Rabbit red blood cell lysis assay

The assay was performed as previously described (197). Briefly, LAC was cultured in 5 mL of TSB for 8 h with the indicated treatments, centrifuged, and the supernatants were filtered through a 0.2-μm HT Tuffryn membrane (Pall). Serial 2-fold dilutions of the supernatant were incubated at 37°C for 1 h in a 4% solution of rabbit red blood cells (rRBCs). Lysis was assessed spectrophotometrically at OD_{450}. The data were analyzed by nonlinear regression fit to a four-parameter logistic curve and represented as the HA_{50}, which equals 1/the dilution required for 50% complete lysis.

AgrC constitutive reporter assay

The *agrBDCA* operon was amplified from strain LAC using the primers AgrB+RBS 5′–KpnI (GTTGGTACCCAGTGAGGAGAGTGGTGTAAAATTG) and AgrA 3′-SacI (GTTGAGCTCCTTATTATATTTTTTTAACGTTTCTCACCGATG) and ligated into pRMC2 (198). To make a variant with constitutive AgrC activity, we chose the AgrC R238H mutation, which was previously shown to have similar activity in the presence and absence of the AIP2 inhibitor and maximal activity in the absence of AIP1 (43). The AgrC R238H variant was generated by the QuikChange (Agilent Technologies) site-directed mutagenesis method, using the primers AgrC R238H fwd (CAACGAAATGCGCAAGTTCCATCATGATTATGTCAATATC) and AgrC R238H rev (GATATTGACATAATCATGATGGAACTTGCGCATTTCGTTG). To build a destination strain for assessing alpha-hemolysin production, we selected an *agrC* transposon mutant (NE873) from the Nebraska Transposon Mutant Library (199) and integrated the pLL29 plasmid at the phage 11 attachment site to confer tetracycline

resistance (200). The above-described pRMC2 constructs were transformed into this strain to make reporters AH3469 (AgrC WT) and AH3470 (AgrC R238H). To test OHM, AH3469 and AH3470 were grown overnight with 10 μg/mL Cam and were diluted 1:500 into 5 mL of fresh medium with 10 μg/mL Cam and 0.025 μg/mL anhydrotetracycline. AIP2 control, OHM, or DMSO (vehicle) was added to each strain at the concentrations indicated. The cultures were grown at 37°C and shaken at 220 rpm for 6.5 h. The bacteria were pelleted by centrifugation, and the alpha-hemolysin-containing supernatants were passed through a 0.2-μm HT Tuffryn membrane (Pall). Rabbit red blood cell lysis assays were conducted as above but with an rRBC concentration of 1% and 25% supernatant (vol/vol) to yield complete lysis. The values are presented as the mean relative lysis compared to that with vehicle treatment.

Eukaryotic cytotoxicity

A549, HEK293, or HepG2 cells were seeded in a 96-well tissue culture plate at 2.5×10^4 cells per well and incubated at 37°C with 5% CO_2. XTT [2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] and phenazine methosulfate (PMS) were purchased from Sigma-Aldrich (St. Louis, MO). The XTT assay was previously described (201). After 24 h, the spent medium was removed, and fresh medium containing the indicated drug concentrations or vehicle was added to the cells and incubated for an additional 24 h. To avoid potential interference with the absorbance readings due to the red color of OHM, drug-containing medium was replaced with 100 μL of 0.3 mg/mL XTT with 0.015 mg/mL PMS in Hanks' balanced salt solution (HBSS) and incubated for 1 h. Cell viability was assessed by the metabolic reduction of

tetrazolium measured at OD_{490}. The data are presented as the percentage of viable cells compared to that in the vehicle control.

EMSA and flow cytometry-based AgrA$_C$ promoter binding assays

Escherichia coli expressing the AgrA C-terminal DNA binding domain (AgrA$_C$) along with a 6-histidine tag was generously provided by Chuan He (University of Chicago, Chicago, IL) and purified as previously described (202). Electrophoretic mobility shift assays (EMSA) were performed as previously described (55), with purified AgrA$_C$ and *agr* P2 promoter, and a 16-bp duplex DNA probe with a 3′ 6-fluorescein (P2-FAM) (Integrated DNA Technologies, Coralville, IA). The duplex DNA contained the high-affinity LytTR binding site located in both *agr* P2 and P3 promoters (203). Briefly, 2 μM AgrA$_C$ was incubated for 10 min at room temperature (RT) with vehicle or the indicated concentrations of OHM in Tris-acetate-EDTA (TAE) buffer with 10 mM dithiothreitol (DTT). Next, 20 ng of P2-FAM DNA probe was added and incubated for an additional 10 min. The reaction mixtures were loaded onto a 10% PAGE gel and run at 50 V in the dark for 20 min. DNA migration was assessed by imaging on a FluorChem R system (ProteinSimple, Santa Clara, CA).

For the flow-based AgrA$_C$ promoter binding assays, AgrA$_C$ was biotinylated (AgrAC-BTN) using a Thermo Scientific EZ-Link sulfo-NHS-LC-biotin kit (Thermo Scientific, Rockford, IL), according to the manufacturer's directions. AgrA$_C$-BTN was immobilized on 1-μm-diameter Dynabeads MyOne streptavidin T1 (Life Technologies, Grand Island, NY) (AgrAC-SA), and the beads were suspended in PBS. DNA probe (P2-FAM) was added at a final concentration of 1.6 μM, along with equimolar competing

unlabeled P2, vehicle control, or OHM at the indicated concentrations. OHM-mediated

inhibition of AgrA$_C$-SA binding to DNA probe was measured as decreased mean channel

fluorescence (MCF) compared to that of the vehicle control using an Accuri C6 flow

cytometry system (BD Biosciences).

In silico docking on AgrA$_C$

In silico docking calculations were performed using the Macintosh binary executable of

AutoDock Vina (39). OHM was docked onto the B subunit of the AgrA$_C$ crystal structure

(RSCB Protein Data Bank [www.pdb.org], PDB ID 4G4K) (154, 204) stripped of

heteroatoms. The search box was restricted to the C-terminal region of AgrA$_C$, as

described for 9H-xanthene-9-carboxylic acid (154). Based on initial observations

suggesting that OHM bound to the pocket between the side chains of His200, Arg218,

Tyr229, and Val232, additional calculations were run in which the size of search box was

varied and the side-chain torsion angles for different combinations of residues in the

region were allowed to be flexible. The reported docking solution was obtained by

allowing flexibility in the side chain torsion angles for His200, Arg218, Tyr229, and

Val232 and by using a search box that was large enough to include both the pocket

bounded by the side chains of His200, Arg218, Tyr229, and Val232 and the groove

between Val232 and Lys236. Molecular modeling images were prepared using PDB ID

3BS1 and PyMOL (PyMOL molecular graphics system, version 1.5.0.4; Schrodinger,

LLC).

Surface plasmon resonance analysis

To overcome the potential interference for the oxidative inactivation of $AgrA_C$ during surface plasmon resonance (SPR) analysis, the oxidation-resistant C199S mutation was introduced into the $AgrA_C$ expression construct, as previously described (202), using the QuikChange II XL kit (Agilent Technologies). His-tagged $AgrA_C$-C199S was purified as described previously (55) but without the addition of Tris(2-carboxyethyl)phosphine (TCEP) or DTT during purification.

SPR binding and kinetics analyses were performed on a Biacore X100 instrument (GE Healthcare, Pittsburgh, PA) and evaluated with Biacore X100 evaluation software (version 1.0). His-tagged $AgrA_C$-C199S was immobilized at 10 µg/mL in PBS on a nitrilotriacetic acid (NTA) biosensor with the NTA reagent kit (GE Healthcare). For binding studies, OHM (analyte) was dissolved in running buffer (PBS, 5% DMSO [pH 9]) and applied at a flow rate of 30 µL/min with a 180-s contact time and 300-s dissociation time. The data were fit to a 1:1 binding model after the subtraction of blank injections and the removal of injection spikes from the sensorgrams. NTA biosensor chips were regenerated with the following sequence: two 60-s washes with 350 mM EDTA, a 60-s wash with PBS, and a 60-s wash with 500 mM imidazole, followed by a final 60-s wash with PBS. The analyses were performed at 25°C.

Mouse model of skin and soft tissue infection

The mouse model of skin and soft tissue infection was previously described and was implemented with minor modifications (174). Early exponential-phase *S. aureus* strain LAC was diluted into USP-grade saline (Braun, Irvine, CA) to deliver 5×10^7 to 7×10^7 CFU per mouse. Aliquots of OHM were diluted in 0.5% hydroxypropylmethylcellulose

(HPMC) (pH 11) to deliver 0.2 mg/kg per mouse ($\sim$5 μg). The mice were anesthetized

with 3% isoflurane at 3 liters/min. The bacteria and OHM were mixed 1:1 immediately

before subcutaneous injection into the right flank, at a total volume of 50 μL. The mice

were weighed prior to infection and every day post infection. Additionally, the injection

sites were photographed daily to determine the abscess and ulcer areas with ImageJ

analysis (205). On day three or day seven post infection, each mouse was euthanized by

CO_2 asphyxiation, and a 2.25-cm^2 section of skin surrounding the abscess was excised.

The tissue was mechanically homogenized and serially plated on sheep blood agar to

determine the bacterial burden. The tissue homogenates were stored at $-80°C$ until they

were rapidly defrosted at 37°C for cytokine analysis. On day 3 post infection, the

homogenate was centrifuged at 12,500$\times g$ for 10 min and the clarified supernatant

analyzed with a custom-designed multiplex assay (Merck KGaA, Darmstadt, Germany)

using a BioPlex 200 with BioPlex Manager software (Bio-Rad, Hercules, CA). The

abscess tissues collected for hematoxylin and eosin (H&E) staining were fixed overnight

in 10% formalin and embedded in paraffin. Five-micrometer sections were then stained

with H&E and imaged using an Olympus IX70 microscope (Olympus, Center Valley,

PA).

Alpha-hemolysin quantification

For the detection of alpha-hemolysin (Hla) in abscess tissue homogenates by Western

blot assay, clarified homogenates were rapidly thawed and an aliquot electrophoresed on

a 16% Tris-glycine SDS-PAGE gel (Life Technologies, Grand Island, NY) before

transfer to a polyvinylidene fluoride membrane. The membranes were blocked for 1 h at

RT, using TBST (20 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20) with 5% nonfat

dry milk. Hla was detected using anti-Hla antibody (ab15948; Abcam, Cambridge, MA)

at a 1:1,000 dilution and alkaline phosphatase-conjugated secondary antibody. The

immunoreactive bands were developed with nitroblue tetrazolium (NBT)/5-bromo-4-

chloro-3-indolyl-phosphate (BCIP) (Thermo Scientific) and intensity measured using a

FluorChem R system and the AlphaView software (ProteinSimple). Relative intensity

was calculated as the measured intensity divided by the total protein concentration based

on absorbance at 280 nm.

Mouse macrophage killing of S. aureus

Murine macrophage cells (RAW 264.7) were maintained at 37°C in 5% CO_2 in high-

glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, with 100 U/mL penicillin and

100 μg/mL streptomycin. Twenty-four hours prior to the experiments, the RAW cells

were washed with PBS and the medium replaced with DMEM, as described above but

with 2% FBS without antibiotics. Early exponential-phase LAC or LACΔ*agr* was

cultured in 3 mL of TSB at 2×10^7 CFU/mL at 37°C, with aeration for 5 h, with 50 nM

exogenous AIP1 (Biopeptide Co.) and 5 μg/mL OHM or DMSO (vehicle). The bacteria

were centrifuged, washed in PBS, sonicated, and suspended at 1×10^8 to 2×10^8 in DMEM

but with 1% FBS without antibiotics. The bacteria were opsonized overnight at 4°C with

rabbit anti-*S. aureus* IgG at 100 μg/mL (catalog no. YVS6881; Accurate Chemical &

Scientific Co., Westbury, NY). The RAW cells were washed with PBS and suspended at

2×10^7 cells/mL in DMEM with 1% FBS without antibiotics and combined with

opsonized bacteria at a multiplicity of infection (MOI) of 1:1. The cells were centrifuged at $500{\times}g$ for 3 min to initiate contact and incubated at 37°C in 5% CO_2 for 1 h to allow phagocytosis. Lysostaphin (catalog no. L-0761; Sigma-Aldrich) was added at 2 µg/mL for 15 min to kill extracellular bacteria and then removed by centrifugation and replacement with fresh medium. Half of the samples were immediately processed for CFU determination, and the other half were incubated for an additional 4 h before CFU enumeration. Intracellular bacteria were enumerated by preliminary dilution into PBS–0.1% Triton X-100, followed by sonication and plating onto blood agar.

Human PMN assays

PMNs were purified from normal healthy venous blood, as described by Nauseef (206). The purified PMNs were suspended in HBSS without divalent cations at $\leq 3{\times}10^7$ cells/mL and kept on ice until use.

PMN phagosomal killing of *S. aureus* was conducted as previously described, with the following alterations (207). Prior to opsonization, early exponential-phase LAC or LACΔagr was cultured in 3 mL of TSB at $2{\times}10^7$ CFU/mL at 37°C, with aeration for 5 h, with 50 nM exogenous AIP-I (Biopeptide Co.) and 5 µg/mL OHM or DMSO (vehicle). The bacteria were centrifuged, washed in PBS, and opsonized at $5{\times}10^6$ CFU/mL in HBSS, with divalent cations supplemented with 20 mM HEPES, 1% human serum albumin (HSA), and 10% pooled human serum. Following a 20-min incubation with tumbling at 37°C, the bacteria were pelleted, washed in PBS, and resuspended in HBSS with divalent cations supplemented with 20 mM HEPES. The PMNs and opsonized bacteria were combined at an MOI of 1:1 and incubated for 10 min at 37°C. The

extracellular bacteria were removed by centrifugation at 500×g for 5 min, followed by the

resuspension of infected PMNs in HBSS with divalent cations supplemented with 20 mM

HEPES and 1% HSA. The infected PMNs were incubated at 37°C for 120 min, and the

aliquots were removed at 0, 30, 60, and 120 min. The aliquots were diluted into PBS–

0.1% Triton X-100 to lyse the cells and then serially diluted and plated on blood agar for

CFU enumeration.

The lysis of PMNs by the *S. aureus* supernatant was conducted as previously

described, with minor modifications (55). Briefly, LAC was cultured in 3 mL of TSB for

5 h with 5 µg/mL OHM or vehicle, centrifuged, and the supernatants were filtered

through a 0.2-µm HT Tuffryn membrane (Pall). The supernatants were stored at −80°C

and thawed on ice prior to use. The PMNs were washed with PBS and resuspended in

RPMI supplemented with 10 mM HEPES and 1% HSA. PMNs at a density of 3×10^6

cells/mL in 100 µL were added to 100 µL of RPMI, RPMI with 10% TSB (vol/vol), or

RPMI with 10% *S. aureus* supernatant prepared as described above. The PMNs were

incubated at 37°C and 5% CO_2 for 2 h. Following incubation, the supernatants were

collected by centrifugation at 3,000×*g* for 5 min and assessed for lactate dehydrogenase

(LDH) release, according to the manufacturer's specifications (CytoTox 96

nonradioactive cytotoxicity assay; Promega Co., Madison, WI). Triton X-100 was added

at a final concentration of 0.1% (vol/vol) as a 100% lysis control, while cell-free RPMI

with 5% TSB served as a blank. The data are normalized to 100% lysis control.

Statistical analysis

Statistical evaluations were performed using GraphPad Prism version 5.04. The *in vitro* data were analyzed by the two-tailed Student's *t*-test, and the *in vivo* data were analyzed by the Mann-Whitney U test for nonparametrics. The results were considered significantly different at a *P* value of <0.05.

Results

ω-Hydroxyemodin is a universal inhibitor of S. aureus quorum sensing

Our original report focused on OHM inhibition of QS using an *S. aureus* strain derived from the USA300 *agr*-I isolate LAC (140). However, isolates from all four *agr* alleles contribute to disease in humans. Therefore, to be of maximum utility, a QS inhibitor must antagonize QS by all four *agr* alleles. To address this, we assessed the ability of OHM to inhibit quorum sensing by isolates of all four *agr* types using reporter strains expressing yellow fluorescent protein (YFP) under the control of the *agr*::P3 promoter. OHM inhibited QS by all four *agr* types at concentrations that do not impact bacterial growth (**Figure 3.A**). Importantly, at concentrations required for *agr* inhibition, OHM was nontoxic to human alveolar (A549), kidney (HEK293), and hepatocyte cell lines (**Figure 3.B**). Also as expected based on the inhibition of *agr*::P3 promoter activation, OHM decreased the transcription of the *agr* effector RNAIII and *agr*-regulated virulence factors, including phenol-soluble modulin alpha (*psmα*) and alpha-hemolysin (*hla*) (**Figure 3.A**). OHM also inhibited the production of Hla, as demonstrated by the red blood cell lysis assay (**Figure 3.B**). Therefore, these data demonstrate that at concentrations that are noncytotoxic to eukaryotic cells, OHM is a universal inhibitor of *S. aureus* QS.
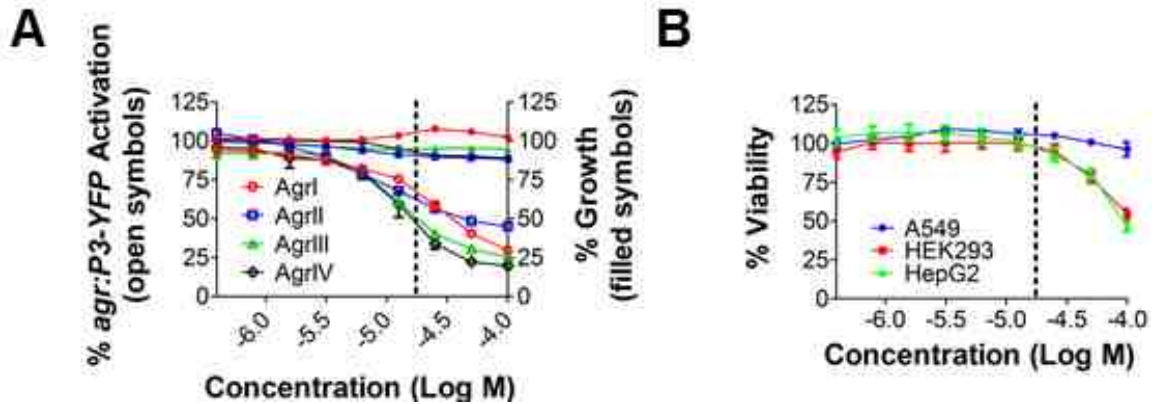
Figure 3.3 – ω-Hydroxyemodin inhibits *S. aureus* quorum sensing by all four *agr* alleles. (A) Effect of OHM on *agr*::P3 promoter activation (open symbols) and cell growth (closed symbols), measured by flow cytometry and OD_{600}, respectively, for *agr*-I (red circles), *agr*-II (blue squares), *agr*-III (green triangles), and *agr*-IV isolates (black diamonds). (B) Percent cell viability of A549 (blue circles), HEK293 (red squares), and HepG2 (green triangles) cells measured by XTT assay after 24 h of incubation with the indicated concentrations of OHM. The dashed vertical lines indicate the concentration used for the *in vitro* assays. Data are shown as means ± SEM. The experiments were performed in triplicate or quadruplicate.
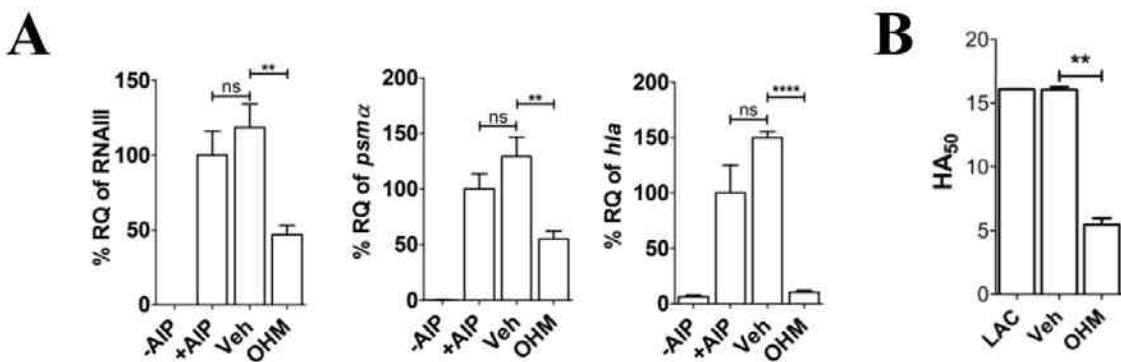
Figure 3.4 - OHM inhibits transcription and translation of *agr*-regulated genes. (A) Quantification of RNAIII, *psmα* and *hla* by qRT-PCR relative to 16S following a 2 h incubation of USA300 isolate LAC (2 x 10^7 CFU/mL) with 50 nM AIP1 and either 5 μg/mL OHM or vehicle control. Data are represented as the fold increase relative to 16S as compared to inoculum bacteria, and normalized to broth with exogenous AIP. (B) Effect of 5μg/mL OHM on expression of α-hemolysin (Hla) assessed via the rabbit red blood cell lysis assay. HA50 is the bacterial supernatant dilution factor required for lysis of 50% of the RBCs. Data are the mean ± SEM of triplicate samples. ns, not significant, ** $p<0.01$, ****$p<0.0001$, by Student's *t*-est.

ω-Hydroxyemodin antagonizes AgrA function

The ability of OHM to antagonize QS by all *S. aureus agr* alleles pointed to a target that is well conserved in the system. Therefore, we first focused on AgrC, the receptor histidine kinase activated by AIP binding. To determine whether OHM disrupted AgrC activation, we tested OHM for the inhibition of *agr*-mediated Hla expression determined by the lysis of rabbit RBCs, using an *agr*-I isolate expressing constitutively active AgrC (R238H (43)). Whereas the addition of inhibitory AIP (AIP2) reduced Hla expression by *S. aureus* expressing wild-type (WT) but not constitutively active AgrC, OHM inhibited Hla expression by both isolates (**Figure 3.A**). These results support a mechanism of action whereby OHM intracellularly inhibits *agr* signaling, downstream of AgrC activation.

The response regulator AgrA functions downstream of AgrC, and we and others have shown that small molecules that target AgrA disrupt QS (55, 154). Therefore, to further address the mechanism of action of OHM, we evaluated potential OHM binding sites on the crystal structure of the C-terminal AgrA DNA binding domain (AgrA$_C$) (154, 208). The most favorable binding site for OHM was near the AgrA$_C$-DNA interface (**Figure 3.B**). Docking studies positioned OHM in a pocket between the side chains of H200 and Y229, with Y229 recently identified as a major contributor to maximal AgrA activity (209), and three residues, R218, S231, and V232, which make direct interactions with bound DNA in the AgrA-DNA crystal structure (203). Given this, together with observations that OHM is within hydrogen bonding distance of R218 and that naturally occurring mutations at R218 result in *agr*-negative phenotypes (210), we predicted that OHM would inhibit AgrA binding to promoter DNA.
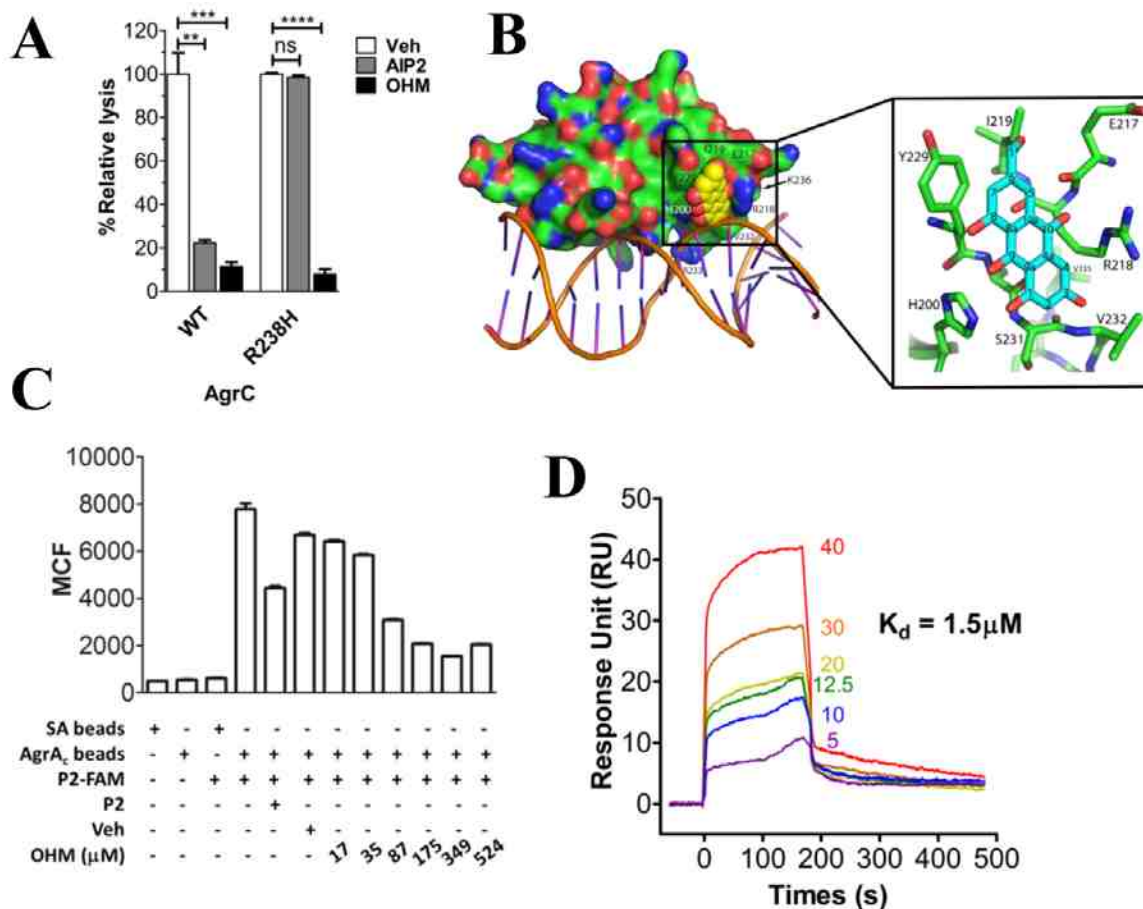
Figure 3.5 - ω-Hydroxyemodin inhibits AgrA binding to promoter DNA. (A) Effect of OHM on *agr*::P3 promoter activation assessed by rabbit red blood cell lysis for AgrC-WT isolate AH3469 and AgrC-R238H isolate AH3470. The data are the mean relative lysis ± standard error of the mean (SEM) compared to the vehicle control. The experiments were performed in triplicate. ns, not significant. (B) Space-fill model of AgrA$_C$ showing potential binding site for OHM. Inset, ball-and-stick representation of OHM binding site. (C) Flow cytometric bead-based assay to determine the effect of OHM on the binding of P2-FAM to biotinylated AgrA$_C$ immobilized on streptavidin beads (SA beads). Unlabeled P2 binding to immobilized AgrA$_C$ in competition with P2-FAM was included as a specificity control. The data are the mean ± SEM ($n = 3$). (D) Surface plasmon resonance analysis of OHM binding to 6-His-tagged AgrA$_C$-C199S immobilized on a nitrilotriacetic acid biosensor. The 50% inhibitory concentration (IC$_{50}$) was calculated as described in Materials and Methods. K_d, dissociation constant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P \leq 0.0001$ by Student's *t*-test.

To test this, we expressed AgrA$_C$ and measured binding to fluorescently labeled duplex *agr* promoter DNA encompassing the high-affinity binding site located in the *agr* P2 and P3 promoters (P2-FAM). As expected, OHM demonstrated a dose-dependent inhibition of AgrA$_C$ binding to *agr* promoter DNA by an electrophoretic mobility shift assay (EMSA) (**Figure 3.A**). In addition, we developed a bead-based assay to measure transcription factor binding to target DNA using flow cytometry. Biotinylated AgrA$_C$ was immobilized on streptavidin beads (SA beads), and the binding to promoter DNA was measured by flow cytometry. As expected, OHM again demonstrated a dose-dependent inhibition of AgrA$_C$ binding to *agr* promoter DNA (**Figure 3.C**). Furthermore, OHM bound directly to immobilized AgrA$_C$, as shown by surface plasmon resonance (SPR) analysis (**Figure 3.D**). Finally, because the amino acid sequence in the predicted OHM binding site of *S. aureus* AgrA is highly conserved with that of *S. epidermidis* AgrA, we postulated that OHM would also inhibit *agr* signaling by *S. epidermidis*. As expected, OHM significantly inhibited *agr* activation by *agr*-I *S. epidermidis* (**Figure 3.B**). Together, these data strongly suggest that OHM inhibits *agr* signaling by binding to AgrA and blocking AgrA function.
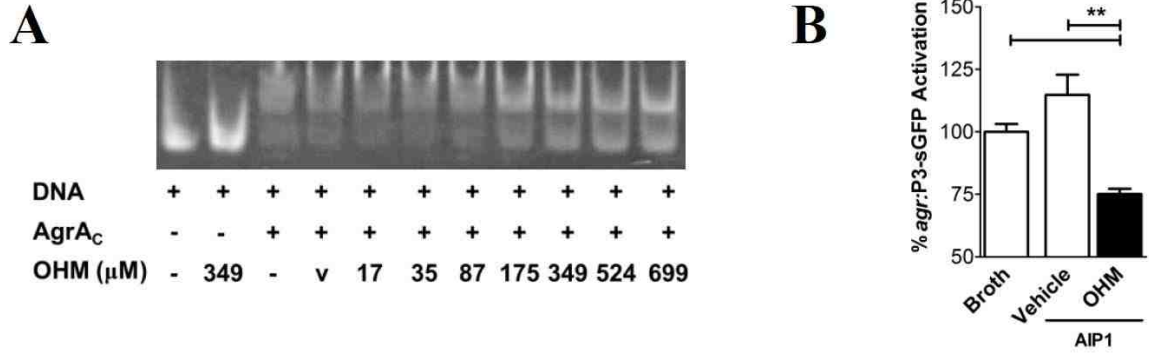
Figure 3.6 – OHM inhibits the AgrA-DNA interaction and inhibits *S. epidermidis* quorum sensing. (A) Effect of OHM on the electrophoretic mobility shift of the AgrA DNA-binding domain (AgrA$_C$) and P2-FAM complex ('v' indicates vehicle control). (B) Effect of OHM on *S. epidermidis agr*::P3 promoter activation measured by flow cytometry normalized to media control (Broth). Data are the mean ± SEM of experiments performed in triplicate. ** $p<0.01$, by Student's *t*-test.

To begin to address the specificity of OHM for *agr* inhibition, we used qPCR to evaluate the effects of OHM on transcription of a series of *agr*- and non-*agr*-regulated genes involved in virulence, the stress response, metabolism, and drug efflux and resistance (44, 55, 211, 212) (**Table 3.**). With respect to virulence genes, OHM treatment resulted in a slight yet nonsignificant increase in the transcription of *spa*, which encodes protein A and is negatively regulated by *agr* (44). In contrast, the expression of the enterotoxin gene *set7* decreased with OHM in LAC but not LACΔ*agr*, and OHM had no effect on the expression of the *saeR* component of the SaeRS virulence regulator. Likewise, the transcription of genes involved in the stress response (*asp23*, *crtM*, and *clpB*) was not altered by OHM, suggesting that OHM does not induce a general stress response in LAC under the conditions tested (213–217). Among the metabolism genes examined, OHM had no significant effect on the transcription of the genes involved in electron transport (*atpG* and *sdhA*). However, OHM treatment significantly decreased the transcription of *murQ*, an N-acetylmuramic acid 6-phosphate lyase, in both LAC and LACΔ*agr*. Although this protein, which is involved in cell wall recycling, is dispensable for growth in *E. coli* (218–220), its contribution to the growth of Gram positive pathogens is less clear. However, the absence of bactericidal or bacteriostatic effects with OHM treatment suggests that MurQ is not required for growth under the conditions tested. In addition, OHM treatment did not increase the transcription of genes examined for their potential to contribute to drug efflux or resistance. Therefore, although there are some non-*agr* effects, these results suggest that OHM is not a general inhibitor of transcription or energetics, nor is it a general inducer of drug efflux.

Table 3.2 – Transcriptional analysis of the *agr* specificity of OHM.

Focus	Gene	*agr* regulation/association	Fold change in gene expression (vehicle/OHM treatment)[a]			
			LAC	*P* value	Δagr^b	*P* value
Virulence	*spa* (SAUSA300_0113)	Neg	<2		ND	
	set7 (SAUSA300_0396)	NA[c]	−2.84	0.0019	<2	
	saeR (SAUSA300_0691)	Pos	<2		ND	
Stress response	*asp23* (SAUSA300_2142)	NA	<2		ND	
	crtM (SAUSA300_2499)	NA	<2		ND	
	clpB (SAUSA300_0877)	NA	<2		ND	
Metabolism	*atpG* (SAUSA300_2059)	NA	<2		ND	
	murQ (SAUSA300_0193)	Pos	−4.99	<0.0001	−8.551	<0.0001
	sdhA (SAUSA300_1047)	NA	<2		ND	
Efflux/antibiotic resistance	*norA* (SAUSA300_0680)	NA	<2		ND	
	mdrA (SAUSA300_2299)	NA	<2		ND	
	NaMDR gene[d] (SAUSA300_0335)	NA	−3.23	0.0015	−2.26	0.0076

[a] Values are shown if ≥2-fold.

[b] Assay performed with Δ*agr* strain if ≥2-fold difference with OHM treatment of LAC. ND, not done.

[c] NA, not applicable.

[d] NaMDR, Na⁺-driven multidrug efflux pump.

Furthermore, together with the above demonstrations of (i) OHM-mediated *agr* inhibition in a whole-cell assay, (ii) OHM-mediated inhibition of AgrA$_C$ binding to *agr* promoter DNA by both EMSA and bead-based assay, and (iii) the direct binding of OHM to AgrA$_C$ shown by SPR (**Figure 3. and Figure 3.6**), these results are consistent with a mechanism whereby OHM predominantly functions as an inhibitor of *agr* activation.

ω-Hydroxyemodin attenuates S. aureus SSSI

Invasive *S. aureus* SSSIs require *agr*-regulated virulence factors (44, 56, 131, 166, 168). Therefore, we assessed the efficacy of OHM in an established mouse model of *S. aureus* SSSI (174). Over the course of a three-day infection with the USA300 isolate LAC, a single 5-μg dose of OHM administered at the time of infection significantly inhibited abscess (**Figure 3.A, B**) and ulcer (dermonecrosis) formation (**Figure 3.A, C**), as well as morbidity at day one post infection (assessed by weight loss) compared to that of the vehicle-treated controls (**Figure 3.D**). In contrast, no differences were observed between the OHM and vehicle-treated mice infected with LACΔ*agr* (**Figure 3.A** and data not shown), demonstrating the specificity of OHM for disrupting *agr* signaling without directly impacting the host. Importantly, the single OHM treatment reduced the day three and day seven post infection bacterial burden at the site of infection in LAC-infected (**Figure 3.E**) but not Δ*agr*-infected mice (**Figure 3.7F**), suggesting that mice were better able to combat the infection in the absence of *agr* signaling.
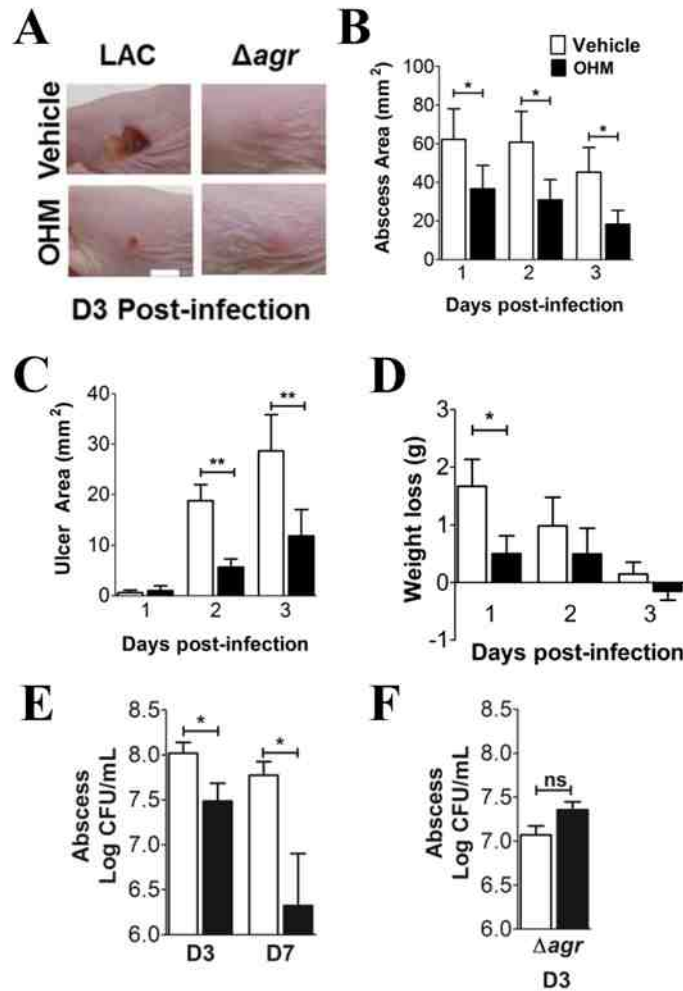
Figure 3.7 - ω-Hydroxyemodin limits abscess formation and dermonecrosis and promotes bacterial clearance in a mouse model of *S. aureus* SSSI. SKH1 mice were subcutaneously injected with 5×10^7 to 7×10^7 CFU of LAC or Δ*agr* along with OHM (0.2 mg/kg) or vehicle control. (A) Representative images of abscesses and ulcers on day three post-infection (D3) (scale bar = 5 mm). Day 3 post-infection abscess (B), ulcer area (C), and weight loss of LAC-infected mice. (E) Day 3 and day 7 post-infection bacterial burden at the site of infection. The data shown are the mean ± SEM (LAC, day 3, $n = 12$ to 16 mice per group; day 7, $n = 5$ mice per group). (F) Δ*agr* day 3 bacterial burden at the site of infection. $n = 6$ mice per group. ns, not significant. *, $P < 0.05$; **, $P < 0.01$ by Mann-Whitney U test.

We predicted that if OHM treatment supported host-mediated clearance by disrupting *agr* signaling, OHM-treated LAC, but not LACΔ*agr*, would be more readily killed by innate immune cells *in vitro* compared to in the vehicle-treated controls. As predicted, OHM treatment of LAC, but not LACΔ*agr*, resulted in significantly increased intracellular killing by both mouse macrophages (**Figure 3.A**) and human PMNs (**Figure 3.B**) compared to that in the vehicle-treated controls. Furthermore, OHM treatment of LAC but not LACΔ*agr* protected human PMNs from killing by secreted *agr*-regulated virulence factors. PMNs showed significantly increased survival in the presence of supernatant from OHM- versus vehicle-treated LAC (**Figure 3.C**).The increased killing was not a result of OHM-mediated effects on opsonophagocytosis, as the total number of bacteria phagocytosed (**Figure 3.D**) and the percentage of bacteria phagocytosed relative to the total inoculum (data not shown) were equivalent, regardless of whether the bacteria were pretreated with vehicle or OHM. Together, these results demonstrate that OHM supports the host-mediated clearance of *S. aureus* by inhibiting *agr*-mediated virulence.
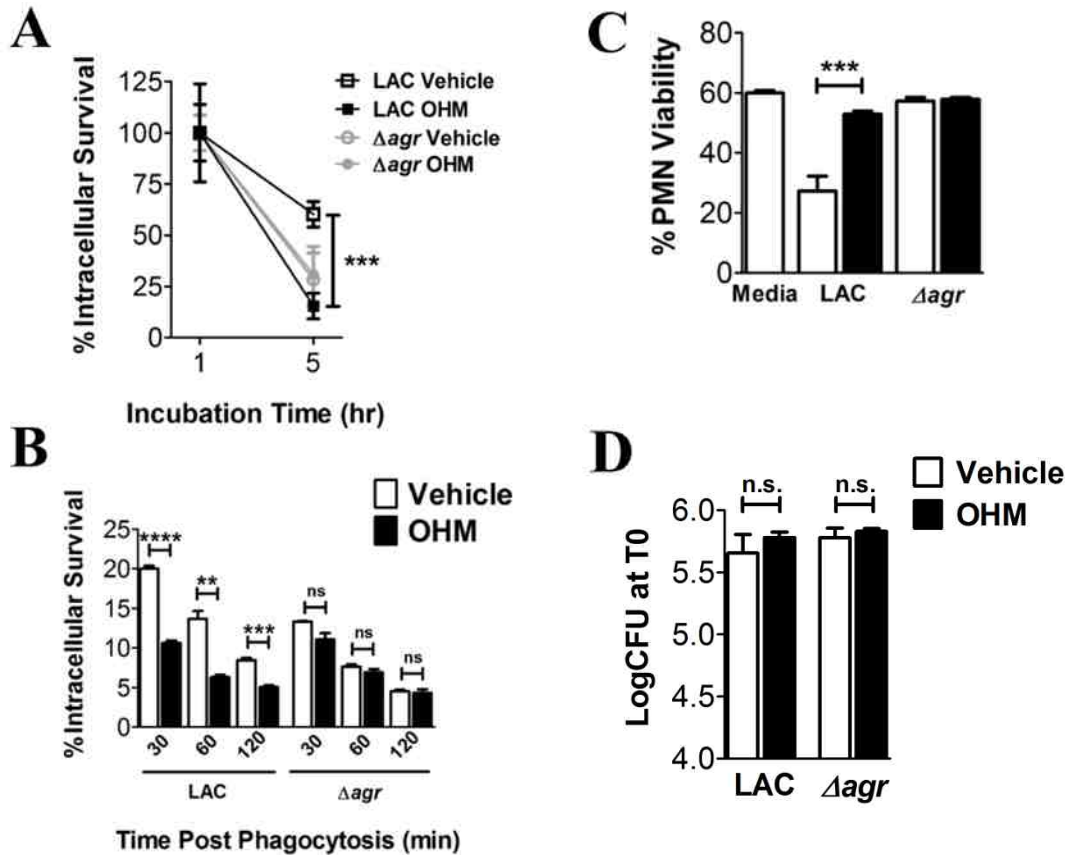
Figure 3.8 - ω-Hydroxyemodin supports immune cell killing of *agr*[+] *S. aureus*. (A) Mouse macrophage (RAW 264.7) intracellular killing of bacteria pretreated with OHM or vehicle control. The data are shown as the mean ± SEM normalized to 100% after 1 h of incubation at an MOI of 1:1. *n* = 6 from two independent experiments performed in triplicate. (B) Human polymorphonuclear leukocyte (PMN) intracellular killing of bacteria pretreated with OHM or vehicle control. The data are the mean ± SEM presented as the percent survival (top) and log CFU reduction (bottom) compared to time zero. (C) Supernatant lysis of human PMNs, assessed by LDH release, after 2 h of incubation with sterile supernatant from overnight cultures grown in the presence of OHM or vehicle control. The data are the mean ± SEM, presented as percent PMN viability compared to 100% lysis by Triton X-100. (B and C) (D) Effect of OHM pre-treatment of *S. aureus* on the ability of PMN to opsonophagocytose bacteria. Data are the mean ± SEM, presented as LogCFU of phagocytosed bacteria at time zero. Experiments were performed in triplicate with PMNs from two separate donors. A representative donor experiment is shown. ns, not significant. **, $P < 0.01$; ***, $P < 0.001$; ****, $P \leq 0.0001$ by Student's *t* test.

ω-Hydroxyemodin limits inflammation mediated by S. aureus QS

S. aureus uses a variety of virulence factors, many of which are regulated by the *agr* system, to evade host clearance mechanisms. These virulence factors cause tissue damage and inflammation and facilitate invasive infection (48, 83, 221–223). Therefore, we postulated that the reduction in bacterial burden in the LAC-infected, OHM-treated mice would be associated with reduced tissue damage and reduced local inflammatory cytokine production compared to the vehicle-treated controls. Histological analysis of day three post infection skin sections confirmed the overall reduction in abscess formation and ulceration in OHM-treated mice (**Figure 3.A**). Additionally, skin sections from the vehicle-treated mice displayed a disorganized architecture at both the epithelium-to-necrosis transition (**Figure 3.A**, left inset) and the abscess periphery (right inset) compared to sections from OHM-treated mice. Also as predicted, OHM treatment resulted in a local cytokine profile matching that of LACΔ*agr*-infected mice on day three post infection (**Figure 3.B**), with significant reductions in interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6, but not the anti-inflammatory cytokine IL-10, compared to the vehicle-treated controls. LAC-infected mice treated with OHM also showed reduced transcription of *il-1β*, *tnfα*, and *il-6* at 24 h post infection compared to the levels in the vehicle-treated mice (**Figure 3.C**). Finally, the activation of the NLRP3 inflammasome and the subsequent release of IL-1β is induced by pore formation in the host cell membranes by Hla (46, 177, 178), and the passive transfer of Hla-neutralizing antibodies is sufficient to limit the secretion of IL-1β (181). Therefore, we predicted that OHM treatment would be associated with reduced Hla expression and *nlrp3* transcription at the site of infection. As expected, the OHM-treated mice showed decreased local Hla

expression and decreased transcription of *nlrp3* compared to the vehicle-treated controls

(**Figure 3.D, E**). Together, these data demonstrate that OHM inhibition of *agr* signaling

limits host tissue damage and inflammation during *S. aureus* SSSI.
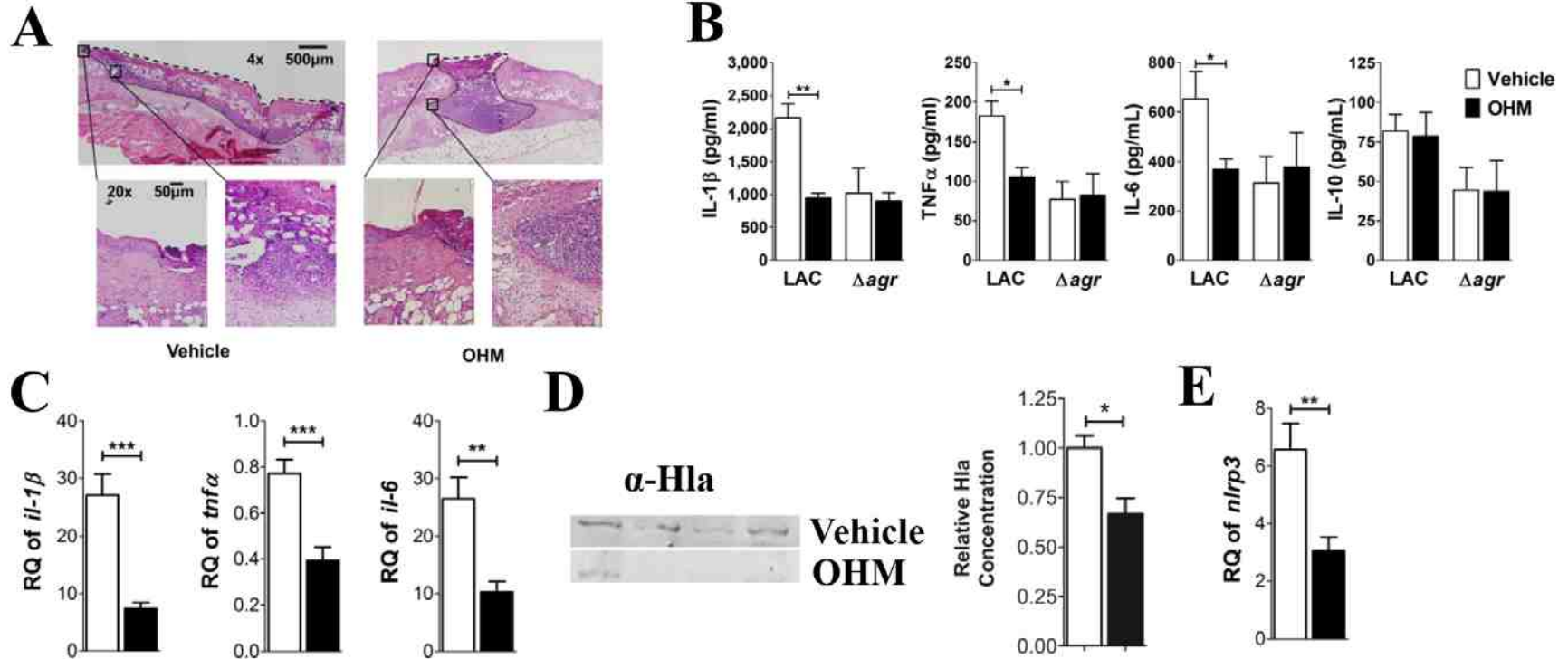
Figure 3.9 - ω-Hydroxyemodin limits pathology and expression of inflammatory cytokines during *S. aureus* SSSI. (A) Top, representative hematoxylin and eosin micrographs of 5-μm sagittal sections of day three post-LAC infection abscess tissue. The abscess area is demarcated by a fine dashed line, while the ulcer surface length is marked by the thick dash line. Bottom, magnification of the transition from normal epithelium to necrotic tissue (left) and organization of the abscess tissue (right). (B) Multiplex analysis of cytokines present in the abscess tissue on day three post infection with LAC or Δ*agr*. (C and E) Quantification of *il-1*β, *tnf*α, *il-6* (C), and *nlrp3* (E) relative to *hprt* in abscess tissue at 24 h post-LAC infection. (D) Western blot analysis and quantification of Hla at the site of infection (day 3) in OHM- versus vehicle-treated mice (*n* = 4 mice/group). The data are the mean ± SEM from infection data, as described in the legend to **Figure 3.**. RQ, relative quantification; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 by Student's *t* test.

Discussion

Recently, the National Institutes of Allergy and Infectious Diseases reported on the current status and future directions for its antibacterial resistance program (9). Two of the strategic approaches highlighted were (i) antivirulence strategies to disarm bacteria to reduce pathogenesis and (ii) approaches to harness the host immune system to better combat infections. Here, we report that OHM, a natural product isolated from the fungus *P. restrictum* (140), addresses both goals by directly inhibiting *S. aureus* QS-dependent virulence while indirectly bolstering the host immune response against *S. aureus* infection. In a mouse model of *S. aureus* SSSI, OHM significantly decreases abscess and ulcer formation and promotes bacterial clearance. Importantly, OHM treatment reduces tissue damage and limits local proinflammatory cytokine production to levels seen in mice infected with the *agr* deletion mutant. Furthermore, OHM treatment enhances immune cell-mediated killing of *S. aureus* in an *agr*-dependent manner. Therefore, these data demonstrate that antivirulence strategies can limit disease by disarming the bacteria while concurrently reducing inflammation and promoting host innate defense. In addition, this is the first polyhydroxyanthraquinone described with *in vivo* efficacy against MRSA infection, adding to the expanse of natural products with the potential to promote human health and advance antibiotic stewardship.

Numerous reviews have addressed the potential role of antivirulence strategies, including the disruption of QS, in combating the antimicrobial resistance crisis (36, 53, 54, 184, 224, 225). We chose to focus on the disruption of *agr* QS as an antivirulence approach to *S. aureus* SSSIs due to their predominance in *S. aureus* disease manifestations, as well as the established contribution of *agr* in facilitating these

infections (3, 12, 13, 44, 56). For example, *agr* deletion mutants (Δ*agr*) are less pathogenic and more readily cleared during SSSIs than are wild-type strains (44, 56), and host innate effectors that disrupt *agr* signaling limit disease in skin infection models (57, 58, 194). Importantly, the sterile supernatant from *agr*$^+$ but not from *agr*-null S. aureus strains is sufficient to cause skin lesions similar to those in an active infection, definitively demonstrating the role of *agr*-regulated secreted virulence factors in skin pathogenesis (131). Here, we used OHM in a prophylactic administration model, similar to that previously reported for the administration of competing AIP or the passive transfer of monoclonal antibodies targeting AIP4 (103, 131), to demonstrate that small molecule-mediated disruption of *agr* signaling *in vivo* results in an "*agr*-null-like" host inflammatory profile.

The disruption of *agr* signaling during SSSIs results in reduced bacterial burden at various time points post infection (55, 56, 131, 168), suggesting that host-mediated bacterial clearance is more effective in the absence of QS. In support of this, we have shown here and elsewhere (55) that *agr* inhibitors enhance some mechanisms of innate immune bacterial clearance against *agr*$^+$ S. *aureus*. The increased bacterial clearance likely results from inhibiting the expression of the *agr*-regulated secretome, which includes virulence factors that target host immune cells. Among these, the phenol-soluble modulins (PSMs) and Hla, in particular, contribute to abscess formation, dermonecrosis, and bacterial burden during SSSIs (117, 166, 168, 226). These toxins target a variety of cell types to suppress both innate and adaptive immunity (227). For example, *S. aureus* utilizes Hla to survive inside neutrophils and macrophages (207, 228–231) and to induce programmed cell death in T cells, B cells, and monocytes (232). In addition, Hla activates

the NLRP3 inflammasome in a variety of cells, resulting in the release of the inflammatory cytokine IL-1β (177–179). Although neutrophils and IL-1β are needed for the ultimate clearance of *S. aureus* SSSI (233, 234), limiting inflammation caused by bacterial toxins clearly benefits the host.

Our molecular modeling studies positioned OHM near R218 of *S. aureus* AgrA. This residue, which is strictly conserved across multiple staphylococcal species (209), is required for *agr* function and contributes to AgrA binding to *agr* promoter DNA (203, 210). Although the potential exists for OHM to drive selection for an alternative amino acid at residue 218, any such mutation would likely result in *agr* dysfunction. The selection for QS-deficient isolates is unlikely to be of significant benefit to the pathogen, as these isolates are severely attenuated, more readily cleared by host defenses, and less effective at initiating infection (56, 168, 202). However, while the residues directly involved in OHM binding have yet to be definitively demonstrated, the question of whether OHM can select for mutation of other residues in the predicted binding site that would prevent OHM function while retaining *agr* activity will require empirical determination.

Along with inhibiting *S. aureus agr* activation, OHM likewise inhibits *agr* signaling by *S. epidermidis*, an important member of the skin microbiome and also an opportunistic pathogen (235). *S. epidermidis agr* regulation drives the mechanisms of resistance to host innate defense (236), suggesting that OHM or related analogues might prove efficacious against *S. epidermidis* infections. However, in its role as a commensal, *S. epidermidis* appears to benefit the host by such means as competing with *S. aureus* for colonization and contributing to overall skin immunity (237–239). Additionally, AIP1

produced by *S. epidermidis* is cross-inhibitory to S. *aureus agr* types I to III *in vitro* (40, 42, 235). Therefore, it is unclear whether OHM-mediated perturbation of *S. epidermidis* QS, during treatment for *S. aureus* SSSI, might result in unwarranted complications, such as increased host-mediated clearance of *S. epidermidis* and/or the loss of potentially beneficial cross-inhibitory AIP. To address these possibilities, it will be important to experimentally determine the *in vivo* implications of disrupting QS in an *S. epidermidis*-colonized host on subsequent *S. aureus* skin infection.

As is the case with existing antimicrobials, QS inhibitors (QSIs) may not be a one-size-fits-all solution. Although SSSIs comprise the vast majority of *S. aureus infections*, this pathogen causes a variety of disease manifestations, including pneumonia, osteomyelitis, endocarditis, and bloodstream infections (BSI). This raises the question of whether the use of QSIs will be universally beneficial. The contribution of *agr* to *S. aureus* pathogenesis has largely been demonstrated in models of SSSIs and pneumonia (44, 56, 103, 131). In contrast, *agr* dysfunction has been associated with persistent bacteremia in hospitalized patients (233, 234), suggesting that QSIs would be best utilized to prevent *S. aureus* invasion prior to BSI. Likewise, the disruption of *agr* is associated with biofilm formation *in vitro* (235), potentially limiting the utility of QSIs for the treatment of infections involving implanted devices, as well as osteomyelitis and endocarditis. Whether OHM and other QSIs would contribute to staphylococcal biofilm formation *in vivo* will require investigation in appropriate animal models of infection. Overall, however, QSIs might be a critical part of the developing arsenal for combating antibiotic resistance either alone, as adjuncts to existing antibiotics, or along with potential vaccines or other approaches to augment host defense.

A substantial portion of the compounds used to fight infections have their origins in natural products (123). Despite clearly representing a wealth of bioactive molecules, natural products have received limited attention with respect to identifying specific inhibitors of *S. aureus* QS that are not bactericidal. To date, other natural products identified as *agr* inhibitors include (i) α-cyperone from the nut grass plant *Cyperus rotundus* (146), (ii) the fungal metabolite ambuic acid (137), and extracts from (iii) the goldenseal plant *Hydrastis canadensis* (148) and (iv) three Italian medicinal plants, *Ballota nigra*, *Castanea sativa*, and *Sambucus ebulus* (147). Among these, OHM is the first to demonstrate *in vivo* efficacy against *S. aureus* QS. Therefore, we predict that targeted testing of structurally diverse natural products will continue to reveal a broad range of antivirulence molecules with the potential to support innate host defense mechanisms and to positively contribute to antibiotic stewardship.

CHAPTER 4: Conclusions

Staphylococcus aureus is a global health threat causing significant morbidity and mortality (14, 15). The exceptional capacity of this pathogen to acquire resistance and the dearth of novel antibacterial therapies has led to the search for alternative treatment strategies. Anti-virulence strategies focused on bacterial disarmament are one such alternative strategy. *S. aureus* regulates over 200 virulence factors with the *accessory gene regulator* operon that encodes a density-dependent quorum sensing system (44). Many of the *agr*-regulated virulence factors are important for invasive infection, and this is further evidenced by the significantly reduced pathogenesis of *agr* deletion mutants in certain infection models (56, 240, 241). Therefore, targeting the *agr* operon represents an attractive approach for prophylaxis or therapeutic intervention.

The work described herein, and summarized in **Figure 4.**, significantly advances the field of anti-virulence strategies to combat *S. aureus* as follows. First, in **Chapter 2**, we report the development and demonstrate the efficacy of the first active vaccine targeting a secreted AIP of the *agr* system. The small size and lability of the AIP molecule has previously proven challenging for active vaccination. The use of stable peptide mimotopes displayed with high valency on a virus-like particle overcomes the AIP shortcomings and proved successful in limiting *agr*-dependent pathogenesis *in vivo*, a significant advance in the field. Second, in **Chapter 3**, we demonstrate that a novel natural product inhibitor of AgrA, ω-hydroxyemodin, leads to bacterial disarmament which limits *S. aureus* pathogenesis and supports host innate defense resulting in increased bacterial clearance. While several natural products *agr* quorum sensing inhibitors have been described, specific mechanism of action studies have not been

described and many of the inhibitors are innately antibacterial, muddling the interpretation of *agr* inhibition. This work is significant for the field of natural products because: (i) it identifies the *agr* target of ω-hydroxyemodin, the response regulator AgrA; (ii) ω-hydroxyemodin has no antibacterial activity and is nontoxic to eukaryotic cells; (iii) prophylaxis was proven efficacious in an animal model of SSSI; and (iv) inhibition of *agr* resulted in a bolstering of the immune response to infection.

The discussion below will address criticisms of the approaches described, experimental approaches to further the two intervention strategies, and finally a discussion on potential clinical utility of quorum sensing inhibitors. Specifically, we will address the suitability of targeting the *agr* operon in terms of resistance development, biofilm enhancement, and the role of *agr* in *S. aureus* virulence regulation. Following that will be a discussion on methodologies to enhance both the mimotope VLP vaccine and the natural product ω-hydroxyemodin. The discussion will conclude with a description of the clinical utility of quorum sensing inhibitors, including populations who would benefit from treatment, potential for *agr* inhibitors in other *S. aureus* diseases, and implications for quorum sensing inhibition for multiple pathogens.
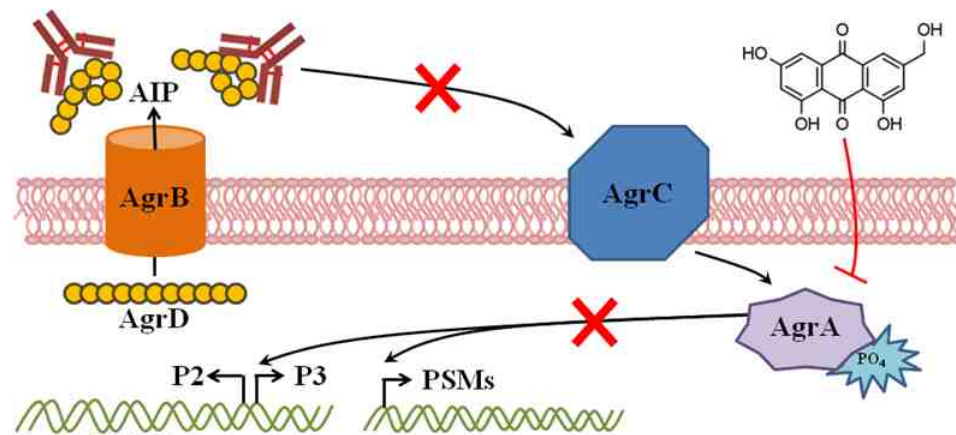
Figure 4.1 - Inhibition of the *S. aureus accessory gene regulator* quorum sensing via vaccination or small molecule inhibition. The *agr* operon is depicted with the inhibitors described in **Chapters 2** and **3** and their protective effects *in vivo*. Mimotope generated antibodies bind the AIP signal preventing activation of AgrC and subsequent *agr* activation. ω-Hydroxyemodin binds the response regulator AgrA preventing interaction and therefore transcription of *agr* promoters. Inhibition via either method results in reduction of disease severity and inflammation *in vivo*. ω-Hydroxyemodin was also specifically shown to enhance immune function in an *agr*-dependent manner.

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Suitability of targeting a*gr* quorum sensing

Although the work reported here makes significant advances in addressing the feasibility
and efficacy of anti-virulence strategies, several questions remain unanswered. There are
three key questions regarding *agr* inhibition as a treatment modality: (i) will resistance to
agr inhibitors develop, (ii) will inhibition of *agr*, which prevents invasion, enhance
biofilm formation and (iii) what is the role of *agr,* as compared to other *S. aureus*
virulence regulators, in virulence regulation during different disease states?

One of the key hypotheses to inhibition of virulence is that because inhibition is
not directly antibacterial, resistance development should be minimal, if existent.
Resistance development is less likely for antibody approaches because the target, AIP
(AgrD), would have to mutate to prevent antibody sequestration. Mutation in AIP would
require concomitant changes in AgrB and AgrC, which makes resistance development
less likely. Resistance to ω-hydroxyemodin is more theoretically possible as it targets a
single protein, suggesting that mutations in AgrA that prevent ω-hydroxyemodin binding
could occur which do not affect the DNA-binding functionality of the protein. Recent
work in the Gram negative organism *Pseudomonas aeruginosa* suggests that QS
resistance may be possible (242–245), but whether these findings will apply to Gram
positive organisms remains to be determined. In contrast to the findings in *P. aeruginosa*,
the study reported by Sully *et al.* attempted to develop resistance in *S. aureus* to a small
molecule QS inhibitor by repeated *in vivo* passage of bacteria with the inhibitor (55). At
the conclusion of the experiment, bacteria passaged with the QS inhibitor were still
susceptible to QS inhibition, but bacteria passaged with an antibiotic control developed
resistance to the antibiotic over the same course of passage (55). In addition to mutations

resulting in resistance to therapeutics, mutations resulting in *agr* inactivation would also render antibodies and small molecules ineffective. Single inactivating mutations in AgrA and AgrC exist in colonizing isolates and clinical isolates from bloodstream infections (210, 246, 247). However, multiple mutations in *agr* are not found, suggesting evolutionary selection for *agr* functionality exists and that these mutation are likely only adaptive for survival in the current host (210). Additionally, transmission occurs only infrequently between immunocompromised hosts, where *agr* may not be necessary, and not stably into general circulation (210). Therefore it is unlikely that treatment induced inactivation of *agr* will lead to dispersal of *agr*-negative strains.

One valid concern of *agr* inhibition is that while inhibition reduces virulence factors resulting in invasive infection, it may favor the formation of biofilms due to the upregulation of adhesive factor expression (38). This is a major concern where biofilm formation is associated with disease, such as ventilator-associated pneumonia, endocarditis, osteomyelitis and implanted medical devices. The role of *agr* in biofilm formation, maturation and dispersal is multifactorial (38, 248, 249). The *agr*-regulated phenol-soluble modulins and many proteases are involved in biofilm formation, maturation, and most importantly dispersal (38, 248–250). Therefore, although *agr* inhibition may in fact increase biofilm formation, the altered nature of the biofilm and the prevention of dissemination may mitigate the concern (250, 251). However, many questions about *agr* inhibition and the effect on biofilm formation have not been addressed experimentally, especially *in vivo*. Until these questions are answered, *agr* inhibitors would be clinically useful where biofilms are of less concern. For example, prophylactically following surgery or trauma, or during an active skin infection,

enhancing adherence while preventing dissemination to the blood and subsequent metastasis may be clinically acceptable. This is especially possible because incision and debridement are current best practices and would help in biofilm clearance (16). Additionally, because *agr* inhibition does not enhance MSSA biofilms (248), inhibition of QS in MSSA strains, which cause almost half of the Staphylococcal SSSI presenting to emergency departments (13), has significant clinical utility.

Another concern is that while inhibition of *agr* could have a significant clinical impact, it is not the only regulatory pathway of importance in *S. aureus*. The two-component signaling *saePQRS* operon also has important roles in toxin and virulence factor production (56, 252). Additionally, *sarA* regulates virulence via *agr*-dependent and *agr*-independent mechanisms (253, 254). These additional systems possess DNA-binding transcription factors (SaeR and SarA) which could be targeted via small molecule inhibition in a similar manner to AgrA. A cocktail of inhibitors could be more efficacious than a single inhibitor as evidenced by the further reduction of virulence in a double *agr* and *sarA* or *saeRS* mutants (56, 255).

Future considerations for mimotope VLPs

One major limitation of mimotope selection, is that it requires an antibody for panning of mimotopes. Therefore, this screening technique is limited to antigens for which antibodies, particularly monoclonals, exist. Despite this requirement, VLPs possess many intrinsic qualities that make them well suited for vaccine development (reviewed in (156)). VLP-based vaccines are already FDA approved for the prevention of viral infection. Due to the nature of the self-assembling repeating capsid, antigens are

presented in a dense, repetitive array suitable for B-cell activation (156). Display of antigens can be accomplished either through genetic insertion in surface exposed loops or by chemical conjugation to exposed amino acids amenable to available chemistries (156). The size of VLPs allow them to be efficiently phagocytosed by dendritic cells resulting in presentation in both MHCI and MHCII contexts (156). VLPs are also relatively easy to produce and are stable over a range of conditions (156). The excellent properties afforded by VLP vaccination suggest that generation of mAb to novel antigens is a necessary, although not convenient, first step for mimotope identification. This is especially true for poorly immunogenic antigens, such as AIPs, that can generate highly-specific and protective antibodies if additional steps are taken.

Another limitation is the *agr* type specificity of the vaccine. A prophylactic vaccine would need to cover all four *agr* types to be of clinical utility. All of VLP-based FDA approved human papillomavirus vaccines are multivalent, suggesting that if mimotopes could be identified for all four AIPs they could be coadministered as a vaccine. Additionally, the ability of VLPs to self-assemble presents an enticing hypothesis for the formulation of an *S. aureus* vaccine. Another possible benefit of VLPs could be whether the multivalent vaccines can be assembled with $T_H17/IL-17$ adjuvants or additional vaccine antigens inside of the capsid, to drive cell-mediated responses or intracellular delivery of antigen, respectively (256).

Future considerations for ω-hydroxyemodin

Pharmacokinetics (PK) is the adsorbtion, distribution, metabolism, and excretion (ADME) of a drug upon administration and it is important to understand for route of

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administration, dosing regimen, and toxicity. ω-Hydroxyemodin is a novel QS inhibitor, and as such the PK has never been assessed and, additionally, understanding the PK of a drug prior to determining its efficacy *in vivo* is of little use. In order to avoid common PK pitfalls, ω-hydroxyemodin was coadministered subcutaneously with bacteria where it was highly efficacious (**Chapter 3**). However, now that efficacy has been established elucidating the PK parameters is essential. Although there are no PK studies for ω-hydroxyemodin, inferences from the numerous studies on the parent compound emodin, an herbal laxative, can inform strategies to enhance ω-hydroxyemodin PK. Importantly, the major metabolite of emodin is ω-hydroxyemodin (**Figure 4.2A**), so these studies have critical relevance (257, 258).

The first PK hurdle for ω-hydroxyemodin, gleaned from research with emodin, is the very poor bioavailability due to rapid glucuronidation and excretion (257, 259, 260). According to the emodin research, glucuronidation would occur on the oxygen of the 3-hydroxy of ω-hydroxyemodin (**Figure 4.2B**). Changing the hydroxyl moiety so that glucuronidation cannot occur would enhance bioavailibity. Bioisosteres, are chemical moieties that share physical and/or chemical properties (261). Bioisosteres of the 3-OH (*e.g.*: -Cl, -F or -CF$_3$, **Figure 4.2B[1]**), could be assessed to identify molecules that are still QS inhibitory but can no longer be glucuronidated (261).

The second PK issue to address is metabolic conversion of ω-hydroxyemodin to genotoxic metabolites. Cytochrome P450 (CYP) oxidation generates several hydroxylated metabolites of emodin, but 2-hydroxyemodin (**Figure 4.2C**) was shown to be the direct-acting mutagen (257, 258). Additionally, CYP oxidation of emodin or ω-hydroxyemodin was required for genotoxicity in *S. typhimurium* (258, 262, 263). The

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widespread use of emodin as a laxative, coupled with the potential genotoxicity, prompted the National Toxicology Program (NTP) to assess emodin for toxicology and carcinogenesis. There was no evidence of carcinogenic activity in a 2-year feed study for male rats and female mice, and only equivocal evidence in female rats and male mice exposed to up to ~200 mg/kg (264). Therefore, although toxic metabolites can be generated *in vivo*, their effects may not be relevant at the doses required for *agr* inhibition *in vivo* (~0.2 mg/kg) based on the NTP studies demonstrating only equivocal evidence at ~200 mg/kg. However, to mitigate the potential for toxic metabolite formation, chlorination at position 2 of ω-hydroxyemodin should protect from CYP hydroxylation at position 2 (**Figure 4.2C[1]**). Since 2-chlorinated ω-hydroxyemodin is available commercially, it would be interesting to assess the effect of 2-chlorination on both QS inhibition and genotoxicity.

Addressing these two key PK complications would greatly enhance the potential for ω-hydroxyemodin to progress towards the clinic. Full PK studies addressing all of the ADME properties for ω-hydroxyemodin specifically would also identify further PK hurdles. Additionally, even if some of the PK complications cannot be overcome, ω-hydroxyemodin may still be useful as a topical or locally administered therapeutic.
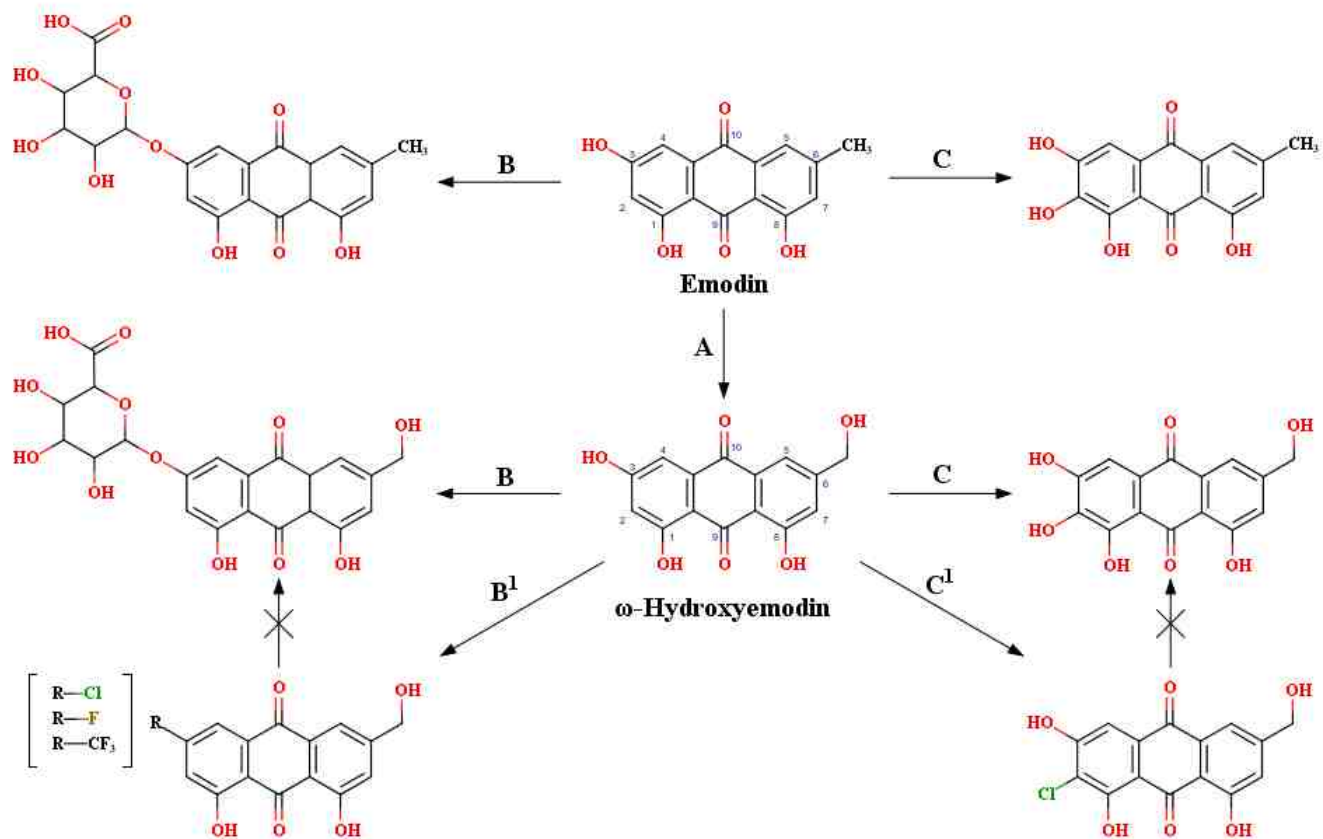
Figure 4.2 – Metabolism pathways of emodin and ω-hydroxyemodin based on studies of emodin. (A) The major metabolite of emodin is ω-hydroxyemodin via CYP hydroxylation of the 6-methyl. (B) Glucuronidation at 3-hydroxy of ω-hydroxyemodin, which results in low bioavailibility. (B[1]) Protection of the 3-hydroxy of ω-hydroxyemodin with chlorine, fluorine or trifluormethyl might prevent glucuronidation. (C) Hydroxylation of ω-hydroxyemodin resulting in the genotoxic 2-hydroxy metabolite. (C[1]) Protection of the 2-carbon of ω-hydroxyemodin with chlorine might prevent hydroxylation and formation of genotoxic metabolite.

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Pharmacodynamics (PD) is the study of drug action or mechanism and is an important consideration for reducing the side-effects of pharmaceuticals. **Chapter 3** demonstrates that ω-hydroxyemodin binds AgrA and prevents activation of *agr* QS. However, there are reports in the literature of emodin and ω-hydroxyemodin (also known as citreorosein) having effects on eukaryotic targets (265–267). Specifically, ω-hydroxyemodin and emodin inhibit inflammation induced via lipopolysaccharide (267) or phorbal 12-myristate 13-acetate activation of mast cells (265, 266). Whether this effect is specific for mast cells or is more generalizable remains to be seen. In **Chapter 3** ω-hydroxyemodin only reduced inflammation to levels seen with *agr* deletion strains. One hypothesis based on these observations is that ω-hydroxyemodin may reduce virulence induced inflammation and this may be a beneficial effect. However, to mitigate potential off-target effects, combinatorial chemistry may be employed to enhance binding to AgrA while limiting eukaryotic effects. In addition to enhancing the PD as described, combinatorial chemistry products could also be assessed for enhancements to the PK.

The power of combinatorial chemistry to improve upon natural product pharmacophores has been extensively demonstrated historically (122, 123, 125). However, identification of the target binding site greatly enhances the power of combinatorial chemistry (122). In **Chapter 3** we demonstrated that ω-hydroxyemodin binds to the C-terminal, DNA-binding fragment of AgrA potentially near the DNA binding site (**Figure 3.5B**). Further refinement of the binding site would greatly enhance combinatorial chemistry approaches. The identification of the binding site via mutagenesis will most likely be difficult. Mutations near the proposed binding site which would prevent ω-hydroxyemodin binding (implicating residues important for binding)

could alter DNA-binding activity. This would require the more challenging SPR technique, in contrast to the facile EMSA, for demonstration of binding. However, the substantial benefit of binding site identification requires that these studies be attempted.

ω-Hydroxyemodin inhibited *S. epidermidis* quorum sensing, and because AgrA is highly conserved amongst staphylococcal species, we hypothesize that ω-hydroxyemodin might also inhibit QS in other species. As most of the staphylococci can cause human disease, demonstrating ω-hydroxyemodin QS inhibition in other species would broaden the clinical utility of ω-hydroxyemodin. Additionally, *agr* belongs to the LytTR family of response regulators, and these regulators are utilized by many pathogens (203). It would be interesting to determine if ω-hydroxyemodin can inhibit other members of the LytTR family.

Clinical utility of QS inhibition

To date, there have been no clinical trials or FDA approved QS inhibitors, although the data reported herein suggest their potential clinical utility. Specifically, virulence factors regulated by the *agr* quorum sensing circuit are important for tissue invasion leading to hematogenous dissemination (14, 15). Inhibitors of *agr* would therefore be useful to prevent severe infection by preventing metastasis from primary to distal infection sites. Specifically, inhibitors of QS would be efficacious as treatments to limit skin infections. Active vaccination would benefit patient populations at risk of developing skin infections, such as patients undergoing elective surgery, patients on hemodialysis, diabetics, and other populations at high risk for infection (prisoners, military personnel, and athletes). Likewise, prophylactic administration of ω-

hydroxyemodin would benefit these same patients, but could also be used following trauma when patients are more susceptible to infection because innate effectors that inhibit quorum sensing are depleted (268). Additionally, *agr* activation occurs more than once during infection (131), suggesting that post-infection therapeutic administration of ω-hydroxyemodin may also be effective. Developing a biomarker to determine *agr* activation status to predict the effectiveness of QS inhibitors would be a substantial benefit in this regard. Additionally, for therapeutic use, although QS inhibitors are designed not to have antibacterial activity and therefore would not prevent infection, their ability to enhance immune-mediated bacterial clearance would promote resolution.

The anti-pathogenesis activity of QS inhibitors that leads to enhanced immune function may even work as an adjunct to antibiotic therapy. Therapeutic treatment with ω-hydroxyemodin and antibiotics may be an additive or synergistic combination due to the immune enhancing effects on bacterial clearance coupled with the direct antibacterial effects of the antibiotics. This may be specifically important for the β-lactams as the *mecA* and *mecR1* genes conferring resistance is positively *agr*-regulated in some strains (44, 47). In fact, synergy was demonstrated between emodin and two β-lactams, although emodin was attributed antibacterial activity in that study which makes interpretation of *agr* inhibitory effects difficult (269). In addition, it is increasingly being recognized that *S. aureus* may persist intracellularly in multiple cell types and that *agr* QS is important for phagosomal survival and escape due to virulence factor production (231, 270, 271). Because AgrA is cytosolic, ω-hydroxyemodin must be able to cross bacterial membranes. An interesting question is, therefore, can ω-hydroxyemodin cross eukaryotic membranes and prevent intracellular QS? This may be one of the mechanisms by which ω-

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hydroxyemodin enhances macrophage and neutrophil killing of *S. aureus*, although this needs to be empirically determined. If proven correct, it would suggest that *agr* inhibition aids infection resolution and prevents persistent intracellular survival. Finally, a cocktail of specific *S. aureus* virulence inhibitors could also be thought of as a 'narrow-spectrum' strategy and one could also imagine the use of a 'broad-spectrum' cocktail against QS in multiple pathogens. A broad-spectrum cocktail should target the antibiotic resistant ESKAPE pathogens as quorum sensing is important in many of these pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanii*, *P. aeruginosa*, and *Enterobacter* species). The 'broad-spectrum' therapy could be administered empirically until the causative pathogen is known, and then narrow-spectrum, specific therapy continued thereafter, as is currently done with antibiotics.

Demonstrating clinical uses beyond SSSI would greatly enrich the clinical impact of *agr* inhibitors. However, the contribution of *agr* to other *S. aureus* disease states is not well understood, so predictions of QS inhibitor effectiveness are not well guided. However, there is evidence to suggest that *agr* is important for infection in pneumonia (185), so assessing the benefit of QS inhibition in this model would be worthwhile. In addition to other *S. aureus* disease states it would be interesting to determine the effectiveness of QS inhibition in immunodeficiency. We hypothesize that QS inhibition might compensate for immunodeficiencies. Animal models of several immunodeficiencies are established which would allow testing of this hypothesis. It will be important to determine under which immunodeficiencies *agr* inhibition can prove efficacious in limiting infection. Finally, others have shown that neutralization of Hla reduces the severity of recurrent infection (226). We predict that because ω-

hydroxyemodin limits Hla production *in vivo*, that adaptive responses will be enhanced, thus decreasing the severity of subsequent infection.

Inhibition of QS alone is not going to solve the antibiotic resistance crisis, but it is another tool that can be used to fight resistance. Additionally, multiple pathogens utilize quorum sensing suggesting inhibitors, or cocktails, would be beneficial. The work in **Chapter 2** and **Chapter 3** demonstrate that inhibition of *S. aureus* QS using an active vaccine or a natural product small molecule limits the severity of infection *in vivo*. Several improvements can be made to either approach, enhancing their ability to move towards clinical use. Finally, understanding the role of *agr* in other disease states would broaden the clinical impact of *agr* inhibitors.

APPENDIX A: Abbreviations

Ab – antibody

ABC Transporter – ATP-binding cassette transporter

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

*agr*I-IV – *accessory gene regulator* type I-IV

ADAM10 – A disintegrin and metalloproteinase domain-containing protein 10

ADME – adsorbtion, distribution, metabolism, and excretion

AI – autoinducer

AIP1-4 – autoinducing peptide 1-4

Als3 – agglutinin-like sequence 3 protein

BSA – bovine serum albumin

BSI – blood stream infection

Cam – chloramphenicol

CAMP – Christie-Atkins-Munch-Peterson factor

CA-MRSA – community associated methicillin-resistant *Staphylococcus aureus*

Can – collagen binding protein

CDC – Centers for Disease Control and Prevention

cDNA – complementary DNA

CFU – colony forming unit

ClfA, B – clumping factor A, B

Coa - coagulase

CP5, 8 – capsule type 5, 8

Csa1A – conserved staphylococcal antigen 1A

CYP – cytochrome P450

DNA – deoxyribonucleic acid

DMEM – Dulbecco's modified Eagle's medium

DMSO – dimethyl sulfoxide

DT – diphtheria toxin

DTaP – diphtheria, tetanus and pertussis vaccine

DTT – dithiothreitol

Eap – extracellular adherence protein

E. coli – Escherichia coli

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

EMSA – electrophoretic mobility shift assay

EpiP – epidermin leader peptide processing serine protease

Erm – erythromycin

EsxA, B – ess extracellular A, B

ESKAPE – *E. faecium*, *S. aureus*, *K. pneumonia*, *A. baumanii*, *P. aeruginosa*, and

Enterobacter species

exoA – exotoxin A

Fab – fragment antigen-binding

FAM – fluorescein amidite

FBS – fetal bovine serum

FDA – Food and Drug Administration

FhuD3 – ferrix hydroxamate-binding lipoprotein

FnBPA, B – fibronection binding protein A, B

GFP – green fluorescent protein

Glc – PNAG fragment

Gt - goat

HA-MRSA – healthcare associated methicillin-resistant *Staphylococcus aureus*

HBSS – Hanks' balanced salt solution

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H&E – hematoxylin and eosin stain

HiB – *Haemophilus influenza* B

Hla – alpha-hemolysin, also α-hemolysin

Hlb – beta-hemolysin, also β-hemolysin

Hld – delta-hemolysin, also δ-hemolysin

HPMC – hydroxypropylmethylcellulose

HRP – horseradish peroxidase

HSA – human serum albumin

HSL – homoserine lactone

IACUC – Institutional Animal Care and Use Committee

IgG – immunoglobulin G, also immunoglobulin gamma

IPTG – isopropyl-β-D-1-thiogalactopyranoside

IsdA, B – iron-regulated surface determinant A, B

KC – keratinocyte-derived chemokine

LB – Luria broth

LDH – lactate dehydrogenase

LOS – length of stay

LTA – lipotechoic acid

LukF-PV, LukS-PV – Panton-Valentine leukocidin subunit F, S

LYST – lysosomal trafficking regulator

mAb – monoclonal antibody

MHCI, II – major histocompatibility complex I, II

MIC – minimum inhibitory concentration

MCF – mean channel fluorescence

MntC – manganese transport protein C

MOI – multiplicity of infection

mRNA – messenger ribonucleic acid

MRSA – methicillin-resistant *Staphylococcus aureus*

Ms - mouse

MSSA – methicillin-sensitive *Staphylococcus aureus*

MWCO – molecular weight cutoff

NADPH – nicotinamide adenine dinucleotide phosphate

NBT/BCIP - nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate

NIAID – National Institute of Allergy and Infectious Diseases

NIH – National Institute of Health

NLRP3 - NOD-like receptor family, pyrin domain containing 3

NTA – nitrilotriacetic acid

NTP – National Toxicology Program

OD – optical density

OHM – ω-hydroxyemodin, also omega-hydroxyemodin and citreorosein

PAGE – polyacrylamide gel electrophoresis

pAb – polyclonal antibody

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PD – pharmacodynamics

PK – pharmacokinetics

PMN – polymorphonuclear leukocyte

PMS – phenazine methosulfate

PNAG – poly-N-acetyl glucosamine

P. restrictum – Penicillium restrictum

PSM – phenol soluble modulin

qPCR – quantitative polymerase chain reaction

QS – quorum sensing

QSI – quorum sensing inhibitors

RAP – RNAIII activating protein

Rb – rabbit

Rh - rhesus

ROS – reactive oxygen species

Rot – repressor of toxins (transcription factor)

rRBC – rabbit red blood cell

RNA – ribonucleic acid

RPMI - Roswell Park Memorial Institute medium

rt - rat

RT – room temperature

RT-PCR – reverse transcriptase polymerase chain reaction

SA - streptavidin

S. aureus – Staphylococcus aureus

SDS – sodium dodecyl sulfate

SdrD, E, G – serine-aspartate repeat D, E, G

SEB, SEC – staphylococcal enterotoxin B, C

SEl-X – staphylococcal enterotoxin-like X

sGFP – superfolder green fluorescent protein

SpA – Staphylococcus protein A

SPR – surface plasmon resonance

SSSI – skin and skin structure infection

STAT3 – signal transducer and activator of transcription 3

Std. Rx. – standard treatment

TAE – tris-acetate-EDTA buffer

TBST – tris-buffered saline

TCEP – tris(2-carboxyethyl)phosphine

TLR2 – toll-like receptor 2

tRNA – transfer ribonucleic acid

TSB – trypticase soy broth

TSST-1 – toxic shock syndrome toxin-1

TT – tetanus toxin

UPLC – ultrahigh-performance liquid chromatography

USP – United States Pharmacopeia

USUHS – Uniformed Services University of Health Sciences

VISA – vancomycin-intermediate *S. aureus*

VLP – virus-like particle

VRSA – vancomycin-resistant *S. aureus*

vWbp – con Willebrand factor binding protein

WT – wild type

XTT – 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

YFP – yellow fluorescent protein

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