Attenuating *Staphylococcus aureus* Virulence Gene Regulation: A Medicinal Chemistry Perspective

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**ABSTRACT:** Virulence gene expression in *Staphylococcus aureus* is tightly regulated by intricate networks of transcriptional regulators and two-component signal transduction systems. There is now an emerging body of evidence to suggest that the blockade of *S. aureus* virulence gene expression significantly attenuates infection in experimental models. In this Perspective, we will provide insights into medicinal chemistry strategies for the development of chemical reagents that have the capacity to inhibit staphylococcal virulence expression. These reagents can be broadly grouped into four categories: (1) competitive inhibitors of the accessory gene regulator (agr) quorum sensing system, (2) inhibitors of AgrA–DNA interactions, (3) RNAIII transcription inhibitors, and (4) inhibitors of the SarA family of transcriptional regulators. We discuss the potential of specific examples of antivirulence agents for the management and treatment of staphylococcal infections.

1. **INTRODUCTION**

The term “antibiotic agent” was initially coined by Waksman as a chemical substance “which has the capacity to inhibit the growth of and even to destroy bacteria and other microorganisms.” To this day antibiotic drug development has essentially held firmly to this definition, focusing on molecules that elicit bacteriostatic or bactericidal effects. The mode of action of such conventional antibiotics largely relies on targeting essential cellular functions, such as DNA replication and protein and cell wall biosynthesis. While the success of these approaches is unquestionable, a significant limitation remains; i.e., conventional antibiotics impose immense selective pressure on bacteria. This coupled with the chronic misuse and overuse of our most potent antibiotics in modern medical and agricultural practices has fueled the rise of multiantibiotic resistant bacteria. These include methicillin resistant *Staphylococcus aureus* (MRSA) which constitutes a major, global public health threat. Although MRSA infections have predominantly been associated with health care settings (HA-MRSA), invasive community-acquired MRSA (CA-MRSA) strains have recently emerged that infect previously healthy individuals.4,5

Clinically significant antibiotic resistance has evolved against virtually every antibiotic deployed, while the development of new antibiotic classes has lagged far behind the requirement for new drugs.6 Many major pharmaceutical companies have withdrawn from the antibiotic discovery field mainly because of the huge economic cost of developing drugs that are likely to become rapidly obsolete through resistance. Consequently, there is an urgent need to identify novel antibacterial targets and develop new agents effective against multiresistant strains that do not rapidly succumb to resistance.

In simplistic terms, bacteria can be separated into two classes, pathogenic and nonpathogenic. Nonpathogenic bacteria derive carbon and energy from the environment, either as free-living or as host-associated commensals or symbionts. In contrast, pathogenic bacteria, at least transiently, may derive their carbon and energy parasitically or destructively from a host organism.7 This is accomplished through the production of diverse virulence factors that protect the pathogen from host defenses while facilitating the colonization and subsequent destruction of host cells and tissues liberating nutrients which sustain pathogen growth. In essence, virulence factors are responsible for the classical and potentially lethal symptoms of infection, such as abscesses, inflammation, and sepsis.7 This raises the question of whether virulence can be attenuated and an infection resolved if the production or action of one or more virulence factors is inhibited.8

Indeed, there is a growing body of evidence indicating that inhibiting virulence factor production can significantly attenuate infection, and thus, developing therapies to “disarm” bacteria is a promising approach to combating infection.9−13 Such an approach has a number of perceived benefits over conventional antibacterial strategies and would create an in vivo scenario that is similar to vaccination, in which the bacteria are eventually cleared by the host’s innate defenses with little to no likely impact on the normal human microbiota.14 Furthermore, in contrast to conventional antibiotic strategies inhibition of virulence factor action/production would attenuate infection via nonbactericidal pathways, and given that most virulence factors are not essential for bacterial viability, in principle, the
blockade of virulence may exert less selective pressure for the generation of resistance. However, there have been recent examples, in laboratory settings, in which xenobiotic/chemical modulated virulence attenuation could be overcome. The lifestyle of pathogenic bacteria revolves around (i) locating a host, (ii) finding a colonization niche, (iii) initiating and establishing an infection, and (iv) dispersal to a new host. For a pathogen to progress from one stage to the next, changes in the sensory input that signal environmental change must be perceived and acted upon, e.g., by the induction of new gene expression. Such changes may result from movement from one environment to another, be due to the actions of bacteria within a given environment, or be a consequence of host responses to bacterial activity. Thus, from a prokaryotic perspective, the successful interaction of bacterial cells with mammalian host tissues depends on a coordinated response to environmental cues, such as nutrient availability, temperature, pH, and bacterial cell population density. It is becoming evident that inhibiting virulence gene expression and thus the ability of bacteria to adapt to the host environment offers considerable potential for attenuating infection.

In this Perspective, we will focus on virulence gene expression in *S. aureus* as an antibacterial target. This Gram-positive pathogen is capable of causing a diverse array of both minor and life threatening, acute, and chronic infections, including boils, pneumonia, toxemia, meningitis, endocarditis, and osteomyelitis. Virulence in *S. aureus* depends on a diverse range of cell-surface associated and secreted exoproducts. The former exoproduct includes fibronectin-, fibrinogen-, and immunoglobulin-cell wall binding proteins and capsular polysaccharides. Among the *S. aureus* secreted exotoxins are α-hemolysin, multiple enterotoxins and toxic shock syndrome toxin-1 (TSST-1), Panton–Valentine leukocidin (PVL) and the phenol soluble modulins, and multiple secreted tissue-damaging exoenzymes. While cell wall proteins are involved in promoting adherence to host tissues and aiding immune evasion, *S. aureus* exotoxins cause tissue damage and many function as superantigens promoting the onset of shock-like syndromes. Staphylococci also readily establish biofilms on host tissues and implanted medical devices, causing chronic infections that exhibit remarkable resistance to conventional antimicrobials. Collectively, these staphylococcal virulence determinants enable the organism to evade host defenses, adhere to cells and the tissue matrix, spread within the host, and derive energy by catabolizing host cells and tissue components.

Figure 1. Schematic of the (a) *agr* system (blue) and (b) the SarA protein family (orange). (a) The *agr* locus is composed of two divergent transcripts called RNAII and RNAIII, driven by the P2 and P3 promoters, respectively. The RNAII transcript is an operon of four genes, *agrBDCA*, which encodes the core machinery of the system. AgrD, the peptide precursor to the autoinducer peptide (AIP), is processed and exported through AgrB and possibly SpSB action at the cytoplasmic membrane. AgrB catalytically functions as a cysteine endopeptidase to afford an AgrB–AgrD acyl-enzyme intermediate, which undergoes intramolecular trans-thioesterification resulting in the release/regeneration of AgrB. At the threshold concentration, AIP binds to the AgrC receptor, a membrane-bound histidine kinase. AIP binding activates the AgrC kinase, resulting in phosphorylation of the AgrA response regulator and activation of the P2 and P3 promoters. (b) The SarA protein family encompasses at least 10 transcriptional regulators that initiate intricate molecular cascades that up- or down-regulate the expression of numerous virulence systems. Activation or repression ultimately affects RNAIII expression which is considered to be the effector molecule of *S. aureus* virulence. Current strategies to suppress RNAIII expression can be grouped into four categories: (1) competitive inhibitors of AgrC, the *agr* histidine kinase receptor, (2) inhibition of AgrA–P2/P3 interactions, (3) RNAIII transcription inhibitors (precise mechanisms undetermined), and (4) transcriptional regulator inhibition.
Energetically, staphylococcal virulence factor expression is expensive and is thus tightly regulated by intricate gene regulatory networks incorporating transcriptional regulators and two-component signal transduction systems (TCSTS).21−33 Studies of S. aureus strains carrying mutations in key regulatory elements have provided significant proof-of-principle that their loss can significantly reduce virulence. For example, strains carrying perturbed or nonfunctional TCSTS, such as saeRS, srrAB, and agr mutants, display reduced virulence in mouse models of skin infection and necrotizing pneumonia.34−37 Likewise, S. aureus strains carrying mutations within transcriptional regulator genes, such as sarA, display a reduced ability to induce septic arthritis and osteomyelitis in murine models of musculoskeletal infection.38−40

Until relatively recently, studies of S. aureus virulence mechanisms have essentially relied on genetic techniques. However, the emergence of crystal structures of key virulence regulators and the discovery of a raft of small molecules displaying virulence inhibitory activities have provided a platform to initiate medicinal chemistry programs. As outlined in Figure 1, which will be explained in greater detail throughout this article, from a medicinal chemistry perspective the most advanced strategies targeting S. aureus virulence gene regulation can be grouped into four categories of inhibitors. These inhibitors are directed toward (1) the sensor kinase AgrC, (2) the transcriptional activator AgrA, (3) RNAIII, and (4) the SarA family of transcriptional regulators.

2. TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEMS

Genome sequencing has revealed that there are at least 16 two-component systems in the chromosome of S. aureus.40 A full account of all TCSTS involved in S. aureus virulence falls outside the scope of this Perspective; nevertheless, an overview of a number of TCSTS known to play key roles in S. aureus pathogenesis is presented in Table 1. These TCSTS allow bacteria to adapt to environmental changes in response to various cues such as nutrient concentrations, cell population density, antibiotics, ionic strength, and membrane disturbances.

Typically TCSTS consist of two key proteins, a sensor, usually a membrane-associated histidine kinase, and a cytoplasmic response regulator that acts at the level of transcription.17 Upon activation of the sensor by the cognate signal, autophosphorylation of a His residue within the cytoplasmic kinase domain occurs followed by phosphor transfer to an Asp residue in the response regulator protein. This promotes binding of the latter to a specific DNA target sequence and leads to activation or repression of the target structural gene(s).

2.1. Accessory Gene Regulator (agr) System. At present the most extensively characterized TCSTS in S. aureus is the global regulatory system known as the accessory gene regulator (agr) which up-regulates virtually all S. aureus toxins as well as multiple exoenzymes (proteases, lipases, and nucleases) while down-regulating the expression of numerous surface protein genes.17,18 This reciprocal regulation facilitates the progression of an infection from the early stages when staphylococcal surface proteins are required to promote host tissue colonization to the later stages when exotoxins are required to combat host immune defenses alongside degradative exoenzymes that facilitate nutrient acquisition.

In contrast to other TCSTS that respond to external environmental cues, the agr system responds to a self-
generated, secreted signal molecule that facilitates the coordination of gene expression at a cell population density level termed “quorum sensing”. This primitive cell-to-cell communication mechanism in *S. aureus* employs cyclic thiolactone peptides known as autoinducing peptides (AIPs) as quorum sensing signal molecules.42,43

Typically, AIPs consist of seven to nine amino acids in which a central cysteine residue is covalently linked to the C-terminal amino acid carboxylate.42,43,47,48 The sequence of the AIPs is highly variable, and on the basis of AIP primary amino acid sequence, *S. aureus* can be subdivided into four different *agr* groups (I–IV). Intriguingly, most cross-group AIP–*AgrC* interactions are inhibitory, with AIPs activating their cognate receptors and competitively inhibiting noncognate receptors. For example, AIP-1 (compound 1, Chart 1) activates its cognate receptor AgrC-1 (EC₅₀ ≈ 28 nM) while competitively inhibiting the noncognate receptors AgrC-2 and AgrC-3 (IC₅₀ ≈ 25 nM and IC₅₀ ≈ 3 nM, respectively).47

As outlined in Figure 1, the *agr* locus consists of two adjacent but divergent transcriptional units (RNAII and RNAIII) under the control of the P2 and P3 promoters, respectively.41 The P3 transcript, a 517-nucleotide termed RNAIII, is the effector of the *agr* response, initiating the production of multiple exoproduct virulence factors. The *agrP2* operon consists of four genes, *agrBDCA*, which are required for the activation of transcription from the *agrP2* and *agrP3* promoters which code for the cytosolic, transmembrane, and extracellular components of this population density-sensing TCSTS.42,43 AgrD is a propeptide that is processed by AgrB to generate the AIP, which is secreted via a mechanism in which the signal peptidase SpsB has been implicated.41 AIPs binds to their cognate AgrC transmembrane receptor, which results in autophosphorylation of the cytoplasmic histidine kinase domain. Subsequent transphosphorylation of AgrA activates transcription from the P2 and P3 promoters, which drives the autoactivation circuitry and up-regulates production of AIP and RNAIII, respectively. Until recently RNAIII was considered to be the primary effector of the *agr* response, although it is now clear that the *agr* regulon can be divided into RNAIII-dependent and RNAIII-independent, AgrA-dependent genes.49

### 2.2. Competitive Inhibitors of AgrC–AIP Binding

As outlined previously, cross-group AIP–*AgrC* interactions are typically inhibitory with AIPs competitively inhibiting noncognate receptors (Chart 1).47 Although the precise evolutionary and physiological relevance of this cross-talk inhibition has yet to be elucidated, it offers significant therapeutic potential. Inhibition of the TCSTS by noncognate AIPs virtually abolishes the production of the enterotoxin C3, lipase,
and toxic shock syndrome toxin-1. Additionally, interference of AIP signaling through the use of competing AIPs or AIP-sequestering antibodies reduces abscess formation in S. aureus skin and soft tissue infections. Together these studies demonstrate that competitive AIP inhibition constitutes a promising therapeutic approach for attenuating S. aureus infections.

These findings stimulated investigations directed toward the development of global inhibitors of all four S. aureus agr groups, and to this end, the AIP macrocycle has been subjected to a number of structure–activity relationship (SAR) studies. Consistent within these studies was the observation that the macrocycle is critical for AIP function while replacement of the thiolactone moiety with a lactone or lactam group virtually eliminates cognate activation but not cross-group inhibition. However, at present perhaps the most significant observation emerged from alanine-scanning of the AIP-1 scaffold which afforded the aspartate to alanine-5 (DSA) variant (compound 2, Chart 1) displaying potent inhibitory effects against all four AgrC subtypes (Chart 1). Further investigations focused on truncated analogues with tr-AIP-1 (compound 3, Chart 1), tr-AIP-2 (compound 4, Chart 1), and tr-AIP-4 (compound 5, Chart 1) which displayed potent inhibitory properties, although tr-AIP-1 still served as a weak AgrC-1 activator. Combining truncation and the aspartic acid to alanine mutation gave the currently accepted D5A (compound 3, Chart 1) which displayed IC₅₀ values of ~0.1–5 nM across all four agr systems.

Building on the identification (Ala⁵)-AIP-1 as a potent global AgrC inhibitor, additional analogues were synthetized, including the aminobutyric acid analogue 7 and the 4-substituted phenoxybutyryl analogues 8 and 9. The rationale for the incorporation of the 4-benzylphenoxyalkanoyl acid synthon was two-fold: (a) structural variants of benzoylphenol are readily accessed by the Friedel–Crafts acylation of substituted benzene using p-methoxybenzoyl chloride and (b) biaryl ketones are photoactivatable and hence the 4-benzylphenoxyalkanoyl-derivatized AIP-1 could be used for photolabeling studies of AgrC. As outlined in Figure 2, the subtle replacement of a methyl with an ethyl group at the endocyclic position 5 (compound 7) increased inhibitory activity against AgrC-2 but resulted in a decreased inhibitory activity toward AgrC-1. However, the 4-benzylphenoxybutyryl analogues (8, 9) displayed decreased potencies against both AgrC-1 and AgrC-2, indicating that the larger aromatic moiety at the exocyclic position is detrimental to activity.

Further examination of the thiolactone scaffold focused on truncated AIP-2 analogues with glycine insertions, N-methylation scan, and alteration to the thioester linker. As outlined in Chart 2, N-methylation of either Ser-6 or -7 residues resulted in reduced inhibitory activities toward AgrC-1 and AgrC-2 (compounds 13 and 14). In contrast, N-methylation of the Leu-8 and Phe-9 residues (compounds 15 and 16) abolished activity against both AgrC-1 and AgrC-2. Substitution of either Ser-6 or Ser-7 (10 or 11) or both Ser residues (12) with Gly residue(s) yielded analogues with improved inhibitory activities against AgrC-1, but the later two changes marginally affected activities against AgrC-2. Unexpectedly, the Ser-6-to-Gly substitution (compound 10) resulted in a 5-fold loss of inhibitory activity against AgrC-2. Likewise, inhibition of both groups I and II AgrC receptors was substantially reduced with substitution of the two adjacent serine residues with a 5-aminopentanoyl linker (compound 17) and with replacement of the thioester bond with an amide (compound 18). Furthermore, removal of the amino group of the cysteine (compound 19) resulted in decreased activity. Thus, in terms of AgrC-1 and AgrC-2 inhibition, these SAR data indicate that the cysteine and two C-terminal hydrophobic residues at endocyclic positions 7 and 8 are crucial for inhibitory activity while the remainder of the molecule appears less important (Figure 3). In the case of noncognate agr inhibition, the serine residues at endocyclic positions 5 and 6 can be replaced with an alkyl linker without dramatic loss of activity.

Despite their peptidic nature, the AIP analogues appear to be relatively stable in physiological conditions and are resistant to many endoproteases, including chymotrypsin, thermolysin, proteinase K, and V8 serine protease (unpublished data from our lab). Nevertheless, in an effort to reduce peptidic character, a series of peptoid–peptide hybrids, or peptomers, derived from the tr-AIP-1 scaffold were investigated. Of the 11 analogues synthesized, one analogue (compound 20, Figure 4) was shown to stimulate biofilm formation, a phenotype linked to AgrC inhibition. However, given the structural diversity and lack of activity associated with this peptomer library, no definitive SAR data could be established. Nevertheless, the peptoid scaffold does show promise for further analogue development.

Thus, present endeavors to develop a global AgrC competitive inhibitor have focused on developing analogues based on the native S. aureus AIP structures. However, it appears that macrocyclic peptides from other bacteria may provide valuable leads. For instance, solonamide A and

![Figure 2](dx.doi.org/10.1021/jm3014635)
solonamide B (Figure 5, compounds 21 and 22, respectively), which were isolated from a marine Photobacterium, display agr inhibitory activity.61 It was speculated that the solonamides may serve as quorum sensing signals for Photobacterium, and the obvious structural similarities with tr-AIP-2 and tr-AIP-3 suggest they may function as competitive inhibitors of the S. aureus AgrC receptor. As previously outlined, structural investigations of the tr-AIP-2 scaffold demonstrated that adjacent leucine and phenylalanine residues are crucial for potent AgrC competitive inhibition while substitution of the thiolactone moiety with a lactone has minimal impact on inhibitory activity.47,53,56 Each of these features is present within the solonamide scaffold. Although IC\textsubscript{50} values were not reported, Northern blot analysis confirmed the agr interfering activity of the solonamides in both S. aureus strain 8325-4 and the highly virulent CA-MRSA strain USA300.61

Thus, ligand-based design of competitive AgrC inhibitors has provided compelling proof-of-principle that S. aureus virulence can indeed be inhibited through chemotherapeutic intervention. A number of the macrocyclic analogues that displayed submicromolar activity against AgrC are stable against numerous endoproteases and attenuate in vivo dermonecrotic infection caused by various S. aureus strains including USA300. However, of the numerous macrocyclic peptide analogues

| Compound | AgrC-1 IC\textsubscript{50} (μM), (95% CI) | AgrC-2 IC\textsubscript{50} (μM), (95% CI) |
|----------|-----------------------------------|-----------------------------------|
| 4 (tr-AIP-2) | 0.26 (0.151–0.237) | 0.23 (0.167–0.225) |
| 10 | 0.189 (0.053–0.048) | 2.194 (0.170–0.334) |
| 11 | 0.209 (0.066–0.089) | 7.880 (4.100–15.200) |
| 12 | 0.319 (0.284–0.357) | 1.050 (0.795–1.380) |
| 15\textsuperscript{e} | > 30 | > 30 |
| 16\textsuperscript{d} | > 30 | > 30 |
| 17 | 1.141 (0.799–1.630) | 50.7 (54.1–47.6) |
| 18 | 1.390 (1.150–1.560) | 167 (96.4–288) |
| 19 | 1.680 (1.310–2.170) | 47.3 (37.3–63.3) |

\textsuperscript{d}IC\textsubscript{50} values for peptides 15 and 16 could not be determined because of lack of inhibition up to the highest concentration tested (>30 μM).
examined, none displayed significant impact on bacterial viability and repeat exposure experiments did not induce resistance. These successes have fueled preclinical investigations of these compounds and inspired investigations of alternative pathways that could be exploited to attenuate virulence.

The existence of an additional agr-linked quorum sensing system (SQS1) in S. aureus has been proposed. However, there is considerable controversy within the field and three independent reports provide compelling evidence that the original work was flawed probably as a consequence of secondary mutations in the strains used. SQS1 was hypothesized to operate upstream of agr, controlling the activity of RNAIII via its own autoregulatory mechanism, involving an autoinducer RNAIII-activating protein (RAP), a sensor histidine kinase (SvrA), and the response regulator protein TRAP (target for RAP, encoded by traP). However, mutation of traP in a number of different S. aureus strains had no effect on either agr expression or virulence and the original data are most likely accounted for by a nonsense mutation in agrA.

Nevertheless, the SQS1 system was reported to be inhibited by a linear peptide known as RNAIII-inhibiting peptide (RIP, sequence YSPSTNF-NH2). An in silico generated pharmacophore of RIP was screened against a library of commercially available small molecules, and a nonpeptidic RIP analogue, hamamelitannin, was discovered. Hamamelitannin (Figure 6) is a natural product found in the bark of Hamamelis virginiana (witch hazel). In a rat graft model, hamamelitannin prevented device-associated infections in vivo, including infections caused by methicillin-resistant S. aureus and S. epidermidis strains. Thus, although hamamelitannin mechanism of action has not been unequivocally delineated, the molecule may still represent a viable lead for the discovery of both virulence and biofilm formation inhibitors of S. aureus.

3. AgrA AS A TARGET

As outlined in Figure 1, RNAIII is the effector of the agr response initiating up-regulation of several exotoxins and enzymes while repressing expression of a range of bacterial cell-surface proteins. Consequently, any reduction of RNAIII transcription via perturbation of the AgrA interaction with the P2 and P3 promoters could provide an additional route to block S. aureus virulence.

AgrA is indispensable to agr P2- and P3-driven transcription, and while no bona fide inhibitors of AgrA–P2/P3 interactions have been reported, the recently reported cocrystallized structure of the DNA-binding domain of AgrA complexed with a DNA pentadecamer duplex has provided a potential platform for structure-based drug design endeavors. The crystal structure of the C-terminal DNA-binding domain, termed AgrA C (residues 137–238), indicates that three amino acid residues His-169 (blue), Asn-201 (orange), and Arg-233 (green) make specific contacts with DNA (Figure 7). Indeed, the importance of residues H169 and R233 in DNA binding was confirmed by alanine mutagenesis and subsequent isothermal titration calorimetry studies.

4. SMALL MOLECULE INHIBITORS OF RNAIII EXPRESSION

Several synthetic and natural product small molecule analogues with agr inhibitory properties including savirin (Staphylococcus aureus virulence inhibitor) (compound 24, Figure 8), benzbrumaronine (compound 25, Figure 8), and a benzo-1,4-dioxane analogue (compound 26, Figure 8) have been described. These compounds were identified from extensive random screening programs, and each inhibits AIP-induced production of RNAIII transcripts and thus virulence factors such as α-hemolysin and lipase.

In terms of in vivo efficacy, the most extensively examined analogue is savirin (24), and this compound was shown to prevent the development of dermonecrotic ulcers following
infection with *agr*^+^ bacteria in an experimental mouse model of skin and soft tissue infection.76 Moreover, repeated exposure of *S. aureus* to savirin either in vivo or in vitro did not induce resistance. Although the mechanism of action of savirin has yet to be elucidated, transcriptome experiments indicate that most of the genes down-regulated in an *agr* positive strain on treatment with savirin were also down-regulated in the absence of *agr*, i.e., in an *agr* mutant.76 At present, the structure has not been subjected to any SAR; however, methods to rapidly produce analogues have been reported,77 thus making savirin an attractive lead.

Benzbromarone, which is traditionally utilized as a gout medication, was also found to reduce abscess formation in mouse models and provided protection against *agr*-II and *agr*-III *S. aureus* infections.75,76 These inhibitory effects were elicited in the *S. aureus* RN6390 strain as well as in NM300, which is closely related to the CA-MRSA USA300 strain.75 Once again, precise mechanism of action has not been elucidated; however, receptor binding studies indicate that benzbromerone may inhibit binding of AIP to AgrC.75 However, it also elicits more global effects on the bacteria, and in addition to inhibiting late stationary phase growth, benzbromerone also inhibits production of staphyloxanthin which is a carotenoid pigment used by the bacteria as protection against oxidants.76

The benzbromerone scaffold has been subjected to preliminary SAR investigations; however, of the 24 analogues examined none displayed increased activity against *S. aureus agr* group I.75 As outlined in Table 2, alterations at R^1^ included a number of aryl substituents as well as the incorporation of alkyl and ester moieties. However, not all permutations of the other two regions were represented while holding R^1^ constant as an aryl group; for instance, compound 29 lacks an aryl group at R^1^ but still displays activity. Nevertheless, a p-hydroxy moiety seemed to be desirable for activity in this region with the dibromo-substituted benzbromerone maintaining its role as the best inhibitor. Alterations to R^2^ included alkyl and aryl substituents; however, only alkyl analogues displayed *agr*-I inhibitory activity. Likewise, alterations to R^3^ of the scaffold were detrimental to activity with benzofuran and aryl

| Compound | R^1^ | R^2^ | R^3^ | Agr-I IC50  |
|----------|------|------|------|-------------|
| Benzbromerone |      |      |      | 0.13 μM     |
| 25       |      |      |      | 0.54 μM     |
| 27       |      |      |      | 1.29 μM     |
| 28       |      |      |      | 0.78 μM     |

substitutions affording only one active compound, 29. Nevertheless, the activity of compound 29 is assumed to result from the inclusion of the sterically bulky dibromomethoxy moiety.

An additional series of small molecules displaying inhibitory activities against *agr* are the 1,3-benzodioxoles and benzo-1,4-dioxanes.74 The lead compound (compound 26) inhibits RNAIII promoter activation with an IC_{50} in the range of 100–200 nM, and maximal effects (90% or greater inhibition) can be achieved at 12 μM in in vitro experimental systems.74 Intriguingly, the lead compound inhibits AIP-induced production of virulence factors α-hemolysin and lipase in *agr* group III but not group I strains of *S. aureus*. Receptor binding studies indicate that it does not significantly inhibit the binding of AIP to the AgrC receptor, the initial step in the bacterial quorum sensing pathway. Additionally, cell viability is unaffected.74

The scaffold was subjected to an extensive SAR study with a total of 44 compounds synthesized and evaluated.74 In general, the 1,3-benzodioxoles (compounds 30–33, Table 3) were the most active of the five scaffolds investigated, particularly when R^1^ was a simple phenyl and R^2^ was a piperidine moiety. Addition of heteroatoms to the piperidine moiety decreased activity with ethers, esters, ketone protections, or ketones leading to loss of inhibition. Additionally, having anything at R^3^ other than a simple phenyl appears to impart a detrimental effect on activity.

Small molecule inhibitors of RNAIII expression have also emerged from natural sources, and among these are the secondary fungal metabolite known as ambuic acid (compound 38, Figure 9). Preliminary data indicated that compound 38 inhibits AIP biosynthesis; however, even at high concentrations it was reported that the inhibitory effect is not substantial. Nevertheless, the inhibitory effect appeared sustained over a period of several hours.78 Thus, the ambic acid scaffold offers a lead to developing indirect inhibitors of the *agr* TCST.

Some of the most intriguing natural compounds displaying *agr* inhibitory activities have emerged from investigations of mixed microbial infections. A number of these studies have focused on chronic infections within the airways of cystic fibrosis (CF) sufferers and led to the discovery that the prolonged growth of *S. aureus* with either *Pseudomonas aeruginosa* or with physiological concentrations of the *P. aeruginosa* exoproduct 4-hydroxy-2-heptylquinoline N-oxide (HQNO, compound 39, Figure 10) selects for typical *S. aureus* small-colony variants (SCVs).79 However, SCVs are well-known for aminoglycoside resistance and persistence in chronic infections, including those found in CF. Evidence suggests that the development of SCVs is in part due to HQNO-mediated repression of the *agr* system. In addition to other unknown mechanism(s) of action, HQNO apparently has the capacity to inhibit *agr* group I with an IC_{50} of 1.3 μM (unpublished data from our lab).

Other studies of *P. aeruginosa* demonstrated that the quorum signal molecule N-(3-oxododecanoyl)-1-homoserine lactone (3-oxo-C12-HSL) (compound 40, Figure 11) also elicits inhibitory effects against *agr* (IC_{50} = 6 μM) and, at high concentrations, staphylococcal growth (100 μM).80 Indeed, exposure of *S. aureus* to different N-acetyl 1-homoserine lactones (AHLs) revealed that 3-oxosubstituted AHLs with C10 to C14 acyl chains inhibited virulence factor production and growth in a concentration-dependent manner, while short-chain AHLs had no effect.80 3-Oxo-C12-HSL inhibited the production of exotoxins and cell wall fibronectin-binding proteins but enhanced protein A expression. Although the biological
mechanism by which 3-oxo-C12-HSL inhibits agr is yet to be elucidated, evidence exists that the molecule may affect SarA functionality and potentially antagonize other membrane-associated regulators, such as the sensor components of arlRS, sacRS, and srrAB. Thus, although 3-oxo-C12-HSL analogues display potential as antivirulence agents, their antagonistic activity on a number of growth and metabolic pathways suggests that resistance may develop easily.

Table 3. Structures and AgrC-3 IC₅₀ Values of the Most Active 1,3-Benzodioxole Analogues Unearthed during an Initial Structure—Activity Relationship Investigation

| Generic Structure | Compound | R¹ | R² | Agr-C3 IC₅₀ |
|-------------------|----------|----|----|------------|
| ![Structure 26](image) | 26 | -Ph | | 0.13 μM |
| ![Structure 30](image) | 30 | -Ph | | 0.16 μM |
| ![Structure 31](image) | 31 | -Ph | | 0.98 μM |
| ![Structure 32](image) | 32 | -Ph | | 1.26 μM |
| ![Structure 33](image) | 33 | -Ph | | 0.98 μM |
| ![Structure 34](image) | 34 | -H | | 1.51 μM |
| ![Structure 35](image) | 35 | | | 0.25 μM |
| ![Structure 36](image) | 36 | | | 0.72 μM |
| ![Structure 37](image) | 37 | | | 1.31 μM |

*Intriguingly these analogues only displayed activity against S. aureus group III.*

Figure 9. Structure of the secondary fungal metabolite, ambuic acid.

Figure 10. Structure of the *Pseudomonas aeruginosa* alkylquinolone, 4-hydroxy-2-heptylquinoline-N-oxide, which displays inhibitory effects against the agr system.
Lactobacillus reuteri RC-14, a human vaginal isolate, produces molecules capable of inhibiting the S. aureus agr system, and initial data indicate that this bacterium can repress the expression of TSST-1. TSST-1 has been associated with essentially all cases of menstruation-associated toxic shock syndrome, and evidence indicates that women with a deficiency of Lactobacillus reuteri RC-14 within the vaginal mucosa are more susceptible to this illness. It is believed that two active compounds involved in this interspecies communication are the cyclic dipeptides cyclo(L-Tyr-L-Pro) (compound 41, Figure 12) and cyclo(L-Phe-L-Pro) (compound 42, Figure 12). Although no IC₅₀ values were reported, competition assays demonstrated that both cyclic dipeptides antagonized the AIP-mediated activation of agr, indicating that they may compete for the ligand-binding pocket on the AgrC receptor.

Figure 11. Structure of the Pseudomonas aeruginosa quorum sensing molecule 3-oxo-C12-HSL which elicits inhibitory action against agr (IC₅₀ = 6 μM) and at high concentrations, staphylococcal growth (100 μM).

Figure 12. Structures of the Lactobacillus reuteri RC-14 derived products cyclo(L-Tyr-L-Pro) (41) and cyclo(L-Phe-L-Pro) (42).

Table 4. Overview of a Number of SarA Homologues and Their Proposed Functions in the Regulation of S. aureus Virulence Pathways

| regulator | virulence effect | proposed interactions and regulations | reference |
|-----------|-----------------|---------------------------------------|-----------|
| SarA      | + An activator of the agrABCD operon activating P2 transcription. SarA is also involved in agr-independent pathways via binding to conserved regions, termed Sar boxes, within the promoters of several cell-wall-associated proteins and exoproteins. | 44, 45, 87, 88 |
| SarR      | – SarR represses P2 transcription and binding of SarR to the sarA promoter represses SarA expression. | 18, 44 |
| SarS      | – Activates protein A (spa) and represses α-toxin (hla) transcription. | 18, 89 |
| SarT      | – Activation of sarT results in up-regulation of sarS, thus leading to hla repression and spa activation. | 18, 44 |
| SarU      | + sarU is repressed by SarT which in turn is down-modulated by agr. Since sarU is an activator of agr expression, this will lead to amplification of the original agr signal. | 18, 44 |
| Rot       | – Represses toxin synthesis and up-regulates cell wall protein synthesis. Rot affects the transcription of 168 genes, many of which reflect an agr minus phenotype. | 90 |
| SarX      | – SarX acts as a negative regulator of agr. Furthermore, MgrA is an activator of sarX, thus implying an additional regulatory loop whereby mgrA can modulate agr expression. | 91 |
| MgrA      | + Regulates cell-wall turnover and activates the production of secreted toxins, proteases and is a regulator of autolysis. The effect of MgrA on autolysis may be mediated by SarV which is a positive regulator of several autolytic enzymes. | 92–94 |
| SarZ      | + A positive regulator of hla expression. A sarZ mutant of RN4220 had attenuated virulence in both silk worm and mouse infection models. | 18, 95 |
| SarV      | – A regulator of autolysis that is repressed by SarA and MgrA. A sarV mutant was found to be more resistant to detergent- or cell wall antibiotic-mediated lysis. | 18, 96 |
SarA was the first member identified as playing a pivotal role in the regulation of virulence genes in *S. aureus*. Transcriptional profiling revealed that SarA modulates either directly or indirectly at least 120 genes including up-regulation of extracellular proteins such as α- and β-hemolysins, TSST-1, staphylococcal enterotoxin B, and fibronectin binding protein. A number of studies have indicated that SarA regulates virulence via *agr*-dependent mechanisms, including binding to the *agr*-P3 promoter, and via *agr*-independent mechanisms including binding to the promoter regions of *hla* (α-hemolysin), *tst* (TSST-1), *sec* (enterotoxin C), and *trxB* (thioredoxin reductase).

Inactivation of SarA afforded strains displaying reduced virulence in several experimental staphylococcal infection models. Furthermore, SarA mutants display a reduced ability to induce septic arthritis and osteomyelitis in murine models of musculoskeletal infection. Although no small molecular SarA inhibitors have been reported, the current crystal structure of SarA and a number of mutagenesis studies have provided a platform for structure-based drug design methodologies. SarA is a typical "winged-helix" DNA binding protein with the helix–turn–helix and the winged regions proposed to interact with the major and minor grooves of target promoter DNA, respectively. Mutations of individual residues within the DNA-binding helix–turn–helix and the winged region, as well as within the metal-binding pocket, implicate basic residues Arg-84 and Arg-90 within the winged region to be critical in DNA binding, whereas acidic residues Asp-88 and Glu-89 (wing), Asp-8 and Glu-11 (metal-binding pocket), and Cys-9 are essential for SarA function. The presence of a sole free-cysteine within the metal-binding pocket is a ubiquitous feature of the SarA protein family, common to SarA, SarS, and MgrA. The cysteine residue is believed to function as an oxidation sensor or redox switch that regulates gene expression. Oxidation of the free-Cys leads to dissociation of the oxidized protein from DNA and thus inhibiting gene expression. Thus, as outlined in Figure 13, two approaches to inhibit the function of the SarA protein family exist: small molecules could be designed to bind (a) within the wing region to inhibit DNA–protein interactions or (b) within the Cys pocket, which would potentially mimic the oxidized or nonfunctional state.

The identification of a number of small molecule MgrA inhibitors clearly demonstrates that the SarA family of proteins is indeed a potential target for chemotherapeutic agents. MgrA positively affects the expression of capsular polysaccharide and nuclease while repressing expression of α-toxin, coagulase, and protein A. In addition to regulating virulence determinants, MgrA also represses the expression of several efflux systems such as NorA, NorB, NorC, and Tet38. Moreover, MgrA is known to play a critical role in *S. aureus* virulence, as an MgrA mutant strain exhibited 1000- to 10000-fold virulence reduction in a mouse model of infection. As outlined in Figure 15, the MgrA monomer possesses the typical winged helix structure consisting of eight α-helices and three β-strands and possesses the ubiquitous single cysteine redox switch (Cys-12) in the dimerization domain. Oxidation of this Cys residue leads to dissociation of the oxidized MgrA from the sarV promoter, thus repressing expression of a number of virulence factors. However, MgrA negatively regulates the expression of efflux pumps, including Tet38, NorA, NorB, and NorC, which account for bacterial resistance to multiple antibiotics, such as tetracycline, norfloxacin, and ciprofloxacin. MgrA is also shown to affect vancomycin resistance, as mutation of MgrA leads to increased resistance of the bacterium to these antibiotics. Thus, this could potentially limit the therapeutic potential of MgrA inhibitors in a clinical setting.

Nevertheless, a recent high-throughput screening program was conducted that identified a number of small molecule inhibitors of MgrA–DNA interactions, including 5,5-methyleneedisalicylic acid (43, MDSA) and a series of 3-aryl-3-(2,5-dimethyl-1H-pyrrol-1-yl)propanoic acids (44–46) (Figure 14). MDSA was found to alter the transcriptional expression of a number of virulence factors including *hla* (α-hemolysin) and *spa* (protein A) while eliciting no negative impact on
bacterial growth. Moreover, an esterified prodrug MDSA analogue was shown to attenuate virulence in a mouse model of infection. The mechanism by which MDSA inhibits MgrA–DNA interactions is currently unknown. However, preliminary computational docking experiments indicate that MDSA may bind around the DNA-binding lobe that is flanked by Trp-48 (Figure 15).\(^{100}\) Nevertheless, these compounds clearly demonstrate the therapeutic potential of virulence transcription factor inhibitors.

![Figure 15. Crystal structure of a monomeric unit of MgrA (PBD accession code 2BV6)](image)

### 6. CONCLUSIONS AND FUTURE DIRECTIONS

The rapid emergence of multiantibiotic resistant bacteria represents one of the greatest threats to human health worldwide.\(^{107}\) Among these superbugs, *S. aureus* presents one of the greatest threats with methicillin-resistant strains such as USA300, killing more Americans in 2007 (∼19,000) than emphysema, HIV/AIDS, Parkinson’s disease, and homicide combined.\(^{107}\)

Against this backdrop the development of new classes of antibiotics is lagging. As a result, we are faced with an urgent need to better exploit the new targets that are emerging from our increased understanding of the molecular basis of bacterial pathogenicity if we are to develop novel prevention and treatment strategies. As outlined throughout this Perspective, there is an emerging body of evidence indicating that inhibiting the ability of *S. aureus* to produce exoproduct virulence determinants significantly attenuates infection. Consequently, developing therapies geared at “disarming” the bacterium is a promising approach for combating infections.\(^{9−13}\)

Currently, the most advanced strategies include inhibitors of the SarA protein family and the agr-TCSTS. Given that the *agr* system is conserved across many different Gram positive pathogens, lessons derived from studying *S. aureus* will undoubtedly be applicable in the development of chemo-therapeutics to treat other problematic Gram-positive pathogens, including *Listeria monocytogenes* and *Enterococcus faecalis*.\(^{108}\) In terms of developing clinically useful agents, much work remains. For example, most of the currently reported in vivo studies have involved coadministering virulence inhibiting compounds with bacteria, and thus, it is not clear whether such agents can effectively attenuate pre-existing infections. Further concerns relating to the propensity of *agr* inhibitors to stimulate biofilm formation must be investigated especially in relation to chronic *S. aureus* infections. This is because *agr* mutants form better biofilms in vitro and *agr* is required for biofilm dispersal.\(^{109}\)

Since planktonic cells are more susceptible to conventional antibiotics, ironically, it has been suggested that combination therapy with AIPs could usefully be employed to disperse biofilms and restore antibiotic susceptibility.

Nevertheless the emerging palette of small molecule inhibitors of staphylococcal virulence gene expression will provide invaluable tools to further probe and manipulate virulence pathways while providing significant benefits over genetic techniques.\(^{110−112}\) The advantages of using small molecule inhibitors are numerous and include the following:

(a) small molecules can be used in a conditional manner allowing for temporal control of a biological system; (b) small molecule perturbation of protein function is generally reversible which allows studies to be carried out on the reversibility of the system; (c) the biological effects of small molecules are generally rapid thus allowing characterization of intermediate/early responses in the system; (d) small molecule effects can be controlled by varying concentrations, thereby allowing the generation of dose–response data. Thus, compounds that are not druglike can be useful tools for temporal and dose–response studies of these systems which may not be possible by genetic manipulation.

Overall, the developments of small molecule inhibitors of staphylococcal virulence expression are still in an embryonic state. However, the proliferation of crystal structures of key virulence regulating proteins and hit compounds over the past 5 years undoubtedly means that medicinal chemists will now play an ever increasing role in developing virulence attenuation strategies. Given the current trajectory of research, it seems that there is much room for optimism that a virulence inhibiting therapeutic agent will be clinically available within the current half of the 21st century, thus providing a vital addition to our rapidly depleting antibiotic arsenal.

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**Notes**

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Paul Williams is Professor of Molecular Microbiology and Head of the School of Molecular Medical Sciences, University of Nottingham, U.K. He is internationally known for his research on quorum sensing and has (co)authored over 280 research and review articles, book chapters, and patents. His current research interests focus primarily on the regulation of gene expression through cell–cell communication (quorum sensing) in pathogenic bacteria with the aim of identifying new targets for novel anti-infective agents. For his quorum sensing research he was awarded the Royal Pharmaceutical Society of Great Britain Conference Science Medal (1992), the Pfizer prize in Pharmaceutical Sciences (1994), and the Society for General Microbiology Colworth Prize Lecture (2007).

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ABBREVIATIONS USED

MRSA, methicillin resistant Staphylococcus aureus; HA-MRSA, health care associated methicillin resistant S. aureus; CA-MRSA, community-acquired methicillin resistant S. aureus; TSST-1, toxic shock syndrome toxin-1; PVL, Panton–Valentine leukocidin; TCSTS, two-component signal transduction systems; agr, accessory gene regulator; AIP, autoinducing peptide; CF, cystic fibrosis; AHL, N-acyl l-homoserine lactone

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