Targeting MgrA-Mediated Virulence Regulation in *Staphylococcus aureus*

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SUMMARY

Increasing antibiotic resistance in human pathogens necessitates the development of new approaches against infections. Targeting virulence regulation at the transcriptional level represents a promising strategy yet to be explored. A global transcriptional regulator, MgrA in *Staphylococcus aureus*, was identified previously as a key virulence determinant. We have performed a fluorescence anisotropy (FA)–based high-throughput screen that identified 5, 5-methylenedisalicylic acid (MDSA), which blocks the DNA binding of MgrA. MDSA represses the expression of α-toxin that is up-regulated by MgrA and activates the transcription of protein A, a gene down-regulated by MgrA. MDSA alters bacterial antibiotic susceptibilities via an MgrA-dependent pathway. A mouse model of infection indicated that MDSA could attenuate *S. aureus* virulence. This work is a rare demonstration of utilizing small molecules to block protein-DNA interaction, thus tuning important biological regulation at the transcriptional level.

INTRODUCTION

Staphylococcus aureus is a common human pathogen that causes a wide variety of diseases, ranging from minor skin infections to life-threatening endocarditis, pneumonia, blood infections, and toxic shock syndrome (Archer, 1998; Lowy, 1998). This pathogen can infect almost every tissue in the human host. Its versatility is mostly due to the production of various virulence factors, such as hemolysins, leukocidins, and immune modulators. More troublesome, strategies that fight staphylococcal infection have deteriorated in the past several decades owing to the emergence of multiple methicillin-resistant strains (MRSAs) and more recent vancomycin-resistant strains. MRSAs, which are isolated in up to 60% of community and 80% of hospital infections, have rendered the entire class of β-lactam antimicrobials obsolete as therapeutic agents (Centers for Disease Control and Prevention, 2007). Although vancomycin

has long been considered as the last resort therapy for MRSAs (Fridkin et al., 2005), vancomycin-resistant strains with intermediate (VISAs) or full resistance (VRSAs) to vancomycin and other glycopeptide inhibitors of cell wall synthesis have caused infections for which traditional antibiotic treatment have been in vain (Chang et al., 2003; Walsh, 1993; Weigel et al., 2003).

Virulence suppression represents an alternative approach to combating infections (Balaban et al., 1998; Gresham et al., 2000; Ji et al., 1997). The mode of action of conventional antibiotics largely relies on targeting essential cellular functions, such as DNA replication, protein synthesis, and cell wall synthesis. Because of bactericidal (bacterial-killing) or bacteriostatic (growth-inhibitory) effects, overuse of these antibiotics imposes selective pressures on bacteria and thus leads to the emergence of drug-resistant strains. Given that most virulence factors are nonessential for bacteria, in principle, antimicrobial agents that are designed for inhibiting microbial virulence without inhibiting growth presumably exert less selective pressure for the generation of resistance (Clatworthy et al., 2007). The expression of virulence factors is coordinately regulated by multiple two-component systems and global transcription factors. MgrA in particular plays a key role in the regulation of the expression of major virulence factors in *S. aureus* (e.g., capsular polysaccharide, protease, α-toxin, nuclease, and protein A) (Ingavale et al., 2005; Luong et al., 2006; Truong-Bolduc et al., 2005). MgrA is a member of the MarR (multiple antibiotic resistance regulator)/SarA (staphylococcal accessory regulator A) family proteins and controls expression of ~350 genes (Luong et al., 2006). We have shown that MgrA is critical for *S. aureus* pathogenesis in vivo, and an *mgrA* mutant strain exhibits 1000–10,000 fold virulence reduction in a mouse model of infection (Chen et al., 2006). We have also revealed that MgrA acts as a redox-switch via its sole and unique Cys12 to regulate gene expression. Oxidation of this Cys leads to dissociation of the oxidized MgrA from DNA and thus attenuation of the bacterial virulence. The regulation of MgrA could also be affected by environmental stimuli, as shown by a previous study in which the transcriptional expression of *mgrA* was down-regulated by salicylate (Riordan et al., 2007). Given the dramatic role MgrA plays in *S. aureus* virulence, this transcription factor could be a feasible target for developing antimicrobial drugs. Chemically inhibiting DNA binding of MgrA could potentially diminish *S. aureus* virulence. As proof of principle, we demonstrate here that small
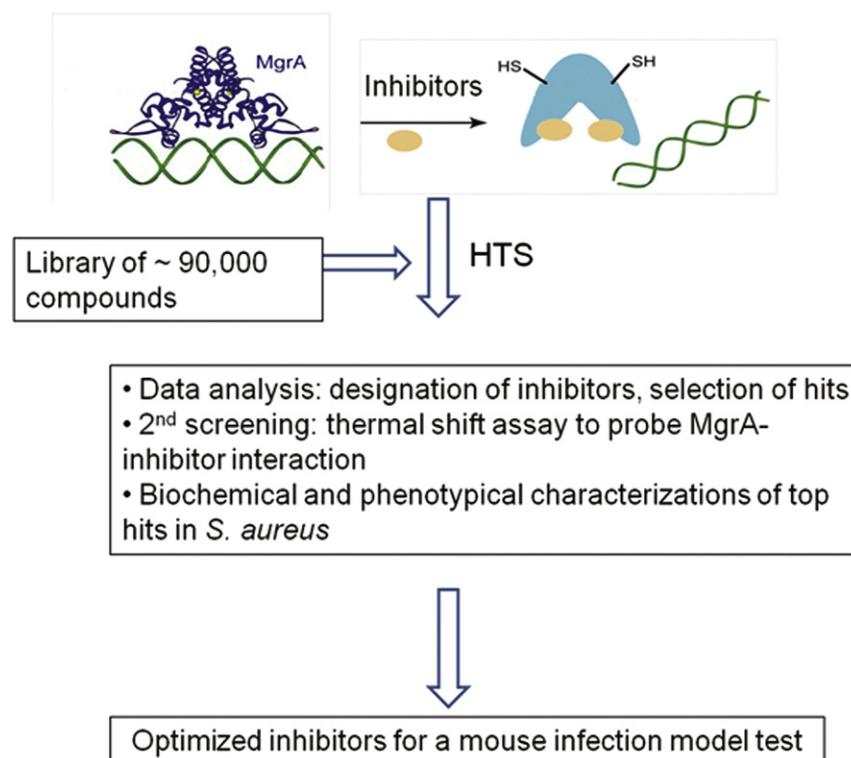
Figure 1. High-Throughput Screening and Hit Characterization Strategy

A fluorescence anisotropy–based assay using a fluorescein-labeled DNA recognized by MgrA was used in HTS (Z' factor of 0.81). Compounds displaying $\geq$50% inhibition on the MgrA-DNA binding were selected as positive hits. The 94 hits were subjected to a fluorescence-based thermal shift assay to further probe their direct interactions with MgrA. Four top hits were evaluated in in vitro experiments, such as EMSA, to confirm their activity in disrupting MgrA-DNA interaction. MDSA, the most effective inhibitor for MgrA, was applied to a series of in vivo characterizations including transcriptional expression analysis on virulence factors, antibiotic resistance assays, and a murine abscess infection model. See also Figure S1.

molecules identified from a high-throughput screen (HTS) are able to disrupt the DNA binding of MgrA and that administration of such a compound leads to a reduced staphylococcal infection in a murine abscess model of infection.

RESULTS

Screening Inhibitors for MgrA

A fluorescence anisotropy (FA)–based biochemical assay that monitors MgrA-DNA binding was optimized for HTS. The DNA probe (5′-TAAACAACA**AGTTGT**CCAAA-3′) containing an MgrA-binding box (AGTTGT) was synthesized and fluorescently labeled with 6-carboxylfluorescein (Manna et al., 2004), a widely used fluorophore with excitation at 494 nm and emission at 521 nm (see Figure S1A available online). According to the Perrin equation, the FA value is proportional to the molecular volume (molecular weight) of fluorescently labeled biomolecules. In this case, the fluorescein-labeled DNA alone generates a small FA value ($\sim$0.8), whereas the formation of MgrA-DNA complex increases the FA value up to $\sim$2.8. The binding affinity of MgrA to this particular DNA probe was determined using this method ($K_d \sim$0.14 μM) (Figure S1B). Translation of this assay into a 96-well format plate yielded a Z' factor of 0.81 with 20 nM DNA and 400 nM MgrA, which is well above the value (0.5) suggested for typical HTS assays (Figure S1C). A total of 88,564 compounds were screened in duplicate against the MgrA-DNA interaction using the NSRB library. Among the small molecules screened, 114 (0.13%) exhibited significant inhibition ($\geq$50%) on the DNA binding of MgrA. Of these, 19 hits are from natural product extracts and tentatively excluded from cherry picks because of a lack of structural and composition information.

The other 95 hits with confident structural information and purity were picked for the subsequent validation and characterization.

Although FA has been widely used to study the interaction of biomolecules and proven to be an effective method for HTS, several caveats must be accounted for when using this type of screen. First, the inherent fluorescent properties of test compounds could interfere with the FA readout and produce false-positive results. Second, in the screen to disrupt protein-DNA interactions, some compounds could simply intercalate into DNA and prevent the binding of MgrA. To rule out these unwanted scenarios, initial screening hits were filtered by a combination of cheminformatics (data analysis to exclude low-confidence inhibitors and autofluorescent false-positive results), the secondary screen based on the fluorescence-based thermal shift assay, and biochemical and phenotypical characterization of top hits in *S. aureus* (Figure 1). The fluorescence-based thermal shift assay, which represents a general method for identification of inhibitors of target proteins from a number of hit compounds, was used to verify the direct interaction between MgrA and selected compounds (Lo et al., 2004). The basis of this assay is that any protein of interest has a characteristic melting temperature because of its intrinsic secondary and tertiary structure, which could be stabilized or destabilized upon ligand binding. This effect is usually reflected by the shift of the melting temperature of the target protein. Sypro orange, an environmentally sensitive fluorescent dye, could be used to monitor the protein-unfolding transition. The 95 compounds obtained from HTS were further tested using this method. Most compounds were either able to increase or decrease the melting temperature of MgrA (data not shown), indicating that these compounds disrupt MgrA-DNA interaction mostly by interacting with the protein rather than intercalating into DNA. Of these, four prominent compounds are listed in Table 1. MDSA, reminiscent of a dimerized salicylic acid, displayed the strongest inhibitory effect on MgrA with an IC_{50} close to $\sim$8.0 μM, according to the FA assay and the electrophoretic mobility shift assay (EMSA)

Table 1. The Structures of Representative MgrA Inhibitors Revealed by FA-HTS and FA-Thermal Shift Assay

HTS Example Active	IC$_{50}$ (μM)	Temperature (°C)
	8	7.7
	30	2.7
	28	3.6
	32	3.5
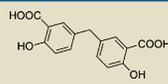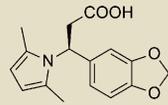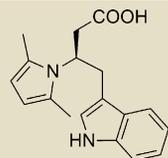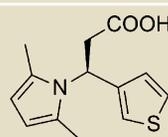
IC$_{50}$ of each compound was determined by fluorescence anisotropy assay and validated by EMSAs (Figure S3). The melting temperature shift (ΔT) is presented as a mean value from two independent experiments (Figure S2). See also Figures S2 and S3.

(Figure 2). In the thermal shift experiment, this compound was capable of increasing the melting temperature of MgrA by 7.7°C (Figure S2). The other three compounds **2, 3,** and **4** fall into the same class of molecule, 3-aryl-3-(2,5-dimethyl-1H-pyrrol-1-yl) propanoic acid, and only differ at the aromatic substituent at the 3 position. All three compounds exhibited good inhibition activities on MgrA, with IC$_{50}$ values ranging from 28 μM to 32 μM. In the thermal shift assay, these three compounds increased the melting temperature of MgrA by approximately 2–3°C (Figure S2), indicating that they might interact with MgrA in a very similar manner. The activity of these compounds to block DNA binding by MgrA was further confirmed by EMSAs (Figures 2B; Figure S3). MgrA was shown to be dissociated from its cognate DNA (*hla* promoter region) in the presence of these small molecules. Consistent with the FA and thermal shift assays, EMSA also showed that MDSA was the most effective compound to disrupt the DNA binding of MgrA, which prompted us to further investigate this compound.

MDSA Alters the Expression of Virulence Factors in an MgrA-Dependent Manner

MgrA is a global transcriptional regulator that controls expression of a number of virulence factors, such as genes encoding α-toxin (*hla*) and protein A (*spa*) (Ingavale et al., 2005). MgrA has been shown to positively regulate the expression of *hla* by binding to its promoter region (Ingavale et al., 2005). To test whether MDSA could abolish the binding of MgrA to the *hla* promoter, EMSAs were performed in the presence of different amounts of MDSA. As shown in Figure 2B, ~8 μM of MDSA could significantly attenuate the binding of MgrA to the *hla* promoter. The presence of MDSA could also alter the expression level of both *hla* and *spa* inside *S. aureus* strain Newman. As

shown in the Northern blotting assays (Figure 2C), the expression level of *hla* in the wild-type Newman was greatly decreased in the presence of 0.2 mM of MDSA. Because MgrA acts as an activator of *hla*, mutation of *mgrA* led to a decreased *hla* expression as expected. The expression of *hla* in *mgrA* mutant was not affected by the presence of MDSA, even up to 0.5 mM, strongly suggesting that MDSA affects the expression of *hla* in an MgrA-dependent manner. In addition to the strain Newman, the inhibition of MDSA on the expression of *hla* was also observed in the MRSA strain USA300 according to western blot analysis (Figure S4). MgrA is known to act as a repressor of the *spa* gene (Ingavale et al., 2005). As shown in Figure 2C, the treatment of wild-type bacteria with MDSA resulted in the increased expression of *spa*, whereas in the *mgrA* mutant strain, the expression of *spa* occurred at a higher level and was not affected by the presence of 0.5 mM of MDSA. Taken together, we have demonstrated that MDSA exerts inhibitory effects on MgrA inside bacteria, thus leading to the altered expression of the *mgrA* regulon.

Binding of MDSA to MgrA

Because we have previously solved the crystal structure of MgrA (Chen et al., 2006), computational molecular docking (AutoDock Vina, vina.scripps.edu) was applied to investigate the possible mode of MDSA binding to MgrA. After running the program, two possible binding sites (α and β) were revealed, and both were equally energetically favored (Figure 3A). Interestingly, both sites are close to the helix-turn-helix DNA-binding motif that is highly conserved among the MarR/SarA family protein (Alekshun et al., 2001), implying that MDSA interacts with the DNA-binding domain of MgrA to interrupt its DNA binding. The docking structure also reveals that MgrA bears a single Trp48 residue that is located ~20.3 Å and ~20.4 Å away from the α and β sites, respectively (Figure 3A). Because MDSA is a fluorescent compound with excitation at 310 nm and emission at 421 nm, we envision that if MDSA does bind to either the α or β position, Förster resonance energy transfer (FRET) should occur between the donor Trp48 (excitation at 278 nm and emission at 330 nm) and the acceptor MDSA. To test our hypothesis, we measured the fluorescence of MgrA in the presence or absence of MDSA. As shown in Figure 3B, MgrA (10 μM) displayed the maximum fluorescent signal at 330 nm in the absence of MDSA. With the addition of MDSA, the peak at 330 nm, corresponding to the emission of Trp48, was decreased, whereas the emission of MDSA at 421 nm was elevated. Because the emission intensity at 330 nm depends on the molar ratio of MgrA-MDSA versus MgrA, the binding affinity of MgrA to the small molecule could be estimated through this measurement (K$_d$ = ~12.8 μM) (Figure 3C). We consider this value a more accurate determination of the binding affinity of MDSA to MgrA.

Far-UV (190–250 nm) circular dichroism (CD) spectroscopy is widely used for monitoring protein secondary structure, whereas near-UV CD spectroscopy is used for comprehending tertiary structure (Beychok, 1966; Greenfield, 2006). To investigate whether the binding of MDSA to MgrA elicits a conformational change, near-UV (250–320 nm) circular dichroism (CD) spectroscopy was used. As shown in Figure 3D, molar ellipticity of MgrA (0.12 mM) was increased slightly at 262 nm but decreased at 285 nm because of the presence of MDSA (0.3 mM), indicating
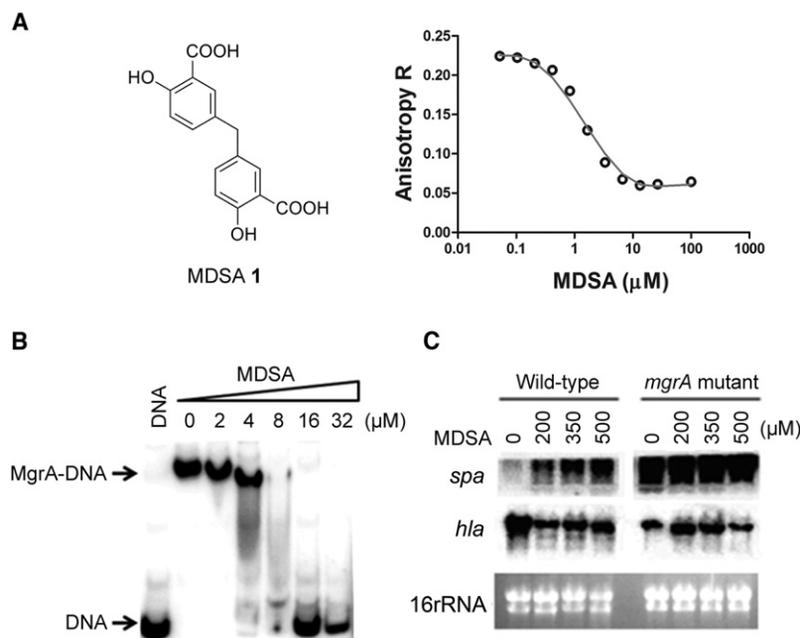
Figure 2. MDSA Inhibits DNA Binding of MgrA

(A) An FA-based binding assay shows dissociation of MgrA (1 μM) from DNA (0.01 μM) induced by MDSA (IC$_{50}$ ~8.0 μM).

(B) EMSA confirming that MDSA induces dissociation of MgrA (0.5 μM) from its cognate DNA. ^{32}P-labeled DNA PCR-amplified from the *hla* promoter region was used.

(C) Northern blot analysis showing that MDSA alters the expression of virulence factors (*spa* and *hla*) regulated by MgrA; 5 μg of total cellular RNA was used, and the ethidium bromide–stained gel of the loaded RNA sample is shown below.

See also Figure S4.

that the binding of MDSA could affect the tertiary structure of MgrA.

SAR Studies of MDSA

Multiple MarR/SarA family proteins, including the *Escherichia coli* MarR, *E. coli* EmrR, *Methanobacterium thermoautotrophicum* MTH313, and *Salmonella typhimurium* SlyA, have been shown to contain potential salicylate-binding sites; however, salicylate associates with these proteins and attenuates their DNA binding only at very high concentrations (e.g., at mM level for MarR) (Martin and Rosner, 1995; Perera and Grove, 2010). Because MDSA appears to be a dimerized salicylate, it is important to ask whether salicylate itself could disrupt the MgrA-DNA interaction. Unlike MDSA that can abolish the MgrA-DNA binding at ~8 μM, up to 250 μM of salicylate showed almost no effect on the DNA binding of MgrA, as shown in Figure 4, indicating that dual phenolic moiety is imperative for the function of MDSA. We tested other modifications. The treatment with 250 μM of 3, 3′-dimethylenebenzoic acid **6**, which lacks the OH group at aromatic ring, was unable to dissociate MgrA from DNA, suggesting that the hydroxyl group is required for the binding. The COOH group is also required for binding; the dimethyl ester of MDSA cannot disrupt the binding of MgrA to DNA. The conformation of MDSA is crucial to its interaction with MgrA. As shown in Figure 4, Olsalazine **8**, an anti-inflammatory medicine that is structurally analogous to MDSA but has a rigid *trans* configuration, could not affect the DNA binding of MgrA at 250 μM level. Collectively, all the structural features of MDSA as well as its flexible conformation are required for its ability to disrupt MgrA-DNA interaction.

MDSA Changes the Susceptibility of *S. aureus* to Antibiotics

MgrA negatively regulates the expression of efflux pumps including Tet38, NorA, NorB, and NorC, which account for bacte-

rial resistance to multiple antibiotics such as tetracycline, norfloxacin, and ciprofloxacin (Kaatz et al., 2005; Truong-Bolduc et al., 2005; Truong-Bolduc et al., 2006). MgrA is also shown to affect bacterial resistance to vancomycin (Chen et al., 2006; Trotonda et al., 2009; Truong-Bolduc and Hooper, 2007). Mutation of *mgrA* leads to increased resistance of the bacterium to these antibiotics. Therefore, MDSA should alter staphylococcal resistance to these antibiotics if it does exert inhibitory effects on MgrA inside *S. aureus*. As shown by the plate sensitivity assay in Figure 5, in the absence of MDSA, the *mgrA* mutant strain displayed stronger resistance to vancomycin (1.2 μg/ml), compared to the wild-type Newman, as expected. The phenotype can be complemented with pYJ335-*mgrA*, which restores vancomycin susceptibility to the wild-type level. In the presence of MDSA (0.2 mM), both the wild-type and complementary strain exhibited an increased vancomycin resistance that is comparable to the *mgrA* mutant strain, implying that MgrA is inhibited in both strains. Similar effects were observed on the plates containing fluoroquinolones (norfloxacin [0.8 μg/ml] and ciprofloxacin [0.3 μg/ml]); the wild-type Newman was more susceptible to fluoroquinolones than the *mgrA* mutant in the absence of MDSA, whereas the growth inhibition of the wild-type Newman caused by norfloxacin or ciprofloxacin was alleviated on the plate containing 0.2 mM of MDSA. The complementary strain carrying pYJ335-*mgrA* showed hypersensitivity toward both norfloxacin and ciprofloxacin, probably because of the elevated level of exogenous *mgrA* under this particular condition. Measurements of minimum inhibition concentration (MIC) for these antibiotics (vancomycin, norfloxacin, and ciprofloxacin) further confirmed our observation from the plate sensitivity assays. As shown in Table S1, the wild-type Newman showed greater resistance toward vancomycin, norfloxacin, and ciprofloxacin in the presence of MDSA (0.2 mM) than its absence, whereas the antibiotic resistance of its *mgrA* mutant was not affected by MDSA. In addition to the strain Newman, the *mgrA* mutant of USA300 also showed increased resistance toward vancomycin in comparison to the wild-type USA300. Similar to Newman, MDSA (0.2 mM) increased the vancomycin resistance of the wild-type USA300, but not its *mgrA* mutant (Table S1). Although the resistance of USA300 toward ciprofloxacin or norfloxacin appears to be unaffected by the mutation of *mgrA*, both of which showed equal resistance (Table S1), the presence of
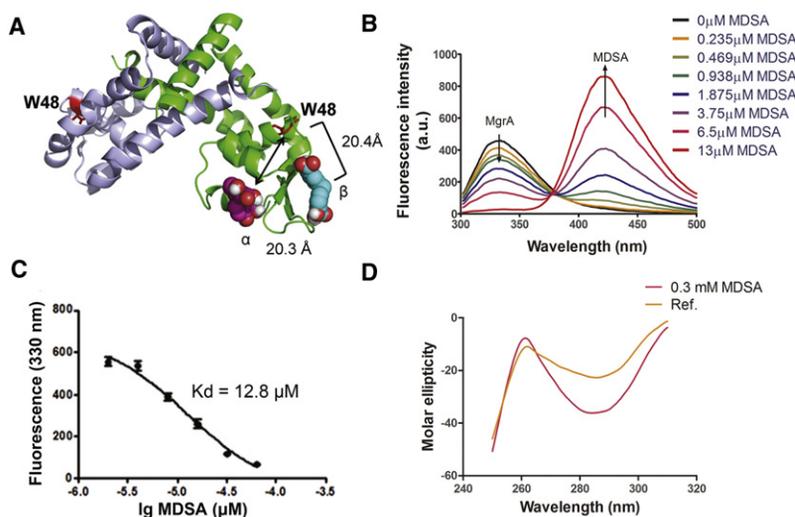
Figure 3. Binding Assays of MDSA to MgrA

(A) Docking models of MDSA binding to MgrA. Molecular docking experiment indicates two plausible binding sites (α and β) for MDSA. The distance between α site and Trp48 is 20.3 Å. The distance between β site and Trp48 is 20.4 Å.

(B) FRET generated between Trp48, the only Trp in the MgrA sequence, and the bound MDSA. Excitation was at 278 nm. Emission of Trp48 in MgrA was at 330 nm. Emission of MDSA was at 421 nm. The fluorescence of the Apo-MgrA (1 μM) was shown in black. FRET experiments were shown in colored bands.

(C) A binding curve of MDSA to MgrA obtained from the FRET experiment; 1 μM MgrA was used. The concentration of MDSA varies from 64 μM to 2 μM. Shown are the mean and standard deviation of three independent experiments.

(D) The near-UV (250–320 nm) CD spectrum of MgrA (0.12 mM) in the absence or presence of MDSA (0.3 mM). Apo-MgrA is shown in yellow. The MgrA-MDSA complex is shown in pink.

See also Figure S7.

MDSA (0.2 mM) could still induce increased fluoroquinolone resistance of not only the wild-type USA300 but also its *mgrA* mutant, implying the existence of a possible *mgrA*-independent pathway affected by MDSA in USA300. Overall, the intrinsic resistance of the *mgrA* mutant of either the strain Newman or USA300 to antibiotics was less affected by MDSA, indicating that MDSA affects *S. aureus* antibiotic resistance via an *mgrA*-dependent pathway. Together, the data suggest that MDSA inhibits MgrA inside *S. aureus*, thereby affecting functions regulated by MgrA.

MDSA Attenuates *S. aureus* Virulence in the Mouse Model of Abscess Formation

Because most virulence factors play nonessential roles in the in vitro growth of *S. aureus*, we envision that the small molecule designed for virulence suppression should not affect the viability of *S. aureus* under normal conditions, yet it should suppress in vivo pathogenesis of *S. aureus*. As shown in Figures S5 and S6, *S. aureus* displayed no growth defect in the presence of up to 10 mM of MDSA. We next employed a mouse model of abscess formation to test the potential virulence suppression function of MDSA. The diacid form of MDSA could suffer from an absorption/distribution (AD) problem once entering the mouse body. Specifically, the negative charge may prevent the molecule from efficiently crossing the tissue-blood barrier to reach infection sites. Previous pharmacokinetic (PK) studies on methyl-salicylate (esterified salicylate) have revealed that methyl salicylate maintains an extremely high permeability through blood-brain barrier and blood-tissue barrier while undergoing rapid and efficient hydrolysis inside animal tissues (Finkel et al., 2009). The liver was shown to be the major site of methyl salicylate hydrolysis (Davison et al., 1961). Because MDSA has high structural similarity to salicylate, we synthesized the dimethyl form 7 of MDSA and envisioned that hydrolysis of **7** in the liver or kidney would lead to accumulation of MDSA, which in turn could potentiate its antistaphylococcal activity inside these organs. To test this idea, we simultaneously injected *S. aureus* and **7** into mice via the retro-orbital route. After 4 days, we observed attenuated abscess formation in kidneys and livers

from mice coadministered with the compound. The number of colony-forming units (CFUs) in the corresponding organs including livers and kidneys was only ~10% of that from mice without treatment (Figure 6). Collectively, these data demonstrate that MDSA is able to prevent *S. aureus* infection in the mouse. Future modifications may improve the in vivo potency.

DISCUSSION

A major challenge facing contemporary medicine is the development of new antibiotics against human pathogens that are resilient to most current antimicrobial therapies (Borlee et al., 2010; Clatworthy et al., 2007; Park et al., 2007; Rainey and Young, 2004; Skaar, 2010; Stringer et al., 2010; Swoboda et al., 2010; Wright et al., 2005). It has been well documented that *S. aureus* has developed multiple resistance mechanisms against almost all known antibiotics, a problem that urgently requires identification of new therapeutic targets and development of alternative strategies for combating *S. aureus* (Fridkin et al., 2005; Lowy, 1998; Walsh, 1993; Weigel et al., 2003). MgrA, a member of the MarR/SarA family transcriptional regulators that controls the expression of many virulence determinants, is essential for staphylococcal virulence in animal model experiments. Therefore, inhibition of the function of MgrA is of great therapeutic potential.

Virulence regulators are promising targets for developing novel antibiotics. However, targeting virulence regulation has not been fully exploited, and only a limited number of examples are present so far (Gauthier et al., 2005; Hung et al., 2005). In *Vibrio cholerae*, an inhibitory small molecule (e.g., virstatin) obtained through HTS has been found to disrupt the dimerization and function of ToxT, a virulence transcriptional regulator, thereby preventing the expression of several critical virulence factors in *V. cholerae* (Shakhnovich et al., 2007). To our knowledge, the strategy of using a small molecule to target transcriptional regulators has yet to be applied to *S. aureus*. Our study represents an early success that demonstrates virulence suppression in *S. aureus* by targeting the MgrA regulator with small molecules.
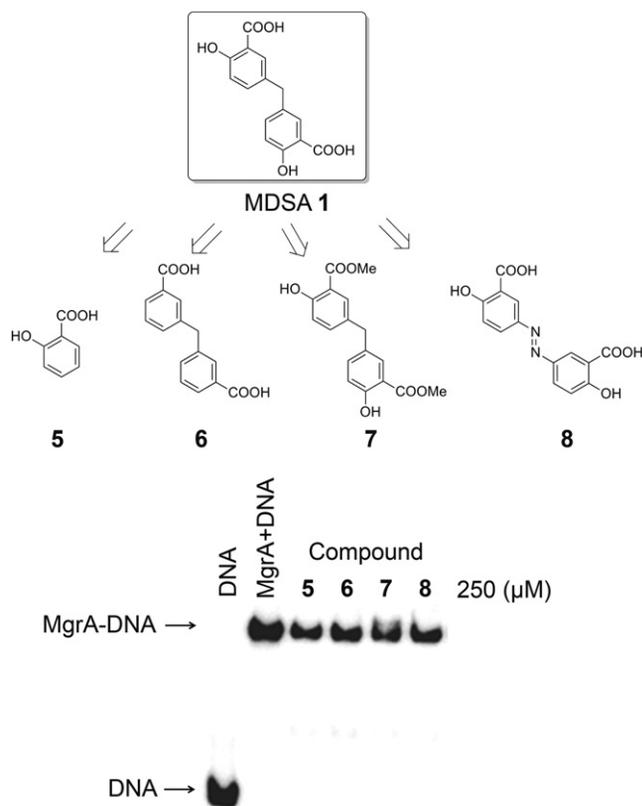
Figure 4. Structure and Activity Relationship (SAR) Studies of MDSA
Four derivatives (salicylic acid **5,** 3, 3-methylenedibenzoic acid **6,** methyl 5, 5-methylenedisalicylate **7,** and Olsalazine **8**) of MDSA are unable to disrupt the MgrA-DNA interaction at concentrations of up to 250 μM.

Structurally, MDSA is a reminiscent of salicylic acid, a prevalent plant hormone that has been shown to affect *S. aureus* in many aspects (Riordan et al., 2007). Previous studies have showed that salicylate induces increased staphylococcal resistance to multiple antimicrobials, such as the DNA topoisomerase inhibitor fluoroquinolones (ciprofloxacin and norfloxacin), the protein synthesis inhibitor fusidic acid, and the DNA-intercalating dye ethidium (Gustafson et al., 1999; Price et al., 2002). Unlike salicylate which reduces the growth of *S. aureus* at 2 mM (Riordan et al., 2007), even 10 mM of MDSA does not inhibit growth of different *S. aureus* strains tested (Figure S5). Although salicylate has been shown to down-regulate the expression of transcription factors *mgrA* and *sarR* while up-regulating *sarA* transcription (Gustafson et al., 1999), our EMSA indicated that a high concentration (250 μM) of salicylate has little effect on the DNA-binding activity of MgrA, which excludes the possibility that salicylate alters *mgrA* transcription through directly interfering its autoregulation (Ingavale et al., 2003).

The small-molecule MDSA might inhibit the DNA binding of MgrA via two possible modes. Because MgrA functions as a dimer, MDSA might disrupt the dimerization of MgrA. However, our gel filtration analysis showed that even 1 mM of MDSA was unable to change the dimeric status of MgrA (Figure S7), which excludes this possibility. The other possibility is that MDSA

directly perturbs the DNA-binding domain of MgrA. Our computational docking experiment has indicated that MgrA bears two possible binding sites for MDSA around its DNA-binding lobe (the HTH motif). This hypothesis was supported by the FRET experiment that showed close proximity of the MgrA-bound MDSA to Trp48 nearby. Potentially, MDSA binds the DNA-binding HTH motif of MgrA, which alters its conformation, thus disrupting DNA binding of MgrA.

Protein-DNA interaction is central to transcriptional regulation in biology. Despite a growing number of examples of small molecules targeting transcription (Chau et al., 2005; Darnell, 2002; Dervan et al., 2005; Kiessling et al., 2007; Lauth et al., 2007; Lu et al., 2005; Rishi et al., 2005; Vassilev et al., 2004), the process of identifying effective small molecules that exhibit activity inside cells is still challenging. In very few examples have small molecules been identified that effectively disrupt protein-DNA interactions. We show here that MDSA is able to disrupt MgrA-DNA interaction and further attenuate staphylococcal virulence expression. The MarR/SarA family proteins present excellent targets because they have long been established as transcription regulators responsive to environmental stimuli such as small molecule ligands, ROS, and pH (Perera and Grove, 2010; Wilkinson and Grove, 2006), of which the intrinsic conformational flexibility and the existence of ligand-binding pocket provide the opportunity to identify inhibitors that can bind these proteins and disrupt regulatory function. It has been recognized that most MarR/SarA proteins comply with a two-state model (Perera and Grove, 2010): the apo-protein compatible with DNA binding and the modified form incompatible with DNA binding owing to ligand binding, or post-translational modifications (PTS), such as oxidation and phosphorylation (Didier et al., 2010; Truong-Bolduc et al., 2008; Truong-Bolduc and Hooper, 2010). Given that MgrA is subjected to multiple PTSs, such as oxidation of Cys12 and phosphorylation at Ser/Thr (Chen et al., 2006; Truong-Bolduc et al., 2008), the binding of MDSA to MgrA might trigger a similar conformational change and preconfigure the protein into the second state that is incompatible with DNA binding. Future study will focus on structurally elucidating the mode of action of MDSA to gain further insight into tuning the function of MgrA with small molecules.

SIGNIFICANCE

MgrA is a global transcriptional regulator essential for the pathogenesis of *S. aureus*. It provides a potential drug target for combating staphylococcal infections. In this study, we identified a small-molecule MDSA that inhibits DNA binding of MgrA through a high-throughput screening. MDSA alters the transcriptional expression of virulence factors, including *hla* and *spa*, both of which are known to be regulated by MgrA. MDSA also affects bacterial antibiotic resistance toward vancomycin, norfloxacin, and ciprofloxacin via an *mgrA*-dependent pathway. Simultaneous administration of esterified MDSA with *S. aureus* could attenuate *S. aureus* virulence, as demonstrated in a mouse model of infection. This study demonstrates that small molecules can be developed to disrupt protein-DNA interactions of transcriptional regulators as a strategy to combat bacterial infection.
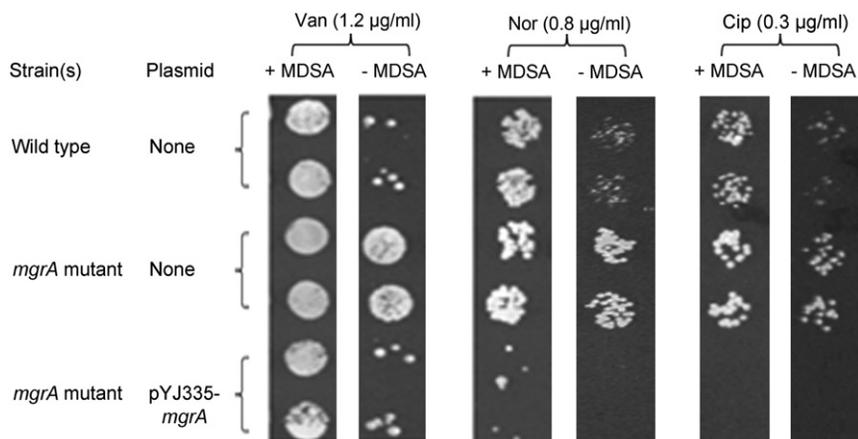
Figure 5. Effects of MDSA on Staphylococcal Antibiotic Susceptibility toward Vancomycin, Norfloxacin, and Ciprofloxacin

The antibiotic resistance levels were tested in the presence or absence of MDSA (200 μM) by a plate sensitivity assay; 10 μl of mid-log culture was diluted 1000-fold in PBS before plating in duplicate. Van, vancomycin (1.2 μg/ml); Nor, norfloxacin (0.8 μg/ml); Cip, ciprofloxacin (0.3 μg/ml). See also Table S1.

EXPERIMENTAL PROCEDURES

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich, and the restriction enzymes were purchased from New England Biolabs. Because all assays were performed under physiological conditions (pH 7.4), compound MDSA was always used in the form of disodium salt unless otherwise noted. Other organic compounds were dissolved in DMSO for biochemical and biological assays.

Ethics Statement

The animal study presented in this report (Approval ID: NW-20) was approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine-Northwest, which complies with the guidelines of the National Institutes of Health.

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains used in this study are listed in Table S2. Unless otherwise mentioned, *S. aureus* strain Newman is the strain used in the study. *S. aureus* cells were grown in tryptic soy broth (TSB). For routine culture of *mgrA* transposon mutants, erythromycin (10 μg/ml) was added, while chloramphenicol (10 μg/ml) was added to *mgrA* mutant complemented with pYJ335-*mgrA*.

Expression and Purification of the MgrA Protein

The expression and purification of His$_6$-MgrA have been described previously (Chen et al., 2006). Briefly, *E. coli* (BL21star (DE3)) carrying pet28a::*mgrA* in LB was grown at 37°C. After reaching mid-log phase (OD$_{600}$, ~0.6), cells were induced with 1 mM of IPTG for 4 hr at 30°C. Cells were harvested and stored at −78°C before use. MgrA was purified by Ni-NTA column following the manufacture's manual (GE Healthcare). The purity of MgrA was determined by SDS-PAGE. Protein was desalted to remove imidazole before subsequent biochemical assays.

Oligonucleotide Synthesis

The oligonucleotide (5′-6-F-TAAACAACAAGTTGTCCAAA-3′) containing 6-carboxyfluorescein was prepared by incorporating the 5′-fluorescein phosphoramidite (6-FAM) (Glen Research, Inc.) at the 5″ end during solid-phase synthesis. The resulting oligonucleotide was purified with denaturing polyacrylamide gel electrophoresis. The labeled oligonucleotide was annealed with its complementary strand and then used for FA-based binding assays.

FA–Based Binding Assays

To determine the binding affinity of MgrA to the labeled DNA, different amounts of MgrA, varying from 0.0015 μM to 6.0 μM, were added into the 96-well format plate containing 20 nM DNA, 10 mM Tris (pH, 7.4), and 25 mM NaCl. The Envision plate reader (475 nm/525 nm) was used to measure FA values. The K$_d$ value was calculated using Microsoft Excel. The measurements were done in triplicate.

Screen of MgrA Inhibitors

A high-throughput screen (HTS) of compound libraries to identify MgrA inhibitors was performed at The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) at Harvard Medical School. The MgrA protein (700 nM, 30 μL) was added to the black polystyrene 384-well plate, except column 24, to which 30 μl of empty control buffer (10 mM Tris [pH 7.4] and 25 mM NaCl) was added. Test compounds (10 ng in 1 μL) were added into each well except columns 23 (negative controls) and 24. The solution was incubated at room temperature for 20 min. Then 20 μL of DNA solution (50 nM) was added to all wells and the mixtures were incubated for another 20 min. The Envision plate reader (475 nm/525 nm) was used to detect FA values. For hit selections, we set up the cut-off range minus positive control (column 24) plus 50% of dynamic range (column 23 minus 24). Ninety-five compounds out of 88,564 were cherry-picked for further validation and characterization.

Fluorescence-Based Thermal Shift Assays

The thermal shift assay was conducted as previously described (Lo et al., 2004). The fluorescent dye Sypro orange (Sigma), an environmentally sensitive fluorophore, was used to monitor the unfolding of MgrA. The basis of this fluorescence-based thermal shift assay is that the protein unfolding exposes Sypro orange to a hydrophobic environment, leading to increased fluorescence of Sypro orange. This assay was performed in the iCycler iQ Real Time PCR Detection System (Bio-Rad). Solutions of 20 μl of MgrA (final concentration, 5 μM), 50 μl of 5X Sypro orange, 2 μl of compound (20 ng), and 28 μl of buffer (100 mM NaCl and 10 mM Tris [pH 7.4]) were added to the wells of the 96-well iCycler iQ PCR plate. The plate was heated from 25°C to 77°C with a heating rate of 0.5°C/min. The fluorescence intensity was measured with Ex/Em: 490 nm/530 nm. Ninety-five compounds from cherry picks were tested in duplicate. The data were processed as previously described (Lo et al., 2004).

FRET Measurement of Small Molecule Binding to MgrA

Various amounts of MDSA ranging from 64 μM to 2 μM were added to the buffer (100 mM NaCl and 10 mM Tris [pH 7.4]) containing 1 μM of MgrA. The change of fluorescence was monitored at 330 nm (MgrA) and 421 nm (MDSA), respectively. Excitation was set at 278 nm.

Circular Dichroism Spectrometry

The MgrA protein (0.12 mM) was incubated with 0.3 mM of MDSA in PBS buffer at 25°C for 10 min. Near-UV region (250–350 nm) CD spectrum was measured at 25°C by AVIV 202 CD Spectrometer (AVIV Biomedical Inc.). The protein sample without the small molecule was also tested as a comparison.

Synthesis of Compound 7

Compound **7** was obtained according to literature methods (Cameron et al., 2002; Guo et al., 2005). Briefly, MDSA (1 g) was dissolved in 20 ml of methanol and then 1 ml of HCl (~10 M) was added. The mixture was stirred and refluxed at 70°C overnight. The resulting product was isolated with silica gel column.

Electrophoretic Mobility Shift Assays

DNA probe was PCR amplified from the *hla* promoter region with primers listed in Table S3. DNA was labeled with ^{32}P at the 5′ end using T4 polynucleotide

Figure 6. Coadministration of the Dimethyl Ester MDSA 7 Attenuates Staphylococcal Virulence in a Murine Abscess Model

The test strains (1×10^7 CFU) mixed with and without compound **7** were administered into 16 mice per strain via retro-orbital injection. Four days later, after measuring weight losses, organs were harvested and bacterial CFU in the organs was measured by the serial dilution method. In the graph, each dot represents a mouse. The group not treated with small molecule is indicated as "WT." The group treated with small molecule is indicated as "15 mg/kg." The statistical difference was determined by Student's two-tailed t test. See also Figures S5 and S6.

kinase (Promega). MgrA (0.4 μM) was incubated with various amounts of test compounds and 2 ng of radioactive DNA probe in 25 μl of the binding buffer (10 mM Tris-HCl [pH 7.4], 50 mM KCl, 5 mM MgCl₂, 10% glycerol, and 3 μg/ml sheared salmon sperm DNA). After 10 min at room temperature, the samples were analyzed by 8% native polyacrylamide gel electrophoresis (100 V for pre-run, 85 V for 85 min for sample separation). The gels were dried and subjected to autoradiography on a phosphor screen (BAS-IP, Fuji).

RNA Isolation and Northern Blotting

To isolate the RNA for Northern blot analysis, all *S. aureus* strains were grown at 37°C overnight in tryptic soy broth (TSB), diluted 100-fold in fresh 10 ml TSB containing various amounts of MDSA (0, 200 μM, 350 μM, and 500 μM) in a 50-ml conical tube (BD Biosciences), and incubated at 37°C with shaking at 250 rpm for 2.5 hr (OD₆₀₀, 0.8). Cells were harvested and disrupted mechanically (Fast Prep FP120 instrument; Qbiogene, Heidelberg, Germany). The RNeasy Mini Kit (QIAGEN) was used for the subsequent RNA purification. RNA concentration and purity were determined by UV absorption at 260 and 280 nm. Northern blotting was performed following previously reported proce-

dures (Lan et al., 2010). Primers used for amplification of DNA fragments in Northern blotting are listed in Table S3.

Antibiotic Susceptibility Assays

Minimum inhibitory concentration (MIC) was determined by the microdilution method in 96-well plates. Antibiotics were serially diluted twofold in 100 μl of LB. Overnight cultures were diluted with 1× PBS to cell density of 10^7 CFU/ml. To each well of the 96-well plate, aliquots of 5 μl were added for a final inoculum of approximately 5×10^4 CFU/well. After incubation at 37°C for 24 hr, the MICs were determined visually.

Plate Sensitivity Assays for Antibiotic Resistance

TSA plates were made with designated amounts of MDSA and the antibiotic (vancomycin, ciprofloxacin, or norfloxacin). The overnight culture was diluted by 100 fold into fresh TSB containing 0.2 mM of MDSA. After 3 hr at 37°C, the mid-log culture was diluted by 1000 fold in PBS. Aliquots (10 μl) of the diluted cultures for each strain were spotted onto the solid media and grown at 37°C for 24 hr. Each experiment was repeated at least three times to ensure consistency.

Mouse Models of Abscess Formation

This assay was performed as described previously (Chen et al., 2006) with slight modifications. To determine the effect of MDSA on the virulence of *S. aureus,* its esterified derivative **7** was used. After measuring the weight of the mice, 1×10^7 CFU of the wild-type Newman strain in PBS mixed with/without small molecules (0.3 mg, the final concentration is 10 mM in the cell culture) were administered to 16 Balb/c mice (Harlan, USA) via retro-orbital injection. The average body weight of mice is ~20 g. Four days after the injection, the mice were sacrificed and their organs (kidney and liver) were harvested. The harvested organs were homogenized; then the CFU of bacteria in the organs were measured using serial dilutions on TSA plates. A Student's t test was performed to evaluate the statistical significance of the data by using Microsoft Excel. As a control experiment to ensure that the same dose of small molecules did not affect bacterial fitness in vitro, the same cell cultures in PBS, after being treated with these compounds for 1 hr at 37°C, were plated on TSA. No growth defects were observed compared to the culture not treated with small molecules.

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