

Identification of small molecules exhibiting oxacillin synergy through a novel assay for inhibition of *vraTSR* expression in methicillin resistant *Staphylococcus aureus*

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Abbreviations

MRSA, Methicillin-resistant *Staphylococcus aureus*; TCRS, two-component regulatory systems; HTS, high-throughput screening; SPR, surface plasmon resonance; Ox, Oxacillin; HK, histidine kinase

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains that are resistant to all forms of penicillin have become an increasingly common and urgent problem threatening human health. They are responsible for a wide variety of infectious diseases ranging from minor skin abscesses to life-threatening severe infections. The *vra* operon that is conserved among *S. aureus* strains encodes a three-component signal transduction system (*vra*TSR) that is responsible for sensing and responding to cell-wall stress. We developed a novel and multi-faceted assay to identify compounds that potentiate the activity of oxacillin, essentially restoring efficacy of oxacillin against MRSA, and performed high-throughput screening (HTS) to identify oxacillin potentiators. HTS of 13,840 small molecule compounds from an antimicrobial-focused Life Chemicals library, using the MRSA cell-based assay, identified three different inhibitor scaffolds. Checkerboard assays for synergy with oxacillin, RT-PCR assays against *vraR* expression and direct confirmation of interaction with *VraS* by surface plasmon resonance (SPR) further verified them to be viable hit compounds. Subsequent structure-activity relationship (SAR) study of the best scaffold with diverse analogs was utilized to improve potency and provides a strong foundation for further development.

INTRODUCTION

Staphylococcus aureus is a common commensal species and a pathogen that is a common cause of skin infections, invasive disease, endocarditis (1) and life-threatening illness. When penicillin-resistant *S. aureus* emerged shortly after penicillin was introduced into clinical practice, the use of β -lactamase insensitive β -lactams such as methicillin and congeners became the mainstay of therapy. However, it was not long before isolates with resistance to methicillin (MRSA) soon became a worldwide healthcare-associated problem. MRSA isolates are also often resistant to multiple classes of other antibiotics, with nearly universal resistance to macrolides and 70% resistance to fluoroquinolones documented in a recent study (2). In 2005, the incidence of invasive MRSA was already greater than any other invasive bacterial agent, with an incidence of 31.8 per 100,000 (3). The recent rapid spread of community-associated (CA) MRSA isolates that emerged in the 1990s has compounded the problem (4). In the US, highly virulent CA-MRSA with the genetic background of USA300 has been reported to be the most prevalent and transmissible MRSA genotype (5-7). The Infectious Diseases Society of America (IDSA), alarmed by the growing problem of antibacterial resistance, and noting the slowing of the industry-driven

pipeline for antibacterial development, launched the 10 X 20 program, calling for 10 new antibiotics by the year 2020 (8).

Two-component signal transduction in bacteria often employs a phosphorelay between a sensor transducing histidine kinase (HK) and a cognate response regulator (RR) DNA binding transcriptional activator referred to generically as two-component regulatory systems (TCRS). VraS and VraR constitute one TCRS among diverse TCRSs that exist in *S. aureus*. These enable the bacteria to respond to and survive diverse environmental stressors. His-Asp TCRSs are not found in the animal kingdom and are distinct from the Ser/Thr and Tyr kinase signaling proteins that are common in mammalian cells. Thus, TCRSs are attractive candidates for development of antimicrobials (9) because inhibitors of His-Asp kinase-RR pairs would have few unwanted side effects in mammals (9). VraS and VraR comprise a TCRS that is required for resistance to cell wall antibiotics including methicillin. Moreover, VraS belongs to the family of intramembrane kinases because its probable signaling domain is buried in the membrane (10).

VraS and VraR are encoded on a 4-gene operon (**Fig. 1**). Boyle-Vavra and colleagues demonstrated that VraS and VraR require a third putative membrane protein for their function that is encoded in the same operon (11), and is annotated as VraT (previously YvqF) (11). It is required by VraS and VraR to elicit the global transcriptional response to cell wall stress and for methicillin resistance (11, 12). VraS is a membrane HK conserved in *S. aureus* that senses stress elicited by cell wall acting antibiotics. The molecular signal is unknown, but VraS responds to this stress by homodimerization and trans autophosphorylation of a conserved His (13). ATP binds to a domain in the cytoplasmic portion of VraS that provides the energy and phosphate required for autophosphorylation. The phosphate is transferred from His to a conserved Asp55 in VraR (13). The X-ray crystal structure of VraR demonstrates that in the pseudo phosphorylated state (using Beryllium fluoride, BeF), VraR homodimerizes in both the receiver and dimerization interfaces, which increases the affinity for specific promoters, including its own, thereby initiating transcription (14). This information provides a major advance in our understanding of VraS and VraR, benefiting potential structure-based optimization of inhibitors that bind to VraS or VraR. It will also allow for identified inhibitors to be co-crystallized. The analysis of the VraR crystal structure revealed that phosphorylation induced dimerization creates a deep hydrophobic pocket in the receiver domain interface that could be a unique target site for compounds that could interfere with VraR dimerization and reduce the affinity of VraR for its target promoters (14).

The VraS, VraR and VraT proteins are each a potential target for small molecule inhibitors to act as potentiators for Oxacillin (Ox). Ox is a methicillin congener used clinically instead of methicillin. We have undertaken a translational project that seeks to identify

potentiators of methicillin and related beta lactam compounds such as Ox for the therapy of MRSA infection. Our strategy is to interfere with expression of the *VraTSR* global regulatory system that is activated by Ox and required for the optimal expression of resistance to Ox and other cell-wall acting antimicrobials such as vancomycin and daptomycin (12, 15-17). In this work, we performed HTS using a MRSA cell-based assay with an antimicrobial focused library of compounds to identify inhibitors of *VraTSR* that would increase the susceptibility of a MRSA strain to Ox and improve the efficacy of Ox therapy.

MATERIALS AND METHODS

Antimicrobial Focused Life Chemicals library.

We have previously described the detailed construction of our Life Chemicals library (18). Briefly, compounds were first selected to be 'Rule of 5' compliant, with one exception tolerated (19) and subsequently filtering out of compounds with known reactive functionalities and toxicities. Compounds were prepared in a 96 well format at 1 mM concentration of each compound suspended in 100% DMSO.

Test Strain Construction.

The test strain was generated using a USA300 MRSA clinical isolate (isolate 923) that we used previously to characterize *vraTSR* expression (15, 20) as a parent strain. To produce a reporter for *vra* operon transcription, the *vra* promoter (P_{vra}) was PCR-amplified from strain 923 using primers P_{vra} -F (5'-aaagaattctgaaggtatggtattagctattg-3' and P_{vra} -R (5'-aaaggatccggtgatgctgatgatggttg-3') containing *EcoRI* and *BamHI* restriction sites (underlined) and inserted into pXen-1 (Caliper Life Sciences) in the compatible restriction sites present upstream of the *Photorhabdus luminescens luxABCDE* operon (*lux*). This *lux* operon encodes all of the components required for luminescence, thus obviating the need for addition of exogenous substrate (21). The ligation was transformed into an *E. coli* strain TOP10 (Invitrogen), and colonies were selected on media containing ampicillin (100 µg/mL). The resulting plasmid (p P_{vra} -*lux*) was confirmed by PCR and agarose gel electrophoresis and electroporated (22) into the restriction-negative, methyl transferase+ *S. aureus* host strain RN4220 (23), before being transformed into the restriction positive clinical strains. To create the MRSA reporter strain, the p P_{vra} -*lux* plasmid was isolated from strain RN4220 and transformed into MRSA isolate 923. *S. aureus* strains containing p P_{vra} -

lux were cultured in the presence of chloramphenicol (Cm) at 10 µg/mL to maintain the reporter plasmid.

Primary HTS screening.

13,840 compounds were screened for their ability to inhibit growth of the MRSA test strain in the presence of oxacillin, and to inhibit P_{vra} -lux-dependent luminescence to test the expression of *vraTSR*. Each test compound was diluted to a final concentration of 10 µM in wells of a 96 well plate that each contained 99 µL of the reporter strain (923 pP_{vra} -lux) diluted 1:100 in tryptic soy broth (TSB) or TSB containing oxacillin (2 µg/mL) and Cm. Plates were incubated at 37°C and luminescence was recorded at 4 hours and the optical density at 600 nm (OD_{600}) was recorded at 24h post-inoculation. For each 96-well plate assayed, 80 compounds were tested, and the remaining 16 wells contained the following controls: positive control for growth and luminescence for test strain incubated with 2 µg/mL Oxacillin, negative control for growth for test strain incubated with 20 µg/mL Oxacillin, and negative control for luminescence for test strain without Oxacillin. The procedure of P_{vra} -lux luminescence consisted of inducing P_{vra} -lux in growing cells by placing oxacillin in the growth medium and then detecting decreased luminescence when a test putative inhibiting compound is added.

Following the initial single data point primary screening, compounds that inhibited growth and P_{vra} -lux luminescence were reinvestigated. The reliability of detecting P_{vra} -lux inhibition was improved by recording luminescence at 15-minute intervals for 24 h instead of a single time point and by using 4 replicates. Cultures were prepared in 96-well microtiter plates as described for the primary assay with the test wells containing the test strain incubated with Ox (2 µg/mL) combined with 10 µM of the compound. For controls, each plate contained 16 wells inoculated with the test strain in TSB alone and 31 wells with the test strain in TSB containing Ox (2 µg/mL). Growth inhibition of cultures was expressed as relative growth (RG) defined as the ratio of the OD_{600} of the culture grown in the presence of test compound and Ox to that of Ox alone. Compounds that produced a mean RG < 0.5 were categorized as “possible potentiators”. Similarly, inhibition of pP_{vra} -lux expression was expressed as relative luminescence (RL), the ratio of the maximum LU value produced by the test strain grown in the presence of test compound and Ox to that of the test strain grown with Ox alone. Compounds were categorized as possible “ P_{vra} -lux repressors” (mean RL of < 0.5). The two equations for RG (1) and RL (2) are shown below.

$$RG = \frac{Abs_{test} - Abs_{negative}}{Abs_{positive} - Abs_{negative}} \quad (1)$$

$$RL = \frac{LU_{test} - LU_{negative}}{LU_{positive} - LU_{negative}} \quad (2)$$

Filtering compounds based on the structure and molecular properties.

The rapid elimination of swill (REOS) filter is similar to the “rule of 5” filter, including property filters for MW, hydrogen bonds, charge, etc and some additional functional group filters to remove reactive and otherwise undesirable moieties (24). The Pan Assay Interference filters (PAINS) eliminates compounds containing rhodanines, phenolic Mannich bases, hydroxyphenylhydrazones, alkylidene barbiturates, etc., that are observed as frequent hits in high-throughput assays (25). Both REOS and PAINS with default parameters were used for filtering out compounds with poor lead-like properties. Additionally, compounds that were observed to be common false positives based on chemotypes (26) that can interfere with firefly luciferase were also eliminated through substructure query in Canvas (27, 28) using default parameters.

Secondary screen by checkerboard susceptibility testing.

Checkerboard MIC testing was performed to measure synergy of the test compounds with Ox, and the MIC assay conditions were in accordance with the method in CLSI M07-A8 (29). Ox was tested in 2-fold serial dilutions (1 - 64 $\mu\text{g/mL}$), and each hit compound was also tested in 2-fold serial dilutions that encompassed the MIC of the compound in the presence and absence of Ox. Both Ox and test compounds were prepared as 4X of their final concentration in TSB containing 2% NaCl. To create an 8 x 12 checkerboard, 250 μL of each concentration of Ox and each concentration of compound were dispensed across 2 x 48-well culture plates. A series of increasing concentrations of Ox alone and compound alone were also included to determine the MIC of Ox and test compound alone. All wells were inoculated with 0.5 mL of a 1×10^6 cfu/mL suspension of isolate 923 in TSB, and plates were incubated overnight at 37°C followed by evaluation of turbidity visually at 24h. The fractional inhibitory concentration index (FIC_i) was determined by equation (3) where $cMIC_{ox}$ is the MIC of Ox when used in combination with compound, MIC_{ox} is MIC of Ox alone, $cMIC_{com}$ is MIC of compound when used in combination

with Ox, and MIC_{com} is MIC of compound alone. An $FIC_i \leq 0.5$ was considered to be synergistic (30). For compounds not showing MIC, the highest concentration tested was used, and it was noted that the FIC_i is less than the resulting value, following standard practice (31).

$$FIC_i = \frac{cMIC_{ox}}{MIC_{ox}} + \frac{cMIC_{com}}{MIC_{com}} \quad (3)$$

Confirmation of *VraR* inhibition via qRT-PCR.

The qRT-PCR was used to confirm the effect of a compound on $P_{vra-lux}$ inhibition on *vraR* expression in the presence and absence of Ox. An overnight culture of strain 923 in TSB was diluted 1:100 in TSB and incubated at 37°C with shaking, and after 1 h, the test compound (final 10 µM) and Ox (2 µg/mL) were added and the culture was incubated for an additional hour. Bacteria were then incubated with lysostaphin (Ambi, 200 µg/mL) to release nucleic acid for 10 minutes at 25°C (11). cDNA was generated using the high-capacity cDNA Archive Kit (Applied Biosystems), and qRT-PCR was performed using a duplex reaction of molecular beacons and primers obtained from Integrated DNA Technologies. *vraR* expression was detected using the primers *vraR*-Forward (5'-tcgtcgcttctacacatccatgt-3') and *vraR*-Reverse (5'tagttggggaaggcgcttctggta-3') with the *vraR* probe (5'6-FAM/ttgccaaag/ZEN/cccatgagt tgaagcca/3IABkFQ) in a duplex assay with a *gyrB*-Forward primer (5'-aacggacgtggtatcccagttgat-3), *gyrB*-Reverse primer (5'-ccgccaattaccaccagcatgt-3') with the *gyrB* probe (5'Cy5/aaatgggacgtccagctgtcgaagtt/3IAbRQSp) as the endogenous control. For *sgtB*, *sgtB*-Forward (5'-cagctgataacatgccagagta-3') and *sgtB*-Reverse (5'-tgcacatctctgtcgctaac-3') were used with *sgtB* probe (5' 6-FAM/agggtgcctt/Zen/tatttcaatggaagatgaacg/3IABkFQ). The ABI Prism 7300 (Applied Biosystems) was used for qRT-PCR reactions with the following parameters: melt at 95°C for 30 seconds, anneal at 55°C for 30 seconds and elongate at 72°C for 30 seconds. Relative quantification (RQ) was done using the comparative $\Delta\Delta Ct$ method facilitated by the ABI Prism 7300 Sequence Detection Software (version 1.2.3, Applied Biosystems). Stimulation of *vraR* expression by a compound was defined as an RQ value ≥ 2 relative to the reference sample. Repression of Ox-induced *vraR* expression and *vraR* neutral values were defined as an RQ value ≤ 0.7 and > 0.7 and ≤ 1.5 , respectively, relative to the reference.

Determination of the dissociation equilibrium constant (K_D) by SPR.

His-tagged VraS-C (cytoplasmic domain, residues 70-345), VraR (full-length, residues 1-209), and VraT (cytoplasmic domain, residues 120-233) were cloned into pET28a between BamHI and HindIII and over-expressed in BL21(DE3) cells. Over-expressed proteins were purified by HisTrap affinity and Superdex 75 size exclusion columns and prepared in PBS buffer containing 10 mM phosphate, pH 7.4, 2.7 mM KCl, 137 mM NaCl, 0.05% Tween-20, and 0.5 mM TCEP. The CM5 sensor surface was first activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxy succinimide (NHS) mixture using a Biacore T200 instrument (GE Healthcare). VraS-C, VraR and VraT were diluted in 10 mM sodium acetate (pH 4.0) and immobilized to flow channels 2, 3 and 4, respectively, followed by ethanolamine blocking on the unoccupied surface area. The unmodified surface was used on the first channel as a blank control. Test compound solutions with a series of increasing concentrations (0 – 100 μ M at 2-fold dilution) were applied to all four channels at a 30 μ L/min flow rate at 25 °C in a SPR binding buffer consisting of 10 mM HEPES, 150 mM NaCl, 0.05% Tween-20, 1 mM MgCl₂, 0.05 mM EDTA and 0.5 mM TCEP. Sensorgrams were analyzed using Biacore T200 evaluation software V3.0, and response units at each concentration were measured during the equilibration phase for steady-state affinity fittings. Equation (4) is shown below, where y is the response, y_{max} is the maximum response and x is the analyte protein concentration. All data were referenced with blank channel RU values.

$$y = \frac{y_{max} \cdot x}{(K_D + x)} \quad (4)$$

Inhibition of VraS kinase.

A kinase assay was developed to simultaneously monitor auto-phosphorylation activity of VraS and its phospho-transfer to VraR using the commercially available ADP-Glo assay kit (Promega) (32). The VraS cytoplasmic domain with both kinase and ATP-binding domains was over-expressed in *E. coli* and purified. 5 μ L of 3X (250 nM final concentration) VraS enzyme prepared in kinase assay buffer (50 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl₂, and 0.5 mM DTT) was distributed in a low-volume 384-well plate. 5 μ L of a series of increasing concentrations of compound solution was added and incubated for 10 min at room temp. Enzyme reaction was initiated by adding 5 μ L of 3X ATP (30 μ M) plus VraR (3 μ M) and kept for 45 minutes. An equal volume of ADP-Glo reagent was then added to quench the reaction for 40 min followed by addition of kinase detection reagent as described in the company protocol. Luminescence signal was

measured by an Optima microplate reader (BMG LABTECH). The IC₅₀ values were calculated by fitting with the Hill equation (5), using Sigmaplot 12.0 where *y* is percent inhibition, *x* is inhibitor concentration, *n* is the slope of the concentration–response curve (Hill slope), and *V*_{max} is maximal inhibition.

$$y = V_{max} \left(\frac{x^n}{IC_{50}^n + x^n} \right) \quad (5)$$

Bactericidal Activity

Bacteria were diluted to a density equivalent to a 0.5 McFarland standard in 0.9% saline and then suspended in 1 mL plain Mueller Hinton broth (MHB) (cation adjusted) or MHB containing Ox and/or compound at the indicated concentrations shown in parenthesis (µg/mL) in 24 well cell culture plates to yield a final concentration of ~0.6 x 10⁶ CFU/mL. The plates were then incubated at 37°C, and the bacterial counts were ascertained at 0, 2, 4, 8, and 24 h after appropriate dilution on TS and overnight incubation.

Selectivity.

The essential penicillin binding protein PBP1, the other essential two component regulatory system, Walk and WalR, and an unrelated protein PurC were cloned in pET15b between NdeI and BamHI with a N-terminal His₆-tag. All four proteins were over-expressed in BL21(DE3) bacterial expression cells and purified by a similar method to VraS-C. SPR binding analyses were performed with these four additional proteins against the main lead compounds in a similar manner as the K_D determination.

HepG2 Toxicity assay.

Cultured HepG2 cells were plated at 2 x 10⁵ mL⁻¹ and grown to confluent monolayers. Media was removed and replaced with fresh media containing controls and test compound dilutions. After 24 hours of compound exposure, triplicate wells are tested for viability using 0.5 mg/mL filter sterilized thiazolyl blue tetrazolium bromide in phosphate buffered saline (PBS) pH=7.0 and were incubated for 1 hour at 37°C. The reagent was aspirated, and the cells were lysed and formazan crystals solubilized by the addition of 0.04 M HCl in isopropanol with 0.5% SDS for 10 minutes at room temperature. Absorbance was measured using a BMG PolarStar Optima plate reader. Background absorbance was subtracted and triplicate wells were averaged. The percent

reduction in viability for all groups was calculated from equation (6). 50% inhibitory concentrations (IC₅₀) were calculated using InSTAT software by linear regression.

$$y = \left(\frac{\text{Media control} - \text{Experimental}}{\text{Media control}} \right) 100 \quad (6)$$

Cytochrome P450 Inhibition assay.

Inhibition of cytochrome P450 3A4 was measured using the standard substrate BOMCC using the Select Screen P450 Profiling assay (Life Technologies). The assay uses P450 Baculosomes® which are microsomes prepared from insect cells infected with recombinant human P450 isozyme and rabbit NADPH-cytochrome P450 reductase. Inhibition of cytochrome P450 activity is measured by the ability to prevent formation of fluorescent signal from a substrate. Inhibition is determined by comparing the fluorescence intensity of wells containing test compound to those containing a blank diluent. IC₅₀ values were calculated from dose response curves.

RESULTS AND DISCUSSION

High-throughput screening (HTS) and hit selection

The antimicrobial focused Life Chemicals library of 13,840 compounds was screened by High-throughput screening (HTS) using the MRSA cell-based assay. The overall screening and hit validation process is summarized in **Fig. 2A** along with the number of hits from each step. A specifically built USA300 MRSA clinical isolate (isolate 923) was used for HTS in order to select only oxacillin (Ox) potentiators. Lux transcriptional fusion with the *vra* operon promoter is induced only in the presence of Ox. Even when P_{vra} was present upstream of *luxABCDE*, lux was silent when Ox was not present. Thus, P_{vra}-lux was induced only when Ox was added to the culture medium, and the inhibition of P_{vra}-lux expression was expressed as relative luminescence (RL) in the P_{vra}-lux luminescence experiment. Each plate contained a total of eight positive controls, four negative controls in the absence of Ox, and another four negative controls in the presence of Ox. The inhibition effect of each compound on cell growth and induction of P_{vra}-lux was monitored by single data point measurement for initial primary HTS (Z'-factor of 0.5 ± 0.1), produced 369 hit compounds with RL below the cut-off value of 0.5. Of 369 compounds, 280 compounds were subsequently confirmed in quadruplicate with luminescence signal measurements at every 15 min for 24 hours. The next step was to use computational filtering to

eliminate false positives and potential toxic and promiscuous compounds. Among the 280 compounds, thirty compounds were filtered out by the REOS filter; an additional 6 compounds were eliminated by the PAINS filter, and about 200 compounds were eliminated as possible false positives based on luciferase interfering chemotypes. Oxadiazoles are known to interfere with firefly luciferase signal, and there are 220 oxadiazole-amide class (almost 80%) among the 280 compounds. However, the top five oxadiazole-amides with high inhibitory activity were still retained for the follow-up assays. 35 non-oxadiazole hits with maximum structural diversity that passed the REOS/PAINS filters were selected, resulting in a total of 40 compounds for the secondary assay.

Hit validation by testing synergy and inhibition of *vraR* expression

The 40 compounds that passed computational filters were further analyzed for synergy with Ox by testing with two-dimensional checkerboard assays using the MRSA isolate 923 (11). Checkerboard assays were performed in 96-well plates, with increasing oxacillin concentrations along one dimension and increasing test compound along the second direction. We also tested whether the inhibition of luminescence detected by hit compounds was an artifact due to interference with lux rather than expression of P_{vra} , which would have given a false positive in the screen. To this end we tested the ability of the compound to inhibit light production from a transcriptional fusion between lux and the promoter from the constitutively expressed pyruvate dehydrogenase gene (*Ppdh-lux*) in *S. aureus* (RN4220). Compounds were eliminated from consideration if they inhibited luminescence expressed from *Ppdh-lux*. Of 40 hit compounds, 22 compounds exhibited synergy with oxacillin, defined as having an FIC ≤ 0.5 , and six best synergistic compound structures are shown in **Fig.2B**. These 22 compounds were further validated by monitoring repressed *vraTSR* expression using qRT-PCR, resulting in five confirmed hits with differing chemical scaffolds that truly inhibit *vraR* expression and the *sgtB* gene that is known to be strongly *vraTSR*-dependent (11). It is important to note that since *vraT*, *vraS* and *vraR* are encoded on the same mRNA, the *vraR* probe we used is a proxy for the entire operon. These five hits contained a triazine diamine, two sulfonamides, a 4-(1-(3-bromophenyl)cyclohexyl)phenol, and a urea, three of which were chosen for further studies. Our most important consideration in hit selection was the degree to which the Ox MIC was lowered by the compound since we are seeking potentiators of Ox and not necessarily compounds with inhibitory activity of their own. Although compound F2645-0188 (an oxadiazole) did not inhibit the expression of *vraR* and *sgtB*, we still included it for follow-up studies because it did decrease

Ox MIC at 16-fold, which is similar to F5882-3050 (urea) and F2619-0556 (sulfonamide). Quality control data of F5882-3050 indicated that the active compound is a di-substituted urea, Lee-3464 (**Fig. 2B**), rather than the structure provided by the vendor. This will be discussed in further detail in the following section. The MICs of Ox in strain 923 were lowered by at least 16-fold for all five compounds, three of which were subjected to qRT-PCR *vraR* and *sgtB* expression analysis (**Fig. 3**). Compound F5882-3050/Lee-3464 decreased both Ox induction of *vraR* and *sgtB* significantly ($P < 0.001$), whereas F2619-0556 slightly decreased them (**Fig. 3B** and **3D**). Interestingly, F2645-0188 increased both Ox induction of *vraR* and *sgtB* ($P < 0.05$), suggesting a mechanism different from the action of F5882-3050/Lee-3464 (**Fig. 3F**). In addition, these three compounds decreased the Ox MIC 8 to 64-fold in three additional MRSA strains (Data not shown). The remaining three compounds in **Fig. 2B** were not pursued further due to limited availability of analogs and structures that were less amenable to medicinal chemistry improvement.

Confirmation of direct interaction and enzyme activity inhibition of VraS

To further clarify whether these lead compounds directly interact with the *vra* operon targets, we performed binding assays between the lead compounds and the purified VraS cytoplasmic domain with both kinase and ATP-binding domains using surface plasmon resonance (SPR). The binding affinities of F5882-3050/Lee-3464 and F2645-0188 to VraS were similar at 14.4 μM and 12.1 μM , respectively, while that of F2619-0556 was slightly weaker at 25.2 μM (**Fig. 4A**), confirming direct interaction. We also developed a kinase assay to simultaneously monitor auto-phosphorylation activity of VraS and its phospho-transfer to VraR using the commercially available ADP-Glo assay kit (32). Half maximal inhibitory activity (IC_{50}) of F5882-3050/Lee-3464, F2619-0556 and F2645-0188 were determined to be similar at 9.7 μM , 7.0 μM and 4.0 μM , respectively. These three hit compounds were classified to three distinct scaffolds, urea, sulfonamide and oxadiazole (**Fig. 4A**). In order to improve potency of validated lead scaffolds, 15 additional commercially available urea and 20 oxadiazole compounds were purchased and investigated. One additional urea compound, F0017-0250 and four oxadiazoles (F1374-2739, F2518-0327, F1374-0033, and F1374-0037) were identified as good Ox potentiators from this process. In **Fig. 4B**, qRT-PCR results are shown for the extent to which one of the urea analogs, F0017-0250, inhibits induction of *vraR* and *sgtB* expression by Ox. In the presence of Ox, the *vra* operon expression is induced about 29-fold, as expected and expression of the *vra* operon in the presence of Ox was inhibited 67 % by F0017-0250. We focus here on the urea compounds as the primary leads since they exhibit the highest amount of *vra* operon inhibition among our lead

compounds. The urea compound F0017-0250 had a binding affinity (K_D) of 7.7 μ M for VraS, and kinase inhibitory activity (IC_{50}) was determined to be 13.2 μ M, showing similar activity to the original urea lead F5882-3050/Lee-3464 (**Fig. 4C**). Fitting curves of F0017-0250 with VraS are shown in Supplementary Fig. S1 to determine binding affinity (K_D) and IC_{50} values as examples.

Re-synthesis of lead compounds

Two lead compounds (F0017-0250 and F2619-0556) were re-purchased while F5882-3050 was re-synthesized for further validation. The re-synthesized F5882-3050 had no synergy with Ox, no inhibitory activities on VraR and SgtB expressions, and no binding to VraS. The inactivity of the re-synthesized F5882-3050 indicated that the original activity resulted from an impurity or mis-assigned compound structure of the commercial source. Therefore, the identity and purity of both the original and synthesized batches of F5882-3050 were evaluated by LC-MS analysis. During this analysis, it was found that the original batch of F5882-3050 was not pure and primarily contained a compound with a higher molecular weight than expected. This compound appeared to be a di-substituted analog of urea; to confirm this, the di-substituted indazole was synthesized (Lee-3464 in **Fig. S2**, synthesis depicted in **Scheme S1**). The synthesized Lee-3464 exhibited synergy with Ox (FIC_i = 0.28) and also showed binding to VraS-C at K_D value of 7.7 ± 5.0 μ M, consistent with activity of the commercial sample. LC-MS analyses of F0017-0250 and F2619-0556 confirmed that their purity was above 90%, and multiple different batches exhibited similar synergistic effect and inhibitory activity.

Ability of lead compounds to potentiate Ox in multiple MRSA strains

Compounds with an Ox potentiating effect against MRSA strains from diverse genetic backgrounds could be good synergistic agents that could potentially be used in the MRSA infection therapy. After preliminary assays, we narrowed our focus to seven compounds to test in combination with Ox against a panel of up to 105 MRSA strains from diverse genetic backgrounds (**Table 1** and **Table S1**). Two urea compounds (the primary leads), a sulfonamide (secondary lead) and four oxadiazoles (tertiary leads) were tested against the indicated number of MRSA strains from 15 diverse genetic backgrounds. All compounds in **Table 1** showed a decreasing MIC of Ox at least 4-fold (μ g/mL) compared with using Ox alone (range 17-fold to 81-fold). In addition, a mean FIC_i close to 0.5 was achieved for both lead urea compounds, the sulfonamide, and two of the oxadiazoles (F1374-2739 and F2518-0327). Note that the urea

compound Lee-3464 synergized (FIC I \leq 0.5) in 79% of tested isolates). The sulfonamide F2619-0556 synergized in 69% of tested isolates.

Bactericidal Activity

Next, we determined time kill kinetics of at least one compound from each lead scaffold (Lee-3464, F2619-0556, and F1374-0037) to test for bactericidal activity when using the combination of Ox and compound. Tested at its synergistic effective concentration, all three compound combinations decreased CFU by $\sim 10^3$ to $\sim 10^5$ fold compared to un-treated or un-combined treatment after 24 hours incubation, demonstrating that all these three compound combinations potentiate the inhibition of growth (**Fig. 5**). However, of three compound combinations, only Lee-3464 combination resulted in a largest decrease ($\sim 10^3$ fold) in CFU from the start inoculation, while F1374-0037 and F2619-0556 combinations resulted in a ~ 50 -fold and ~ 3 -fold decrease respectively, suggesting that Lee-3464 combination is bactericidal and F2619-0556 combination is bacteriostatic.

Selectivity against other targets.

To address selectivity against other essential targets, we used SPR to measure the interaction of our lead compounds with the essential penicillin binding protein PBP1 and the essential two component regulatory system, Walk and WalR, and an unrelated protein PurC from *Bacillus anthracis*. We find that neither of the ureas nor the sulfonamide exhibited any specific binding to PBP1, Walk or WalR (**Table 2**). Two of the oxadiazoles, F1374-0033 and F1374-2739, exhibit moderate binding to Walk and WalR, perhaps consistent with their observed antimicrobial activity. However, the results indicate that neither our primary nor our secondary scaffold lead compounds exhibit interaction with other proteins that are closely involved in Ox resistance, indicating that there is significant specificity of interaction with VraS, even though these are early selection library compounds. Due to the varied degree of non-specificity, oxadiazoles were not pursued further.

Cytotoxicity and Cytochrome P450 (Cyp450) Inhibition assay

Representative lead compounds were evaluated for cytotoxicity and Cyp450 inhibition (**Table 3**). As shown, all compounds were tested since the IC₅₀ was greater than 5X the synergistic

concentration for each compound tested. As shown in **Table 3**, the criteria of the IC₅₀ being greater than 5X synergy is substantially exceeded by the lead urea, F0017-0250, and by the two oxadiazoles, F1374-0037 and F1374-0327. The sulfonamide, F2619-0056, exhibits Cyp450 inhibition at a concentration about the same as the synergy concentration, and thus will require further structural modification to achieve adequate VraS inhibition selectivity. Thus, we identified a primary and at least two backup lead compounds that synergize Ox activity, and that have multiple characteristics suitable for further drug development, including cytotoxicity IC₅₀ greater than 5X the concentration required for Ox synergy and cytochrome P450-3A4 inhibition IC₅₀ greater than 5X the concentration required to achieve synergy with Ox.

Synthesis of lead compounds and analogs.

A separate set of ~40 new urea analogs were developed for testing using SPR and checkerboard assays. These analogs were of two subseries: 1) urea containing adamantane group and a heteroaromatic group and 2) urea comprised of an trimethylbicyclo[3.1.1]heptan-3-yl) functionality. Of 40 urea compounds, two showed synergy with Ox, one of which is shown in **Fig. 6A**. A similarity search using this active urea compound was performed to identify additional compounds of interest from the St. Jude Children's Research Hospital small molecule library. This provided a set of 159 urea analogs, all of which contain 3,4-dichloroaniline moiety. After initial activity profiling of this compound set, selected analogs were re-synthesized and re-tested to confirm activity (Supplementary data Tables S2 and S3). The active analogs can be categorized into five scaffolds: di-aniline compounds, compounds containing a benzylic substituent, pyridine-containing compounds, piperazine, and piperidine-containing compounds (with some of these scaffolds containing multiple of the above stated substituents). Twelve of the di-aniline compounds had MIC < 12.8 µg/mL. A variety of substituents were tolerated at the *meta* or *para* positions of the aromatic ring of these compounds, whereas those containing an *ortho* substituent were not active. Five of the benzylic compounds had MIC < 12.8 µg/mL and compounds with a *meta* fluorine or *para* fluorine on the benzylic ring displayed good activity. Two analogs from each of the pyridine, piperidine, and piperazine series gave MIC < 12.8 µg/mL. The SAR resulting from these analogs will guide future work to improve compound activity and physicochemical properties, and the resulting SAR is shown in **Fig. 6**.

CONCLUSIONS

We have developed a novel and multi-faceted assay to identify compounds that potentiate the activity of oxacillin, essentially restoring efficacy of oxacillin against MRSA. The potentiator compounds act by suppressing activation of the *vra*TSR operon in the presence of cell wall stress, which also suppresses multiple other downstream cell wall stress genes, restoring bacterial sensitivity to oxacillin and congeners. The assay includes a rapid method for identifying oxacillin potentiators, along with evaluation of *vra*TSR operon suppression and direct confirmation of binding to and inhibition of VraS. Using this assay, we identified compounds with multiple scaffolds that potentiate oxacillin activity. SAR analysis of the initial hit urea series provides a strong foundation for further development.

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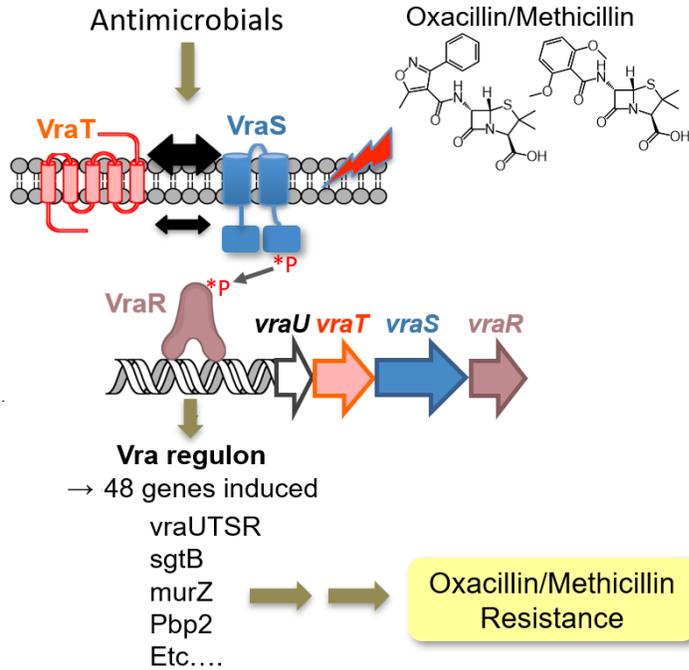


Figure 1. Model for signal transduction between VraTSR and antibiotic resistance. Cell wall stress imparts a signal that leads to phosphorylation of VraS, which in turn leads to phosphorylation of VraR that subsequently activates a regulon of about 48 genes in the cell wall stress response. This figure was modified from the Figure 6 of Boyle-Vavra *et al* (11).

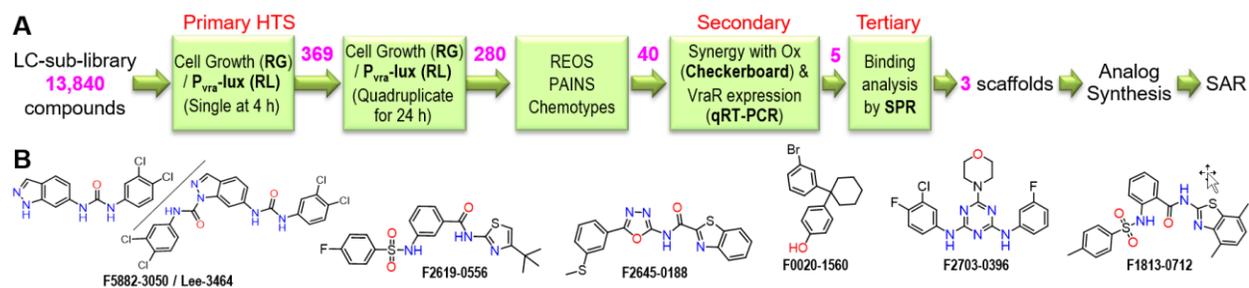


Figure 2. High-throughput screening (HTS) results from Life Chemicals library and hit validation. (A) Schematic of HTS and hit validation process. The numbers in pink indicate the number of compounds that passed each stage of the screen. (B) Structures of six compounds. Quality control analysis and re-synthesis of F5882-3050 determined that the active compound is a di-substituted urea, Lee-3464, rather than the putative structure provided by the vendor.

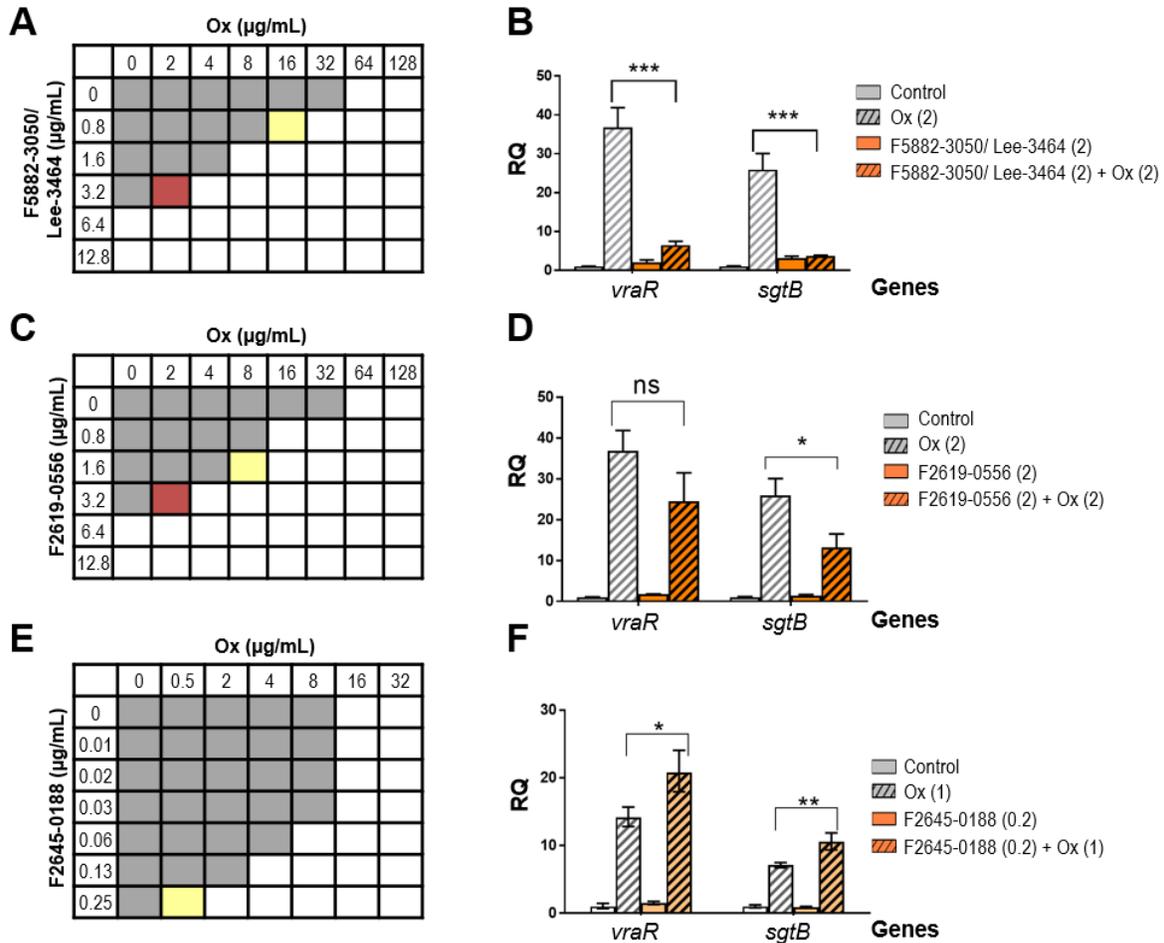


Figure 3. Three HTS lead compound effects on Ox MIC and *vra* operon expression. Checkerboard MIC results of lead compound F5882-3050/Lee-3464 (**A**), F2619-0556 (**C**), and F2645-0188 (**E**). The yellow boxes indicate the ideal FIC index, and brown boxes indicate the concentration of compound with the lowest Ox MIC. The yellow and brown boxes are the same for Fig. 3E. qRT-PCR results of F5882-3050/Lee-3464 (**B**), F2619-0556 (**D**), and F2645-0188 (**F**). qRT-PCR assay results show lead compound effect on *vra* operon expression. Numbers shown in parentheses are concentrations of Ox and tested compounds. The test compounds (0.2 $\mu\text{g/mL}$ or 2 $\mu\text{g/mL}$) and Ox (1 $\mu\text{g/mL}$ or 2 $\mu\text{g/mL}$) were added to the culture after one hour of growth and incubation time was extended for an additional hour before harvesting RNA for the RT-PCR. Statistical significance has been shown on the Figures. ***, $P < 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ns, $P > 0.05$.

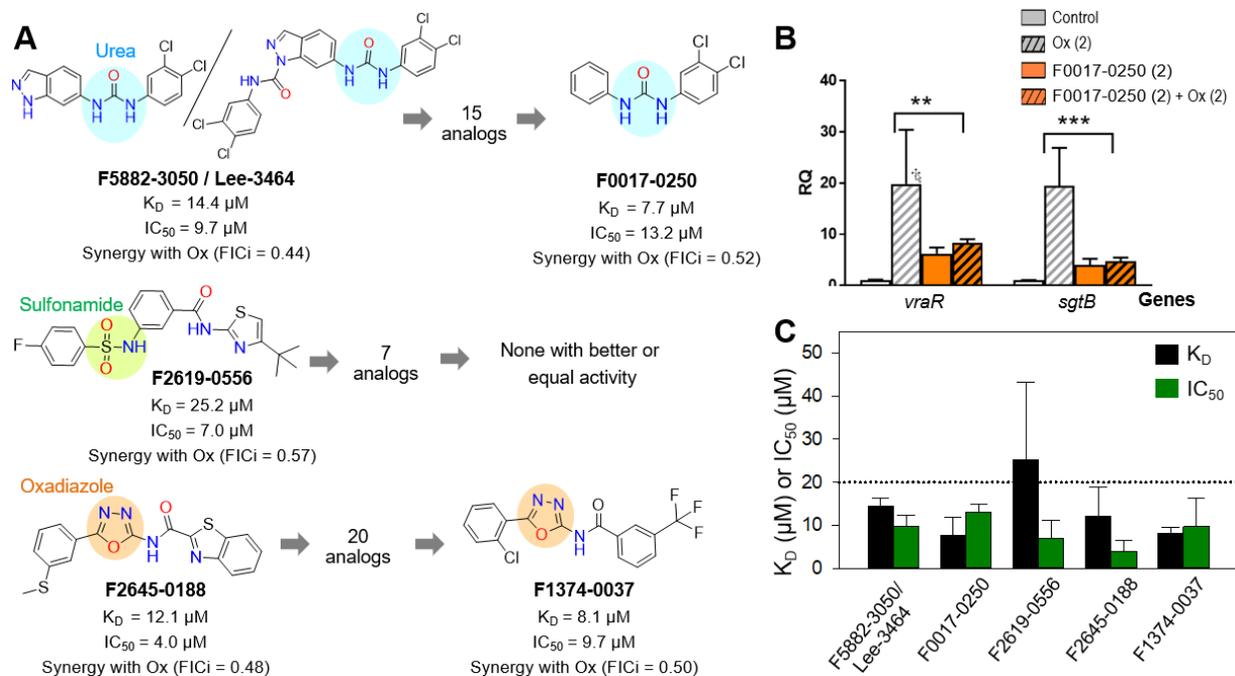


Figure 4. Three main lead scaffolds and analogs. (A) Structures and activities of three lead compounds and newly discovered analogs. (B) qRT-PCR results of F0017-0250. Statistical significance has been shown on the Figures. ***, $P < 0.001$; **, $P \leq 0.01$. (C) The binding affinities (K_D) and VraS kinase inhibitory activities (IC_{50}).

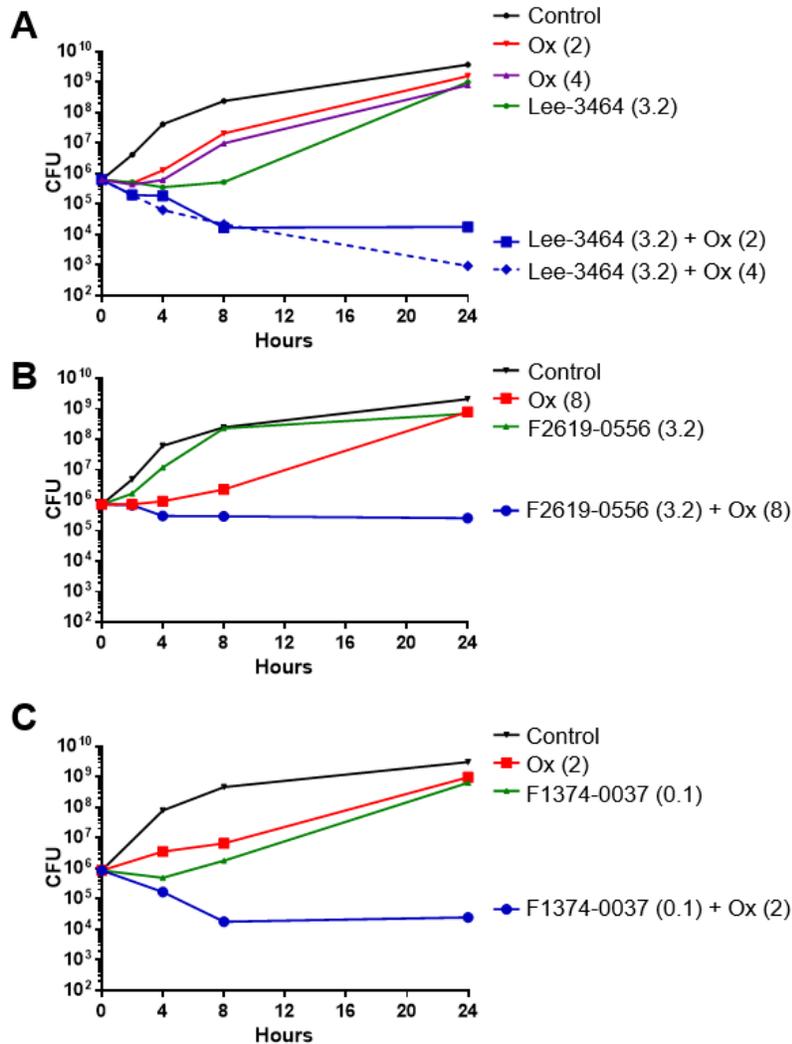


Figure 5. Bactericidal activities. Bacterial kill curves of USA300 MRSA exposed to combinations of Ox + Lee-3464 (A), F2619-0556 (B) and F1374-0037 (C). Bacteria were diluted to a density equivalent to a 0.5 McFarland standard in 0.9% saline and then suspended in 1 mL plain Mueller Hinton broth (MHB) (cation adjusted) or MHB containing Ox and/or compound at the indicated concentrations shown in parenthesis ($\mu\text{g}/\text{mL}$) in 24 well cell culture plates to yield a final concentration of $\sim 0.6 \times 10^6$ CFU/mL. The plates were then incubated at 37°C , and the bacterial counts were ascertained at 0, 2, 4, 8, and 24 h after appropriate dilution on TS and overnight incubation.

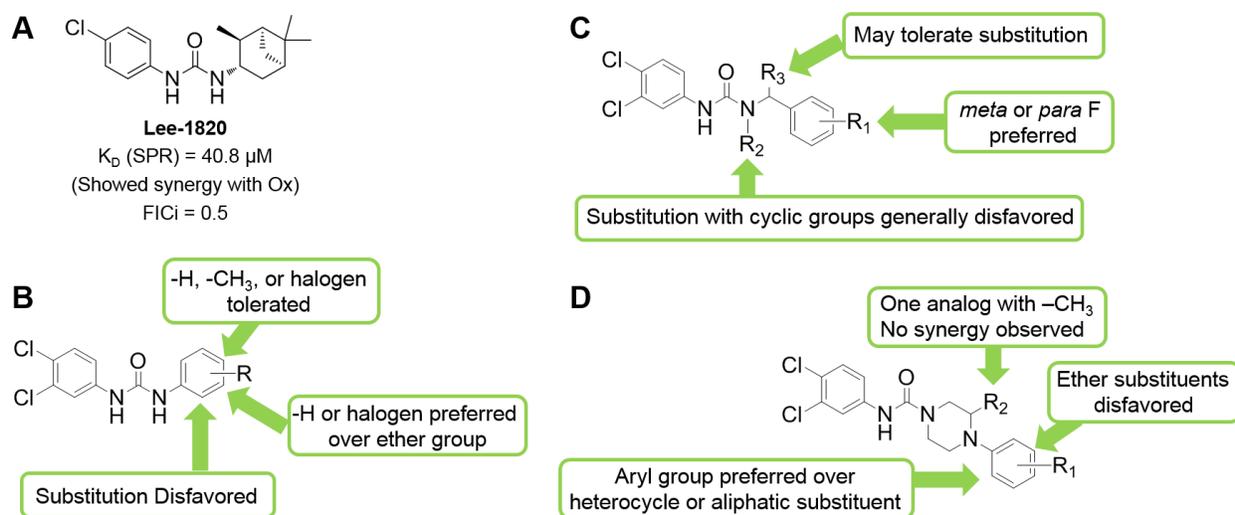


Figure 6. Preliminary SAR of urea scaffold. (A) Slightly different urea scaffold identified from a total of 40 adamantane urea analogs. (B) SAR based on di-aniline urea analogs. (C) SAR based on benzylic urea series. (D) SAR based on piperazine urea series.

Table 1. Checkerboard assays with multiple MRSA strains.

Compound	Scaffold	Number of strains tested	*Ox MIC ($\mu\text{g/mL}$)	\wedge N (%) FIC _i ≤ 0.5	**FIC _i (range)	#Fold decrease MIC Ox
Lee-3464	Urea	105	8	83 (79%)	0.4	30
F0017-0250	Urea	82	4	35 (43%)	0.5 (0.08-1.0)	45
F2619-0556	Sulfonamide	104	16	72 (69%)	0.5 (0.09-1.0)	48
F1374-0037	Oxadiazole	104	8	39 (37.5%)	0.56 (0.07-1.0)	32
F1374-0033	Oxadiazole	12	24	3 (25%)	0.6(0.5-.75)	56
F1374-2739	Oxadiazole	32	6	15 (47%)	0.5 (0.16-1.0)	48
F2518-0327	Oxadiazole	79	2	40 (50%)	0.5 (0.13-1.0)	25

*The median of the low Ox concentration among all strains tested in the checkerboard assay; ** the median FIC index; \wedge Number (%) of strains with med FIC index ≤ 0.5 ; #The average ideal decrease in Ox MIC when used with compound among the tested strains.

Table 2. Selectivity against other targets besides Vra operon proteins (dissociation constant (K_D [μM]) as determined by SPR.

	K_D (μM) by SPR						
	VraS	VraR	VraT	PBP1	WalK	WalR	PurC
F0017-0250	7.7 \pm 4.2	22 \pm 4	NB	NB	NB	NB	NB
Lee-3464	14 \pm 2	39 \pm 6	NB	NB	NB	NB	NB
F2619-0556	25 \pm 18	NB	NB	NB	NB	NB	NB
F1374-0037	8.1 \pm 1.4	NB	NB	NB	NB	NB	NB
F1374-0033	20 \pm 4	NB	29 \pm 13	43 \pm 8	18 \pm 5	35 \pm 16	NB
F1374-2739	70 \pm 12	6.6 \pm 1.8	11 \pm 2	NB	2.6 \pm 1.3	5.1 \pm 1.5	NB
F2518-0327	9.8 \pm 3.0	6.7 \pm 1.8	14 \pm 4	NB	NB	NB	NB

NB: No binding

Table 3. Characteristics of Lead Compounds.

ID	MIC Compound ($\mu\text{g/mL}$)	VraS Binding K_D (μM)	VraS kinase inhibition IC_{50} (μM)	Mean FIC _i	Synergy μM ($\mu\text{g/mL}$)	5X Synergy μM ($\mu\text{g/mL}$)	Cytotoxicity IC_{50} ($\mu\text{g/mL}$)	Cyp450 inhibition IC_{50} (μM)
F0017-0250	3.8	7.7 \pm 4.2	13 \pm 2	0.52	5.2 μM (1.47)	26 μM (7.4)	195 μM (54.8)	>523
Lee-3464	7.7	14 \pm 2	9.7 \pm 2.6	0.44	4 μM (1.33)	20 μM (6.65)	333 μM (106.9)	16
F2619-0556	9.2	25 \pm 18	7.0 \pm 4.2	0.53	6.6 μM (2.9)	33 μM (14.5)	125 μM (54.1)	4

