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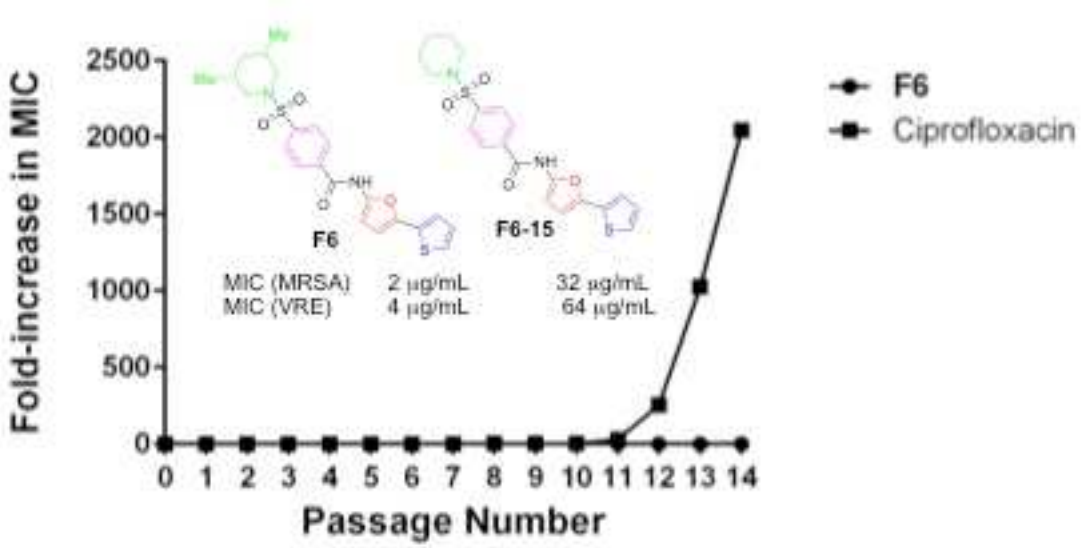

N-(1,3,4-oxadiazol-2-yl)benzamide analogs, bacteriostatic agents against methicillin- and vancomycin-resistant bacteria

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Abstract

Various reports of multidrug-resistant bacteria that are immune to all available FDA-approved drugs demand the development of novel chemical scaffolds as antibiotics. From screening a chemical library, we identified compounds with antibacterial activity. The most potent compounds, **F6-5** and **F6** inhibited growth of various drug-resistant Gram-positive bacterial pathogens at concentrations ranging from 1 μg/mL to 2 μg/mL. Both compounds were active against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA respectively) and vancomycin-resistant *Enterococcus faecalis* (VRE). Resistance generation experiments revealed that MRSA could develop resistance to the antibiotic ciprofloxacin but not to **F6**. Excitingly, **F6** was found to be non-toxic against mammalian cells. In a mouse skin wound infection model, **F6** was equipotent to the antibiotic fusidic acid in reducing MRSA burden.

Keywords: Antibiotic resistance, multidrug-resistant bacteria, bacteriostatic

Highlights

- The antibacterial agent, **F6** possesses potent activity against drug-resistant Gram-positive pathogens.
- MRSA could not develop resistance to **F6**.
- Bacterial burden in skin wound infection could be reduced by **F6**.

Introduction

The discovery and development of antibiotics revolutionized health care in such a way that bacterial infections, which were otherwise deadly, could be treated[1, 2]. However, this was met with a rapid development of resistant bacterial strains that rendered many antibiotics ineffective[3]. Consequently, millions of people are infected with drug-resistant bacterial strains yearly resulting in thousands of deaths. In the US, the Centers for Disease Control and Prevention in 2013 estimated that approximately 23,000 people died from infections caused by drug-resistant

bacterial pathogens at an annual infection rate of about 2 million. The cost to treat such recalcitrant infections exceeds $20 billion per year[4, 5].

It has been suggested that resistance to antibiotics has developed over the years via a myriad of processes including the inordinate use of antibiotics and the lack of development of new antibiotics[3]. The wide gap between emergence of drug-resistant pathogens and the development of novel antibacterial therapeutics has been attributed to the non-profitable nature of the venture (it costs several millions of dollars to conduct clinical trials and the high probability of bacterial resistance emerging against a new antibiotic hinders investment in antibiotic discovery)[2, 3]. Efforts however, need to be directed towards identifying and developing novel structures as antibacterial agents with possibly novel mechanisms of action[2]. It is projected that in the absence of new antibacterial agents, annual mortality rates could exceed 10 million by the year 2050[6].

As noted above, nearly 23,000 fatalities due to antibiotic-resistant infections occurs each year in the US; surprisingly, nearly half of these deaths is linked to one bacterial pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA)[5]. Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) is the principal causative agent for skin and soft tissue infections (SSTIs) in North America[7, 8]. Strains such as MRSA USA300 and MRSA USA400 constitute the most isolated agents in SSTIs[9-11]. Others including USA100 and USA200 have been primarily isolated from hospital-acquired MRSA (HA-MRSA) infections[12]. Diseases including sepsis, endocarditis, and pneumonia could also result from MRSA infection[13, 14]. Clinical isolates of MRSA have been identified that are resistant to several antibiotics. Vancomycin, a glycopeptide antibiotic remains the reference standard for the treatment of multi-resistant MRSA infections[13, 15]. However, there is an emergence of MRSA strains that are resistant to vancomycin including various vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) isolates[15, 16]. When used alone, MRSA strains easily develop resistance to rifampicin, one alternative for treating MRSA infections. Hence rifampicin is usually administered together with a second antibiotic like fusidic acid[15]. Many other antistaphylococcal antibiotics including ciprofloxacin suffer from resistance generation[15, 17]. There is an obvious need for clinicians to be armed with new antibiotics that are less likely to fail due to resistance generation. Consequently, several research groups including ours have programs to understand the mechanisms of resistance and how to inhibit or reverse them.[18-23] Research into the development of promising antibacterial agents with potent activity against drug-resistant bacteria has also increased.[21, 24-27]

We have identified novel structures (**Figure 1**) with potent antibacterial activities against drug-resistant Gram-positive bacteria. In particular, these molecules exhibit potent antibacterial activity against staphylococcal and enterococcal strains including MRSA, VISA, VRSA, and vancomycin-resistant *Enterococcus faecalis* and *E. faecium* (VRE). The most promising compound identified was further evaluated against multiple clinical isolates of MRSA *in vitro* and *in vivo* against MRSA USA300 in a murine wound infection model.

Materials and Methods

Bacterial strains and chemical compounds

All MRSA isolates were acquired from BEI Resources. The remaining bacteria were purchased from the American Type Culture Collection (ATCC). Compounds F3 (cat. no. F0559-0091), F4 (cat. no. F0559-0342), F5 (cat. no. F0559-0343), F6 (cat. no. 0559-0346), F9 (cat. no. 0608-0426), G8 (cat. no. F1821-0760) and G9 (cat. no. F1821-0778) were purchased from Life Chemicals Inc., (Ontario, Canada).

Screening of compounds for antibacterial activity against *S. aureus*

Library compounds and analogs of **F6** were dissolved in DMSO at 10 mg/mL. *S. aureus* was cultured in Mueller Hinton Broth to early exponential phase at which point culture aliquots were incubated with compounds at 16 µg/mL or DMSO in duplicates. The culture was continued at 37 °C for 24 hours. Aliquots (100 µL) of the cultures were dispensed into clear 96 well microtiter plates and OD_{600} was recorded. Percent normalized OD_{600} was obtained by using the equation

$$\%Normalized\ OD_{600} = \left(\frac{X - X_o}{X_T - X_o}\right) \times 100$$

Where for a given compound, X is the OD_{600} of culture with the compound, X_o is that of media only and X_T is the OD_{600} of the DMSO control.

Determination of the MIC and MBC

The minimum inhibitory concentration (MIC) of compounds and control antibiotics (methicillin, linezolid and vancomycin), tested from 128 µg/mL to 1 µg/mL, was determined using the broth microdilution method[28] against the selected bacterial pathogens. Bacteria were cultured in cation-adjusted Mueller Hinton Broth (for strains in Tables 1 and 4) or Brain Heart Infusion broth (for *Enterococcus faecium*) or Tryptic Soy Broth (all other bacteria) in a 96-well plate at 37 ºC for at least 20 hours. The MIC was classified as the lowest concentration where no visual growth of bacteria was observed. The minimum bactericidal concentration (MBC) was tested by spotting 4 µL from wells with no growth onto Tryptic Soy Agar (TSA) plates. Plates were incubated at 37 ºC for at least 18 hours before recording the MBC.

Time-kill analysis

The time-kill analysis was performed as previously described[29]. MRSA USA300 cells in logarithmic growth phase were diluted to 2.92×10^6 colony-forming units per mL (CFU/mL) and exposed to concentrations equivalent to either 6 × MIC (in triplicate) of compound **F6**, linezolid or vancomycin in Tryptic Soy Broth. Aliquots (100 µL) were collected from each treatment after 0, 2, 4, 8, 12, and 24 hours of incubation at 37 °C and subsequently serially diluted in phosphate-buffered saline (PBS). Bacteria were then transferred to TSA plates and incubated at 37 °C for 18-20 hours before viable CFU/mL was determined.

Toxicity profile of F6

Compound **F6** was assayed (at concentrations ranging from 2 µg/mL to 256 µg/mL) against murine macrophage (J774) and human colorectal (Caco-2) epithelial cell lines to determine the potential toxic effect to mammalian cells *in vitro*. Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), non-essential amino acids (1X), and penicillin-streptomycin at 37 °C with CO_2 (5%). J774 cells were cultured in DMEM supplemented with 10% FBS. Upon reaching 85-90% confluency, cells were transferred to all wells of a 96-well tissue-culture treated plate. The cells were incubated in

serum-free medium with the compounds (in triplicate) at 37 ºC with CO_2 (5%) for 24 hours. Cells exposed to equivalent concentrations of DMSO served as the negative control. The assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Promega, Madison, WI, USA) was subsequently added and the plate was incubated for four hours. Absorbance readings (at OD_{490}) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the viability of DMSO-treated control cells (average of triplicate wells ± standard deviation). The toxicity data were analyzed via a two-way ANOVA, with post hoc Sidak's multiple comparisons test ($P < 0.05$), utilizing GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

Multistep resistance selection

To determine if MRSA would be capable of forming resistance to compound **F6** quickly, a multi-step resistance selection experiment was conducted, as described previously[29]. The broth microdilution assay was utilized to determine the MIC of compound **F6** and ciprofloxacin exposed to MRSA USA400 (NRS123) over 14 passages during a period of two weeks. Resistance was classified as a greater than four-fold increase in the initial MIC, as reported elsewhere[30].

Murine MRSA wound infection model

The murine MRSA skin infection was conducted as described in a previous report[31], following the Purdue University Animal Care and Use Committee (PACUC) and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Three groups (n = 5) of eight-week old female BALB/c mice (obtained from Envigo, Indianapolis, IN, USA) were used in this study and received an intradermal injection (40 μL) containing 1.32×10^9 CFU/mL MRSA USA300. After the formation of an abscess/open wound at the site of injection for each mouse, topical treatment was initiated with each group of mice receiving the following: fusidic acid (2%) or **F6** (2%) twice daily for five days. One group of mice was treated with the vehicle alone (petroleum jelly, negative control). Each group of mice was individually housed in a ventilated cage with appropriate bedding, food, and water. Mice were checked at least four times daily during infection and treatment to ensure no adverse reactions were observed. Mice were humanely euthanized via CO_2 asphyxiation 12 hours after the last dose was administered. The region around the skin wound was aseptically excised and subsequently homogenized in PBS. The homogenized tissue was then serially diluted in PBS before plating onto mannitol salt agar plates. The plates were incubated for at least 16 hours at 37 °C before viable CFU were counted and MRSA reduction in the skin wound post-treatment was determined for each group (relative to the negative control).

Results and Discussions

Identification of antibacterial compounds

We developed a program to identify compounds with potent activity against drug-resistant bacterial pathogens. A library of compounds (both commercially available and synthetic compounds synthesized in our laboratory) was initially screened, at a concentration of 16 μg/mL, for their ability to inhibit bacterial growth. Several compounds, which included **F3**, **F4**, **F5**, **F6**, **F9**, **G8** and **G9** (**Figure 1**) were initially screened against *S. aureus*. Compounds **F3**, **F4**, **F5**, **F6** and **G8** significantly inhibited the growth of *S. aureus* (**Figure 2**). Compared to the DMSO control, compound **F9** was not active whilst compound **G9** only slightly inhibited growth (**Figure 2**).

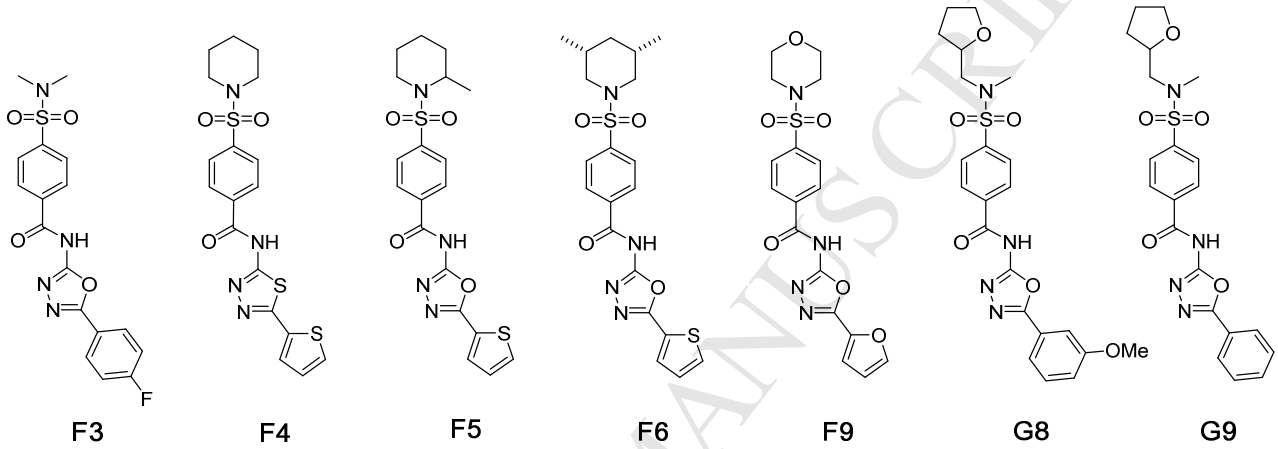


Figure 1. Structures of antibacterial compounds. Note: F6 (*cis* : *trans* = 10:1). Compounds were obtained from Life Chemicals Inc. (Ontario, Canada).

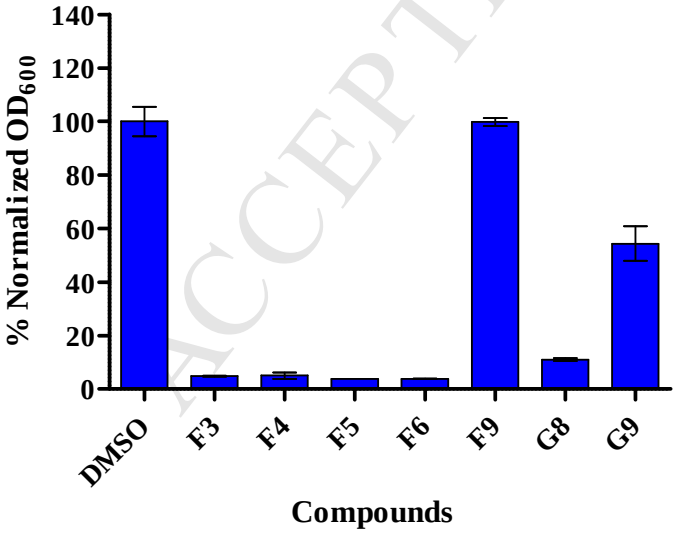


Figure 2. Inhibition of growth of *S. aureus* ATCC 25923 by antibacterial compounds. *S. aureus*, at early exponential growth, was treated with either DMSO or 16 µg/mL of compounds and OD600 measured after 24 h. Error bars represent standard error of the mean of duplicates.

To further characterize the antibacterial properties of the active compounds, we determined their minimum inhibitory concentrations (MIC) against a clinically-relevant panel of Gram-positive bacterial species including MRSA, vancomycin-sensitive *E. faecalis*, VRE and *Listeria monocytogenes*. Based on their activity from the growth inhibition experiment, we determined the MIC only for compounds **F3**, **F4**, **F5**, **F6** and **G8**. The compounds inhibited growth of all strains tested, at concentrations ranging from 2 to 32 µg/mL (**Table 1**).

The presence of methyl substitution on the cyclohexyl moiety of compounds **F4**, **F5** and **F6** is the only structural difference present between the compounds. With the two methyl substitutions, compound **F6** was the most potent compound identified followed by **F5** which has one methyl substitution and then **F4** which has an unsubstituted cyclohexyl moiety (**Figure 1** and **Table 1**). This implies that the substitution on the cyclohexyl moiety may be important for antibacterial activity.

The most potent compound, **F6**, was observed to inhibit growth of *S. aureus* (including MRSA), *E. faecalis* (including VRE), and *L. monocytogenes*, at concentrations ranging from 2 to 4 µg/mL. Compound **F6** and the antibiotic vancomycin were equipotent against *S. aureus* and MRSA (MIC = 2 µg/mL). Impressively, compound **F6** was greater than 32 times more potent than methicillin against MRSA. It was also observed that **F6** was more potent than vancomycin against a strain of *E. faecalis* resistant to vancomycin, with the MIC of **F6** more than 31-fold lower than that of vancomycin.

Table 1. MIC (µg/mL) of compounds screened against a panel of Gram-positive bacterial pathogens.

Test agents	**Bacterial Strains**				
	***S. aureus* ATCC 25923**	**MRSA ATCC 33592**	***E. faecalis* ATCC 29212**	**VRE (*E. faecalis*) ATCC 51575**	***L. monocytogenes* ATCC 19115**
F3	16	16	32	32	32
F4	16	16	32	32	16
F5	8	8	16	16	16
F6	2	2	4	4	4
G8	32	32	32	32	32
Vancomycin	2	2	2	>128	1
Methicillin	2	>128	ND	ND	ND

ND represents not determined

F6 is bacteriostatic against drug–resistant Gram-positive bacteria

Having observed the potent activity of **F6** against a single isolate of MRSA and VRE, we proceeded to confirm the compound's potent antibacterial activity against additional strains of MRSA, VISA, VRSA, and VRE (**Table 2**). Compound **F6** was found to be active against the selected panel of clinical isolates of MRSA at a concentration of 2 µg/mL (**Table 2**). Of note, MRSA USA300 and MRSA USA400 are the main culprits isolated from MRSA skin and soft-tissue infections in North America[10, 11]. Additionally, **F6** (MIC of 2 µg/mL) retained its potent antibacterial activity against clinical isolates of *S. aureus* and *E. faecium* exhibiting high-level resistance to vancomycin (MIC > 128 µg/mL), an agent of last resort for treatment of most MRSA infections[32]. Linezolid was potent against most clinical isolates of MRSA and VRSA at ≤1 µg/mL (**Table 2**). However, linezolid was inactive against MRSA NRS119, a strain isolated as linezolid-resistant; **F6**, in contrast retained its potent activity against this strain (MIC = 2 µg/mL). Interestingly, compound **F6** appears to be a bacteriostatic agent as its minimum bactericidal concentration (MBC) value exceeded >128 µg/mL. This was similar to the results obtained for linezolid, an antibiotic known to exhibit bacteriostatic activity *in vitro* against MRSA[33, 34].

Table 2. The minimum inhibitory concentration (MIC, in µg/mL) and minimum bactericidal concentration (MBC, in µg/mL) of **F6** and select antibiotics.

	F6		**Linezolid**		**Vancomycin**	
Bacterial Strain	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
MRSA NRS119	2	>128	32	32	≤1	≤1
MRSA NRS123 (USA400)	2	>128	≤1	64	≤1	≤1
MRSA NRS384 (USA300)	2	>128	≤1	64	≤1	≤1
MRSA NRS385 (USA500)	2	>128	≤1	2	≤1	2
MRSA NRS386 (USA700)	2	>128	≤1	128	≤1	≤1
MRSA NRS387 (USA800)	2	>128	≤1	128	2	2
VISA NRS1	2	>128	≤1	1	4	4
VRSA VRS12	2	>128	≤1	32	>128	>128
***E. faecium* ATCC 700221 (VRE)**	2	128	≤1	64	>128	>128

As observed from Table 2, the MBC of **F6** was generally >128 µg/mL, several folds above the MIC, an indication that the compound was bacteriostatic. We sought to further ascertain whether **F6** was indeed bacteriostatic. From time-kill analysis using MRSA USA300, at 6× MIC of **F6** (12 µg/mL), we observed that **F6** caused a 2.01-$\log_{10}$ reduction in MRSA USA300, which was just slightly higher than the 1.85-$\log_{10}$ reduction observed with linezolid after a 24-hour incubation period. On the other hand, the bactericidal antibiotic vancomycin completely

eradicated the MRSA USA300 inoculum within 12 hours. These observations imply that **F6**, just like linezolid, exhibits *in vitro* bacteriostatic effect against MRSA USA300 (**Figure 3**).

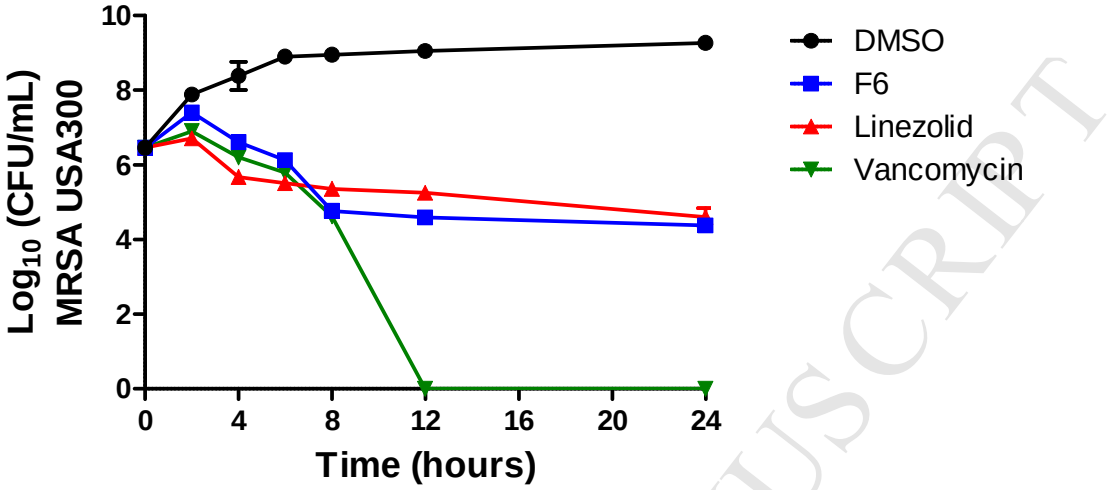


Figure 3. Time-kill analysis of **F6** against MRSA USA300 using linezolid as a control antibiotic. MRSA USA300 was incubated with **F6** (12 µg/mL) or linezolid (6 µg/mL) vancomycin (6 µg/mL) or DMSO and the number of cells estimated at the indicated time points. Experiment was performed in triplicates.

F6 is not active against Gram-negative bacteria

We next moved to investigate whether **F6** would be effective against Gram-negative bacterial pathogens as well. Hence, we determined the MIC of **F6** against a selected panel of clinically-relevant Gram-negative bacterial pathogens. Compound **F6**, was not active against *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* BW25113. The lack of activity against Gram-negative bacteria appears to be due to **F6** being a substrate for efflux. This can be seen by the shift in the MIC observed for compound **F6** against wild-type *E. coli* BW25113 (MIC > 128 µg/mL) in comparison to a mutant strain (*E. coli* JW5503-1) where the AcrAB-TolC multidrug-resistant efflux pump is knocked out (MIC for **F6** improves to 2 µg/mL). A similar result was observed with linezolid and erythromycin, two antibiotics known to be substrates for the AcrAB-TolC efflux pump in Gram-negative bacteria[35, 36].

Table 3. MIC of **F6** against selected Gram-negative bacterial pathogens.

Bacterial Strain	**Test agents**			
	F6	**Linezolid**	**Erythromycin**	**Colistin**
Acinetobacter baumannii ATCC 19606	128	N.D.	N.D.	≤ 1
Klebsiella pneumoniae BAA-1706	>128	N.D.	N.D.	≤ 1
Pseudomonas aeruginosa ATCC 15442	>128	N.D.	N.D.	≤ 1
Escherichia coli BW25113	>128	>128	32	N.D.

Escherichia coli (Δ*tolC*) JW5503-1	2	8	≤ 1	N.D.

ND represents not determined

MRSA does not develop resistance to F6

One of the major challenges in treatment of bacterial infections is the rapid generation of resistant pathogens. In treatment of MRSA infections, antibiotics like ciprofloxacin fail due to resistance[15, 16]. We performed the multistep resistance selection to evaluate the ability of MRSA USA400 to develop resistance to **F6** *in vitro*. The MIC of compound **F6** remained unchanged over nine passages (**Figure 4**). A one-fold increase in the MIC of **F6** was observed after the tenth passage where after no additional increase in MIC was observed up to the 14th passage. This indicates MRSA is unlikely to form rapid resistance to **F6** *in vitro*, even after multiple passages. In contrast, the MIC of ciprofloxacin, an antibiotic that targets DNA gyrase, increased three-fold after the eighth passage and continued to rapidly increase thereafter. MRSA resistance to ciprofloxacin emerged after the eleventh passage (an eight-fold increase in MIC was observed) (**Figure 4**). By the 14th passage, the MIC of ciprofloxacin increased more than 2000-fold from the original MIC value (0.25 µg/mL). The emergence of MRSA resistance to ciprofloxacin agrees with previously published reports [17, 29, 37].

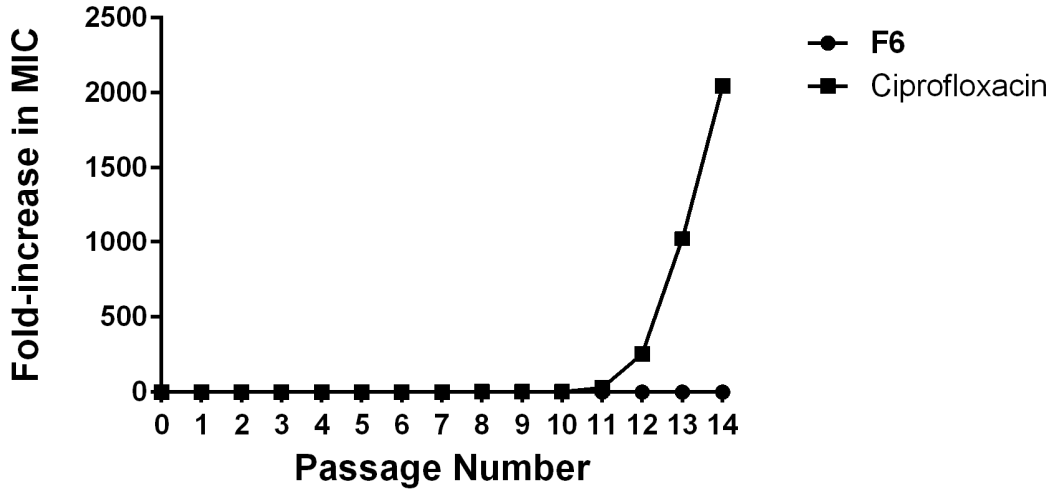


Figure 4: Multi-step resistance selection of compound F6 and ciprofloxacin against MRSA. MRSA USA400 was serially passaged daily over a 14-day period and the broth microdilution assay was used to determine the minimum inhibitory concentration of both **F6** and ciprofloxacin (control antibiotic) against MRSA after each successive passage. A four-fold shift in MIC would be indicative of bacterial resistance forming to the test agent.

F6 is non-toxic against mammalian cells

As earlier stated, MRSA is responsible for SSTIs[7, 8]. Compound **F6** demonstrated *in vitro* potency against several important MRSA strains. Prior to evaluating **F6** in an animal model of MRSA skin infection, we determined the toxicity profile of **F6** against mammalian cells. The compound was incubated with murine macrophage (J774) cells and human colorectal (Caco-2)

cells at concentrations ranging from 2 µg/mL to 256 µg/mL. Compound **F6** exhibited an excellent safety profile against both J774 and Caco-2 cells (**Figure 5**) as the compound was found to be non-toxic up to 128 µg/mL (63-fold higher than the MIC of **F6** against MRSA).

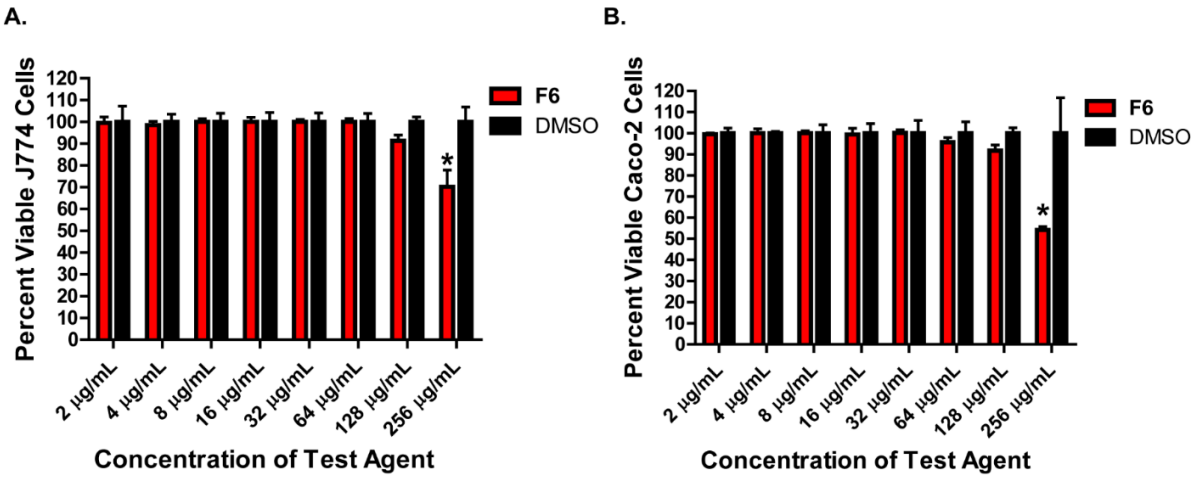


Figure 5. Toxicity analysis of F6 against mammalian cell lines. Percent viable mammalian cells (measured as average absorbance ratio (test agent relative to DMSO)) after exposure to compound **F6** (tested in triplicate) at concentrations ranging from 2 to 256 µg/mL against **A)** murine macrophage (J774) cells, or **B)** human colorectal (Caco-2) cells using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Dimethyl sulfoxide (DMSO) was used as a negative control to determine a baseline measurement for the cytotoxic impact of each compound. Error bars represent standard deviation values for triplicates. A two-way ANOVA, with post hoc Sidak's multiple comparisons test, determined statistical difference (denoted by the asterisk) ($P < 0.05$) between the values obtained for **F6** and DMSO (negative control, used as solvent for the compound).

F6 reduces MRSA burden in mouse skin wound infection

Having determined that F6 was not toxic, an established mouse skin wound infection model[38, 39] was used to assess the *in vivo* efficacy of **F6**. Mice were infected with MRSA USA300, the predominant strain responsible for *S. aureus*-based SSTIs in North America. After the formation of an abscess, the wound was treated twice daily for five days with either **F6**, fusidic acid, or the vehicle (petroleum jelly) alone. It was observed that **F6** (0.59-$\log_{10}$, 72.41% reduction) was as effective as the control antibiotic fusidic acid (0.71-$\log_{10}$, 77.91% reduction) in reducing the burden of MRSA in the wounds of infected mice after only five days of treatment (**Figure 6**). The data garnered from the skin infection mouse model further confirms the potent antibacterial effect of **F6** against MRSA.

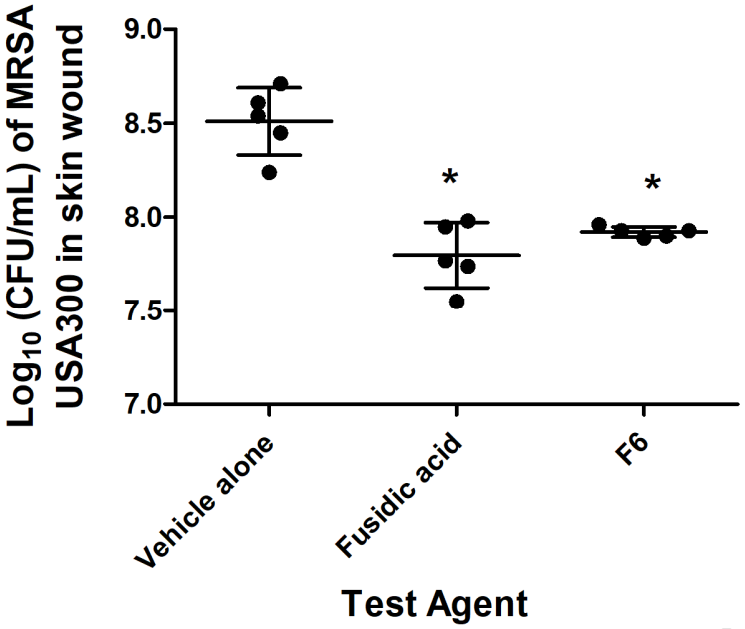
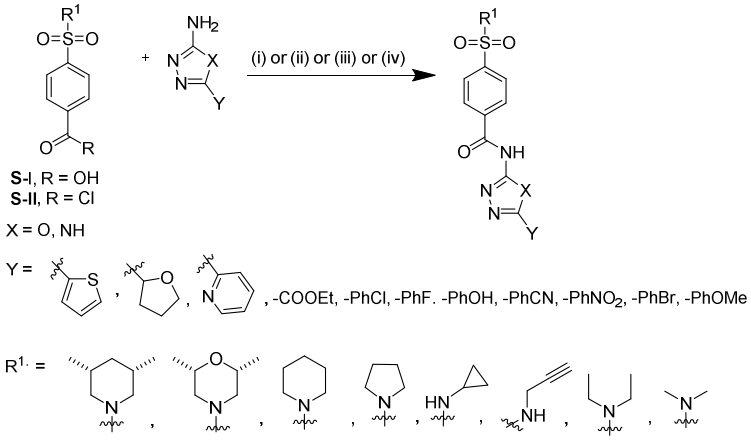


Figure 6. Efficacy of F6 in an *in vivo* mouse skin wound infection model. Average $\log_{10}$ reduction in MRSA USA300 CFU/mL in wounds of mice after five days (two doses per day) of treatment. A one-way ANOVA with post-hoc Dunnet's multiple comparisons found statistical significance (***, $P < 0.05$) between mice treated with fusidic acid and **F6**, compared to mice receiving the vehicle (petroleum jelly) alone.

F6 analogs with potent antibacterial activity

With such impressive antibacterial properties, we wondered whether structural analogs of **F6** could have better activity. We therefore synthesized 20 compounds (**Figure 7**) by making modifications to groups on **F6** and evaluated their ability to inhibit the growth of *S. aureus* at 16 µg/mL (**Figure 8**). For compounds that showed activity against *S. aureus* in the growth inhibition assay, we proceeded to determine the MIC (**Table 4**). It was observed that installation of a morpholine (**F6-1**) instead of a piperidine or deletion of the sulfonamide group (**F6-14**) abolished activity. Growth inhibition was not significantly affected upon deletion of the dimethyl-substitutions on the piperidine (**F6-16**). However, from their MIC values, **F6-15** was not as active as **F6**. These suggested that the 4-((3,5-Dimethylpiperidin-1-yl)sulfonyl)benzamide was relevant for activity. Deletion of the amide linkage between the benzene ring and the oxadiazole ring resulted in compound **F6-4**, which was not active. Also, activity was lost when the oxygen in the oxadiazole ring was replaced with NH (**F6-6**), highlighting the importance of the oxadiazole moiety. We also investigated the importance of the thiophene ring for antibacterial activity. Replacement of the thiophene ring with either a tetrahydrofuran or an acid ester resulted in inactive compounds **F6-2** and **F6-3** respectively. Interestingly, unlike **F6-2** and **F6-3**, replacement of the thiophene ring with a chlorophenyl, bromophenyl, methoxyphenyl, or fluorophenyl resulted in **F6-5, F6-7, F6-12, and F6-13** respectively, which were all found to be inhibit the growth of *S. aureus*. Impressively, the MIC of these compounds against the tested bacterial pathogens ranged from 1 µg/mL to 4µg/mL (**Table 4**).

A.



B.

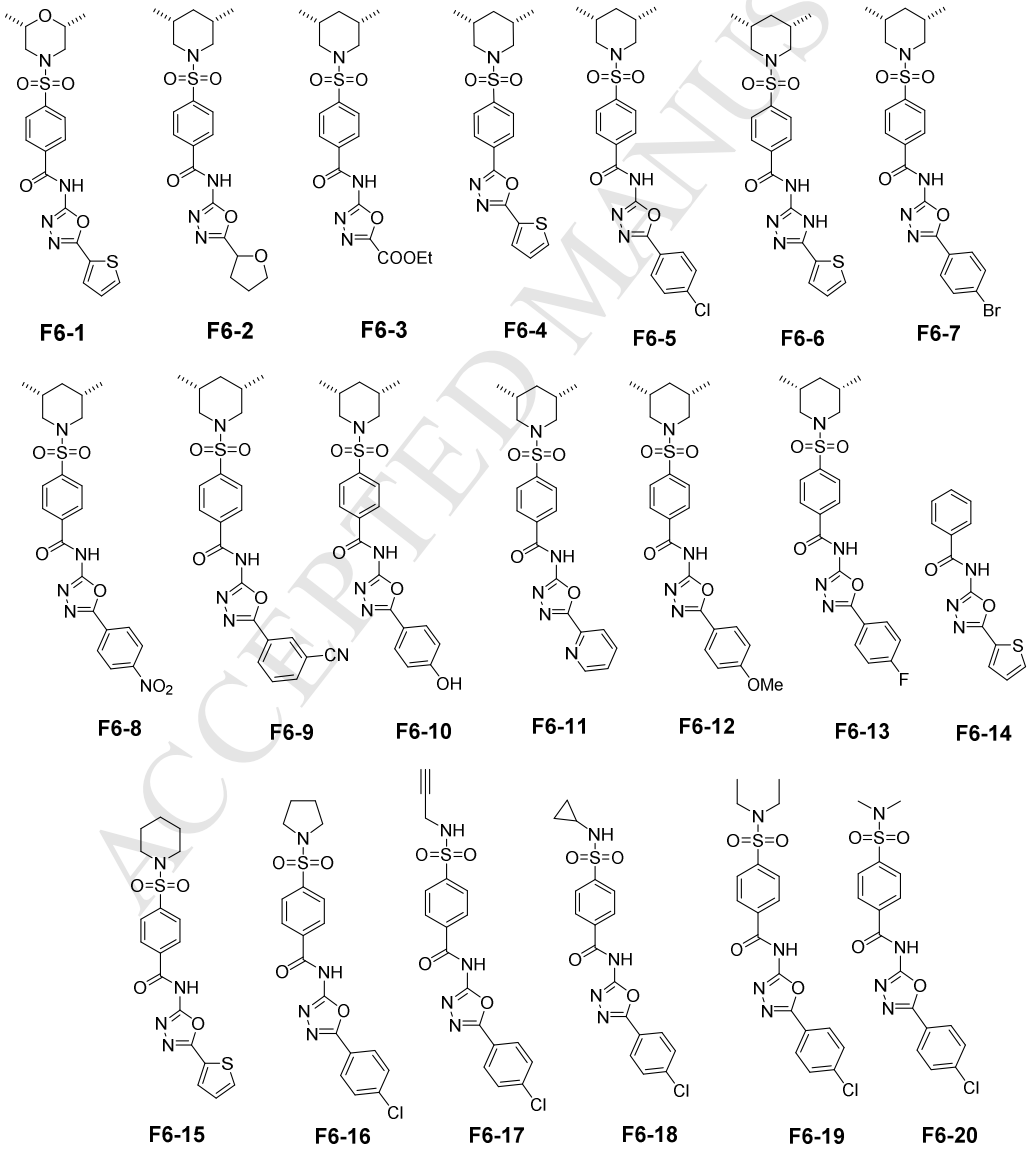


Figure 7. Structural analogs of **F6**, synthesized in our laboratory. **A**. Schematic representation of the synthesis of the analogs studied. Conditions used: (i) MeLi, THF, –78 °C to rt, 14 h; (ii) EDC·HCl, DMAP, CH_2Cl_2, rt, 16 h; (iii) a) T3P, CH_2Cl_2, rt, 1h b) TEA, DMAP, rt, overnight; (iv) BOP reagent, DIPEA, DMF, rt, 16 h; **B**. Structures of analogs synthesized. Note: The starting material **S-I** existed as 4 :1 *cis* to *trans* form. The product obtained as **F6-2** (*cis* : *trans* = 10 :1), **F6-3** (*cis* : *trans* = 4 :1), **F6-4** (*cis* : *trans* = 20 :1), **F6-5** (*cis* : *trans* = 6 :1), **F6-6** (*cis* : *trans* = 13 :1), **F6-7** (*cis* : *trans* = 6:1); **F6-8** (*cis* : *trans* = 5 :1), **F6-9** (*cis* : *trans* = 6 :1), **F6-10** (*cis* : *trans* = 4 :1), **F6-11** (*cis* : *trans* = 6 :1), **F6-12** (*cis* : *trans* = 6 :1), **F6-13** (*cis* : *trans* = 4 :1)

Compared with **F6**, the MIC of **F6-5** (**Table 4**) across the panel of bacterial pathogens tested appeared to be slightly better (**Table 1**). For example, the MIC of **F6-5** against MRSA was 1 µg/mL compared to the MIC obtained for **F6** (2 µg/mL). Furthermore, **F6-5** had an MIC of 2 µg/mL against VRE (*E. faecalis*) and *L. monocytogenes*, compared to the MIC of **F6** (4 µg/mL) against these specific bacterial pathogens. Excitingly, **F6-5** was more active against VRE (*E. faecalis*) than vancomycin.

Given the potency of **F6-5**, we further evaluated the importance of the piperidine moiety whiles maintaining the other portions of **F6-5**. Replacement of the piperidine ring with a pyrrolidine ring yielded compound **F6-16**, with MIC values ranging from 8 µg/mL to 16 µg/mL. Compounds with two alkyl groups on the nitrogen of the sulfonamide, such as the diethyl-substituted **F6-19** and dimethyl-substituted **F6-20** could inhibit *S. aureus* growth**.** However, the diethyl-substituted **F6-19** had better MIC values (16 µg/mL to 32 µg/mL) than the dimethyl-substituted **F6-20** (32 µg/mL to 64 µg/mL). Similarly, analogs with just one alkyl group on the nitrogen of the sulfonamide (**F6-17** and **F6-18**) were less active than **F6-5.** These observations further validate the importance of the dimethyl-substituted piperidine moiety for antibacterial activity.

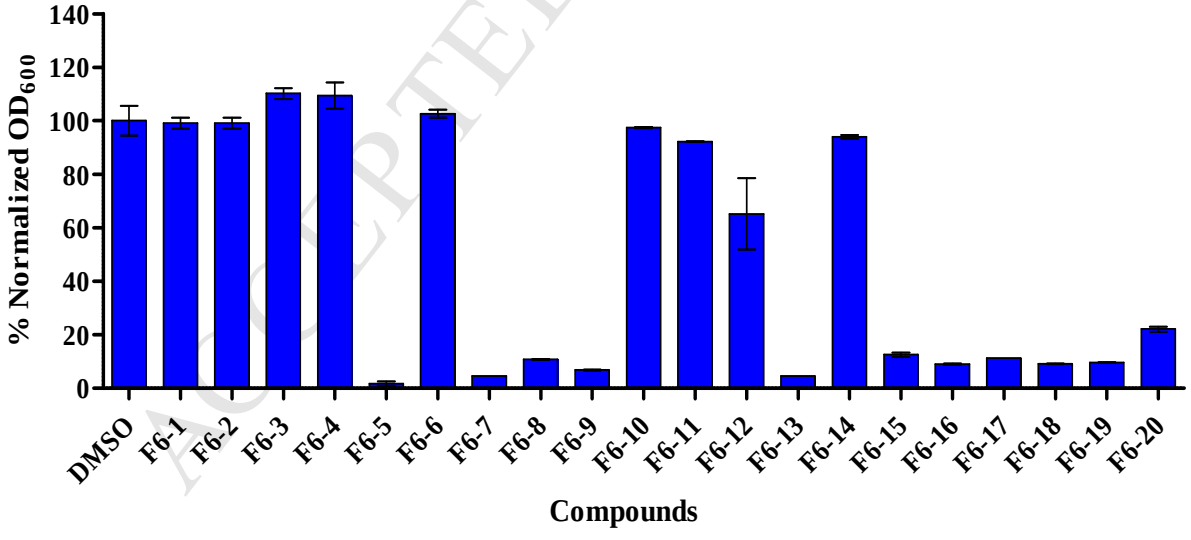
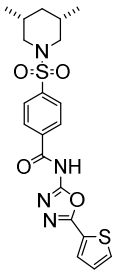


Figure 8. Antibacterial activity of analogs of F6. Compounds were tested at 16 µg/mL for their ability to inhibit *S. aureus* growth. The OD600 of compounds were normalized to that of the DMSO control.

Table 4. MIC (µg/mL) of **F6-5** and vancomycin against a panel of Gram-positive bacterial pathogens**.**

Test agent	***S. aureus* ATCC 25923**	**MRSA ATCC 33592**	***E. faecalis* ATCC29212**	**VRE (*E. faecalis)* ATCC 51575**	***L. monocytogenes* ATCC 19115**
F6-5	2	1	4	2	2
F6-7	2	4	4	4	4
F6-8	32	16	64	32	32
F6-9	16	8	32	16	8
F6-12	4	4	4	4	4
F6-13	4	4	4	4	4
F6-15	32	32	16	64	32
F6-16	16	8	16	16	16
F6-17	64	32	64	64	64
F6-18	16	16	32	16	16
F6-19	16	16	32	16	16
F6-20	64	32	64	64	64
Vancomycin	1	1	2	>128	1



F6- *cis*

Figure 9. Structure of F6- *cis* (*cis* : *trans* = 30 :1).

Thus far the **F6** compound that was initially used for screening was purchased from Life Chemicals Inc. (Ontario, Canada) as a predominantly *cis* isomer (*cis*:*trans* = 10:1). Analogs of **F6**, which were synthesized in our lab were also predominantly *cis* (ranging from 4:1 to 20:1 *cis*:*trans*). To exclude the possibility that the observed antibacterial activities of the compounds were from the minor trans isomer and not the *cis* form, we desired to make at least one of the active compounds (**F6**) with a higher cis/trans ratio than what we had obtained. To do this, we synthesized an isomerically purer **F6** shown in Figure 9, and obtained **F6**-*cis* (*cis*:*trans* is 30:1) (see supporting information). **F6**-*cis* was tested for antimicrobial activity and the MIC was similar to that of the commercially available F6, which had a *cis*:*trans* ratio of 10:1 (compare Table 1 with Table S1, supporting information).

Conclusion

We have identified compound **F6** as a potent antibacterial agent effective against important drug-resistant Gram-positive bacterial pathogens including MRSA, VRSA, VISA, and VRE. It was observed that **F6** was not active against important Gram-negative bacterial pathogens, presumably due to it being a substrate for efflux. Excitingly, resistance was not observed when MRSA was treated with **F6** compared to ciprofloxacin *in vitro*. **F6** was also active *in vivo* in reducing the burden of MRSA in a skin wound infection model in mice. Other compounds like **F3**, **F4** and **F5** were also potent. Through structural-activity relationship (SAR) studies, the relevance of various moieties in **F6** for antibacterial activity was established. Particularly, the 4-((3,5-Dimethylpiperidin-1-yl)sulfonyl)benzamide and oxadiazole amine moieties were required for activity. From the SAR studies, **F6-5** emerged as a slightly more potent analog of **F6** with MIC values ranging from 1 µg/mL to 4 µg/mL.

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Highlights

- The antibacterial agent, **F6** possesses potent activity against drug-resistant Gram-positive pathogens.
- MRSA could not develop resistance to **F6**.
- Bacterial burden in skin wound infection could be reduced by **F6**.