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Antibacterial small molecules that potently inhibit *Staphylococcus aureus* lipoteichoic acid biosynthesis

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Abstract: The rise of antibiotic resistance, especially in *staphylococcus aureus*, and the increasing death rate due to multi-resistant bacteria have been well documented. The need for new chemical entities and/or the identification of novel targets for antibacterial drug development is high. Lipoteichoic acid (LTA), a membrane attached anionic polymer, is important for the growth and virulence of many Gram-positive bacteria and interest has been high in the discovery of LTA biosynthesis inhibitors. Thus far only a handful of LTA biosynthesis inhibitors have been described with moderate (MIC = 5.34 μg/mL) to low (MIC = 1024 μg/mL) activities against *S. aureus*. Here we describe the identification of novel compounds that potently inhibit LTA biosynthesis in *S. aureus*, displaying impressive antibacterial activities (MIC as low as 0.25 μg/mL) against methicillin-resistant *S. aureus* (MRSA). Under similar *in-vitro* assay conditions, these compounds are 4X more potent than vancomycin and 8X more potent than Linezolid against MRSA.

The rise of antibiotic resistance and the increasing death rate due to infections with multidrug-resistant bacteria and *Staphylococcus aureus* (*S. aureus*) have been well documented. *S. aureus,* a Gram-positive bacterial pathogen, is one of the leading causes of community- and hospital- acquired bacteremia[1]. The rise of antimicrobial resistance strains partly contributes to the increasing death rate associated with *S. aureus* infection[2]. Methicillin-resistant *S. aureus* (MRSA) bacteremia is accompanied by higher mortality rates compared to methicillin-sensitive *S. aureus* (MSSA) bacteremia[3]. In 2013, the Center for Disease Control and Prevention (CDC) estimated that bacterial infections kill at least 23,000 annually in the US alone with MRSA being responsible for nearly half of the mortalities[4]. Vancomycin, a glycopeptide antibiotic, is used for the treatment of severe MRSA infections. However, emergence of vancomycin intermediate and resistant *S. aureus* (VISA/VRSA) strains further limits therapy [5]. The discovery of antimicrobial agents with a novel mode of action is vital for the successful treatment of *S. aureus* infections.

The Gram-positive bacteria cell envelope consists of a membrane and a peptidoglycan cell wall with anchored anionic polymers (teichoic acids). Teichoic acids include wall teichoic acids (WTA), which are covalently linked to the peptidoglycan, and lipoteichoic acids (LTA), which are anchored together via a glycolipid[6].

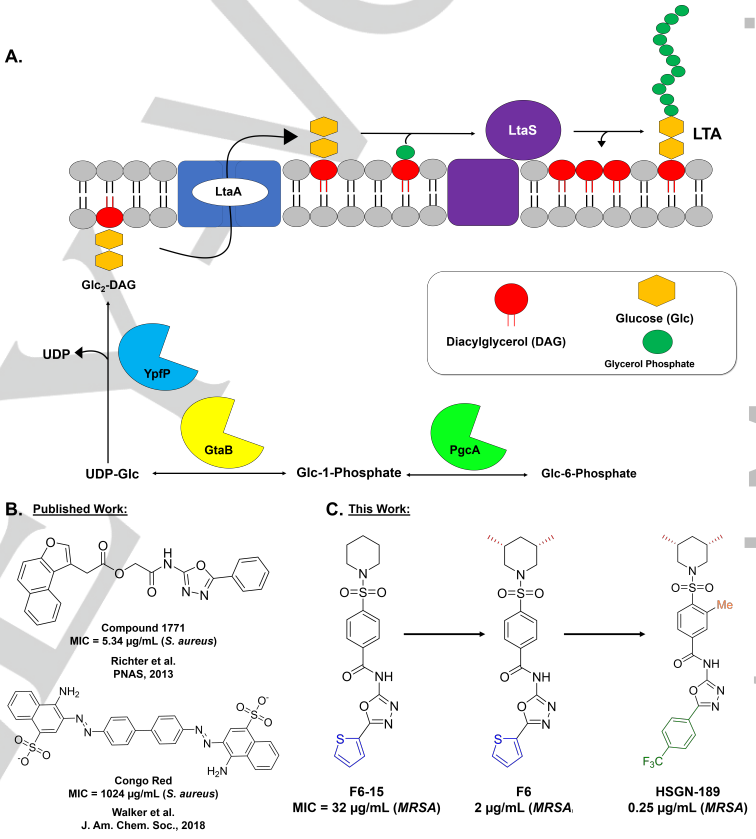

Figure 1. HSGN-189 has potent antibacterial activity and inhibits LTA biosynthesis in *S. aureus*. (**A**) The biosynthesis of LTA takes place at the cell membrane. UDP-Glc is produced by the conversion of glucose-6-phosphate to glucose-1-phosphate by the α-phosphoglucomutase PgcA, followed by activation of UTP:α-glucose-1-phosphate by uridyltransferase GtaB. The glycosyltransferase YpfP transfers two glucose molecules from UDP-Glc to diacylglycerol (DAG), generating the glycolipid Glc_2-DAG. Glc_2-DAG is then displaced to the outer membrane by LtaA. LtaS uses glycerol phosphate as a substrate to repeatedly transfer glycerol phosphate to the Glc2-DAG anchor, producing LTA. (**B**) Previous LTA biosynthesis inhibitors include Compound 1771 and the probe-like molecule Congo Red. These molecules exhibit moderate to low antimicrobial activity with MIC values of 5.34 μg/mL and 1024 μg/mL against *S. aureus* respectively. (**C**) We previously identified **F6-15** as a weak antibacterial agent against MRSA.[11] With further optimization, **HSGN-189** was indentified to be a potent anti-MRSA agent (MIC = 0.25 μg/mL) and LTA biosynthesis inhibitor.

Both polymers are vital components of the cell envelope involved in bacterial growth, replication, colonization and virulence[7]. LTA in *S. aureus,* is composed of a 1,3-glycerol phosphate polymer linked by a diglucosyl diacylglycerol glycolipid anchored to the membrane[6b]. The LTA varies greatly amongst Gram-positive bacteria. Yet, several Gram-positive pathogens, including *Bacillus subtilis, Enterococcus faecalis, and Listeria monocytogenes,* produce the same polyglycerol phosphate polymer as *S. aureus* [6b, 7a]. LTA is synthesized by lipoteichoic acid synthase (LtaS) from phosphatidylglycerol. Depletion of *ltaS* (gene for LtaS) and LTA in *S. aureus* results in growth arrest, cell wall envelope and cell division defects[8]. The essential nature of LTA in *S. aureus*, along with the fact that it is not present in eukaryotic cells, makes LTA an ideal antimicrobial target.

Thus far, there have been efforts to develop potent LTA biosynthesis inhibitors with antibacterial activity by few groups. However, the compounds developed to date are significantly less potent than vancomycin. For example, the first LTA biosynthesis inhibitor, Compound 1771 possessed a minimum inhibitory concentration (MIC) of 5.34 μg/mL against *S. aureus*.[9] Compound 1771 contains an ester moiety, a potential liability due to esterase hydrolysis in blood. In a more recent publication Walker et al. demonstrated that Congo red inhibits LtaS activity[10], however exhibited very low antimicrobial activity (MIC of 1024 μg/mL) against *S. aureus*[10].

Due to the essential nature of LTA, we have been interested in developing antibacterial agents that inhibit LTA biosynthesis. Our group has demonstrated that *N*-(1,3,4-oxadiazol-2-yl)benzamides are potent antibacterial agents with MIC values of 2 μg/mL against MRSA[11]. Here, we report a new generation of *N*-(1,3,4-oxadiazol-2-yl)benzamides, exhibiting MIC values as low as 0.25 μg/mL against MRSA and are more potent than frontline antibiotics used for MRSA infections (4X more potent than vancomycin and 8X more potent than linezolid).

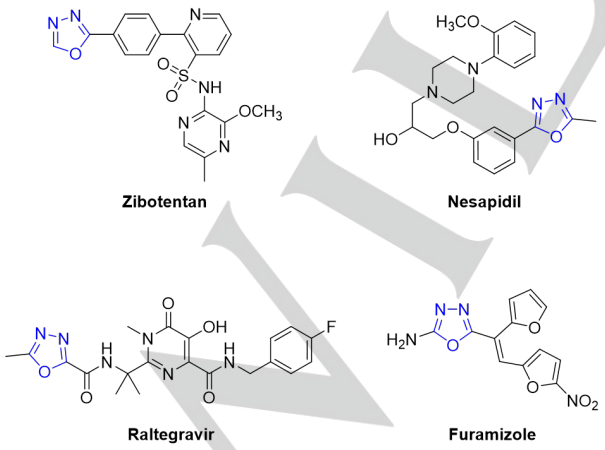

Figure 2. .Clinical compounds containing 1,3,4-oxadiazole unit.

Our group has embarked on the generation of proprietary compounds for evaluation against drug resistant bacteria. As a strategy to increase the chances of advancing a hit molecule to the clinic, we have prepared a library that is enriched with

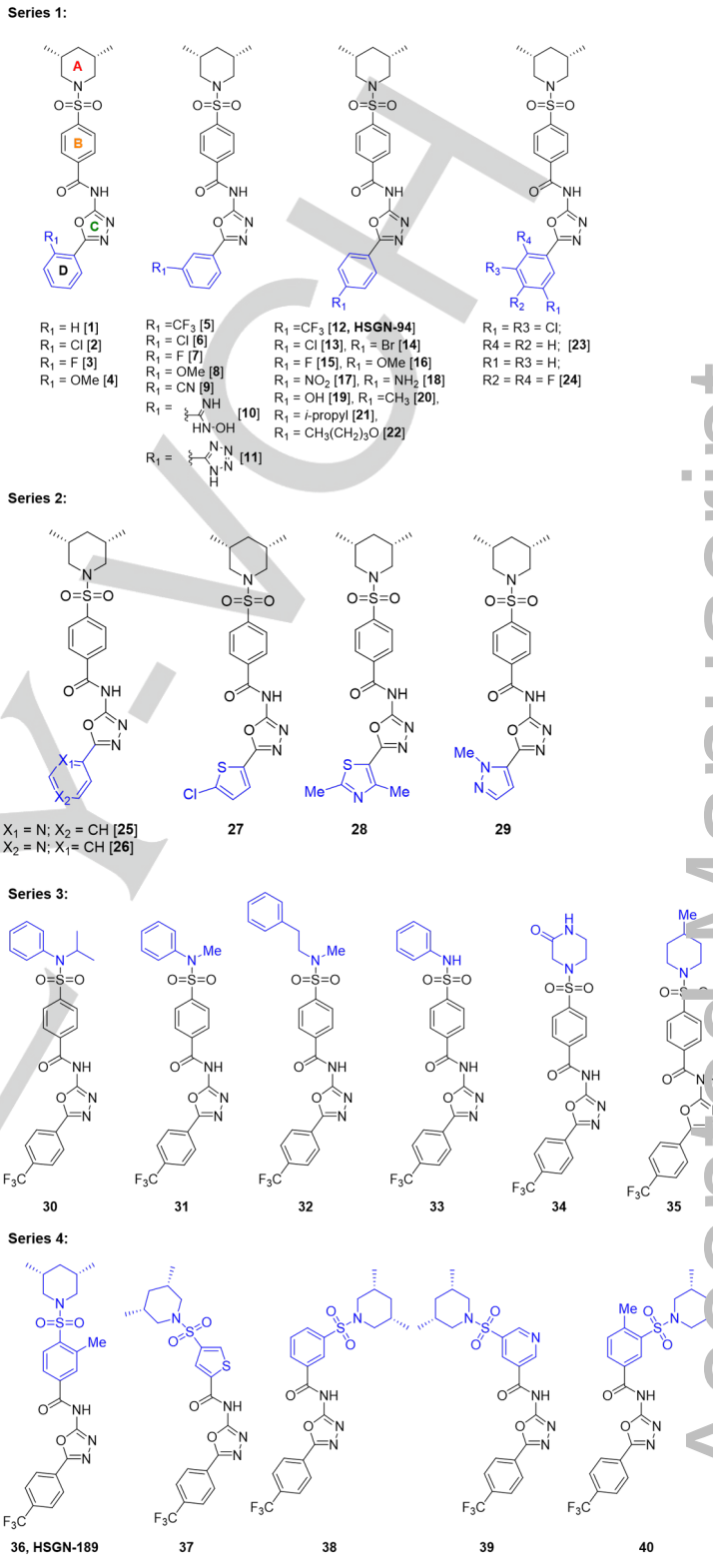

Figure 3. Series of 1,3,4-oxiadizolyl-based compounds synthesized for study.

moieties typically found in other clinical compounds. Several compounds containing the 1,3,4-oxadiazolyl unit have demonstrated interesting biological activities, as exemplified by the drugs such as furamizole[12] (antibacterial), nesapidil[12] (antiarrhythmic), raltegravir (HIV antiviral)[13] and zibotentan (underwent clinical trials for prostate cancer)[14] (see **Figure 2**).

We previously discovered that compound **F6-15** displayed weak antibacterial properties with a MIC of 32 µg/mL against *S. aureus*. Remarkable enhancements in the activity of the lead compound were obtained upon strategic methyl group substitution (the methylation effect)[15]. The installment of the 3,5-dimethyl groups on the piperidine ring gave rise to **F6**, which displayed MIC of 2 µg/mL[11] Notably **F6** was well tolerated in mice and capable of reducing bacterial burden in a wound infection model[11]. While a MIC of 2 µg/mL is respectable, we desired to further optimize this compound by the synthesis of new analogues, which were initially screened for their ability to inhibit the growth of *S. aureus* at 16 µg/mL (ESI, **Figure S1**).

Table 1. MIC (µg/mL) of **HSGN-94, HSGN-189**, analogs, vancomycin, and linezolid against a panel of Gram-positive bacterial pathogens. Experiments were done in triplicate and in all replications the same MIC values were obtained.

Compounds	*S. aureus* ATCC 25923	*MRSA* ATCC 33592	*E. faecalis* ATCC 29212	*VRE* ATCC 51575	*L. monocytogenes* ATCC 19115
F6-15	32	32	16	64	32
F6	2	2	4	4	4
1	4	4	16	8	8
2	32	32	64	64	32
3	2	4	16	8	4
5	2	2	4	8	2
6	1	0.5	1	2	1
7	2	0.5	2	2	2
8	4	2	8	4	4
9	16	8	32	16	8
11	16	16	64	32	32
12 ,HSGN-94	0.25	0.25	2	1	0.5
13	2	1	4	2	2
14	2	4	4	4	4
15	4	4	4	4	4
16	4	4	4	4	4
17	32	16	64	32	32
20	16	8	32	16	16
21	2	1	4	2	2
22	16	16	16	8	16
23	0.5	0.25	2	1	1
24	8	8	32	32	32
26	16	32	64	64	32
27	2	2	64	32	32
28	16	8	32	64	16
29	8	4	16	128	8
30	1	0.5	4	4	2
31	0.25	0.25	2	2	1
32	1	2	8	8	4
33	4	4	8	8	8
35	1	0.5	4	4	2
36, HSGN-189	0.25	0.25	8	8	4
37	0.5	1	16	16	8
38	8	8	>16	>16	>16
39	2	2	4	4	2
40	2	1	8	4	4
Vancomycin	1	1	2	>128	1
Linezolid	2	2	2	2	2

For compounds that showed inhibitory activity, we determined the MIC (**Table 1**). For synthesis of compounds see ESI **Figure S1**. Four types of compounds were made (series 1-4, **Figure 3**). The compounds contained four rings (labeled rings A, B, C and D, see **Figure 3**). Series 1 was made up of compounds with various substitution (halogens, CF_3, CN, OMe, tetrazole, NH_2, OH, Me, hydroxyamidine) to phenyl ring D. Halogen substitutions (especially the Cl, F or CF_3 groups) resulted in the most active compounds. Hydrophilic substituents, such as the NH_2, CN, OH and tetrazole were not active. For the halogen substituents, the position on the ring was also important. For example, the MIC for para-CF_3 (**12, HSGN-94**) was 0.25 µg/mL, whereas that for the meta analog (**5**) was 1 µg/mL against MRSA (**Table 1**). In series 2, we investigated other heteroaromatics, such as pyridinyl (**25** and **26**), chlorothiophenyl (**27**), dimethylthiazolyl (**28**), pyrazolyl (**29**) as ring D. For these compounds, the chlorothiophenyl analog **27** was the most potent (MIC = 2 µg/mL against MRSA). Series 3 explored structure-activity-relationships (SAR) of the sulfonamide moiety (ring A). Here both the methyl substituted piperidine and *N*-substituted aniline substituents were highly active (MIC for compounds **30**, **31**, **32**, and **35** are 0.5, 0.25, 1 and 0.5 µg/mL respectively). Considering that the 3,5-dimethyl piperidine sulfonamide (**HSGN-94**) was one of the best compounds, we proceeded to investigate how substitution of ring B and/or position of the 3,5-dimethyl piperidine sulfonamide (series 4) affected antibacterial activity. Replacement of the phenyl group with thiophenyl (**37**) or pyridinyl (**39**) led to a small reduction in antibacterial activity (MIC = 1 µg/mL and 2 µg/mL for compounds **37** and **39** respectively). Addition of a methyl group to the 3 position of ring B (**36, HSGN-189**) did not effect activity (MIC = 0.25 µg/mL). Changing the position of the 3,5-dimethylpiperidine sulfonamide moiety from *para* to *meta*, (compounds **38** and **40**) on ring B resulted in reduced activity against MRSA (compare MIC of 0.25 µg/mL for **HSGN-94** and **HSGN-189** with 8 µg/mL and 1 µg/mL for compounds **38** and **40** respectively).

HSGN-189 appears more selective than **HSGN-94** (see **Table 1** for comaprision of MICs against other Gram-postive bacteria), thus we proceeded to identify its mode of action. Traditional ways to do this are to generate bacteria that are resistant to the compound and use global sequencing to identify genes that are mutated in the presence of the compound or to use affinity probes to identify binding proteins[16]. Despite many attempts, we have been unable to generate resistant strains towards **HSGN-189** (which looks promising for the eventual translation of this compound or analogs thereof). Given that **HSGN-189** and the known LTA biosynthesis inhibitor, Compound 1771, both contain aryl substituted 1,3,4-oxadiazolyl unit, we investigated the effects of selected compounds on LTA levels in *S. aureus* Excitingly, when we investigated the effects of **F6-15**, **F6** and **HSGN-189** on LTA biosynthesis in *S. aureus*, following the protocol utilized by Walker and Richter, we observed potent inhibition of LTA by these compounds (**Figure 4** and ESI, **Figure S2**). Interestingly, the degree of LTA biosynthesis inhibition correlated with the MIC values, strongly hinting that LTA biosynthesis inhibition is responsible (at least in part) for the antibacterial activities of the compounds. Vancomycin and Congo Red were used as negative and positive controls respectively (see **Figure S2**). Whereas Congo Red reduced LTA biosynthesis in *S. aureus*, vancomycin increased LTA content (see **Figure S2**).

In conclusion, we have identified potent inhibitors of LTA biosynthesis. These compounds potently inhibit MRSA with MIC values that are 4X lower than vancomycin and 8X lower than linezolid, two antibiotics commonly used to treat MRSA infections. However, both traditional antibiotics have many disadvantages. For vancomycin, it is not orally bioavailable and displays nephrotoxicity. Likewise, linezolid can cause serious side effects like bone-marrow suppression, lactic acidosis, peripheral and optic neuropathy, etc[17]. Thus, alternatives to vancomycin and linezolid are needed. Future work will focus on the activities of the potent compounds (MIC less than 0.5 µg/mL) in mice infection models. We will also investigate which of the many enzymes involved in LTA biosynthesis is/are the targets of the described compounds. This work adds to the increasing number of reports that have attempted to address the anti-bacterial resistance issue with novel small molecules[18].

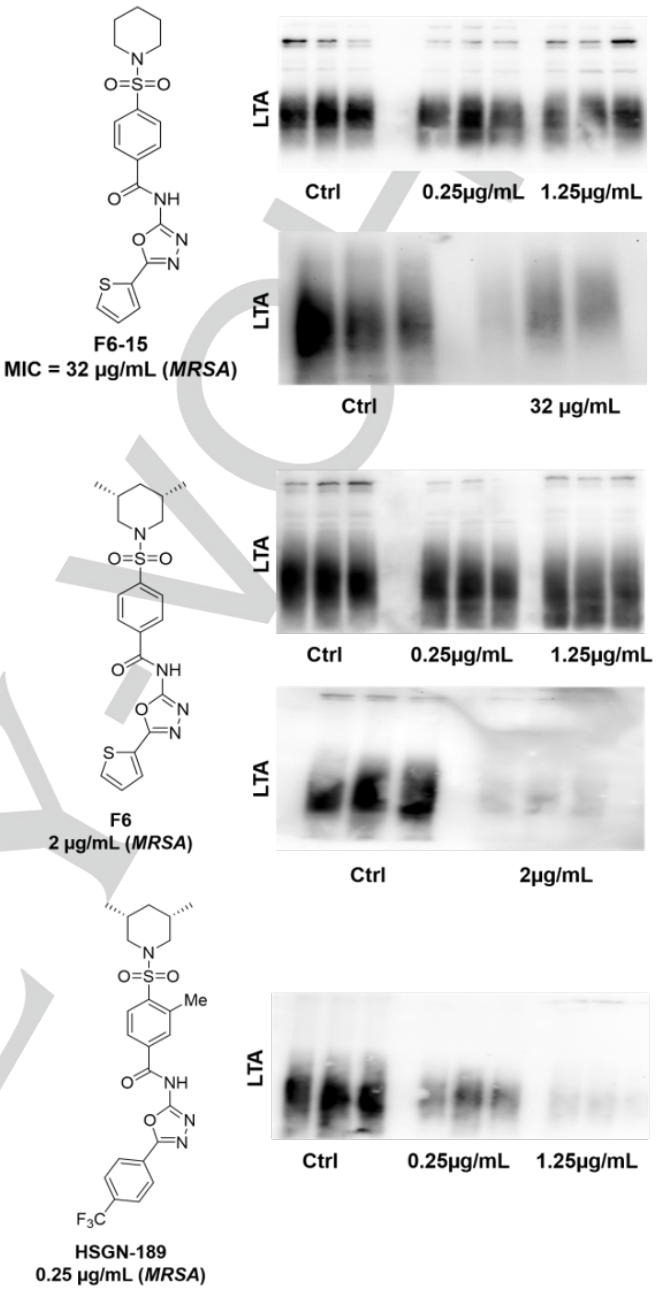

Figure 4. LTA biosynthesis inhibition by 1,3,4-oxadiazolyl –based compounds. The MIC of **HSGN-189** (0.25 µg/mL) is lower than **F6-15** (32 µg/mL) and **F6** (2 µg/mL). At lower concentrations (0.25 µg/mL), only **HSGN-189** significantly inhibited the biosynthesis of LTA. Yet, at the MIC concentration of **F6-15** (32 µg/mL) and **F6** (2 µg/mL), LTA biosynthesis was inhibited.

Experimental Section: For experimental procedures, compound syntheses and characterization data see ESI.

Keywords: LTA biosynthesis inhibitor • MRSA • Antibiotic resistance • 1,3,4-oxadiazole • antibacterial activity

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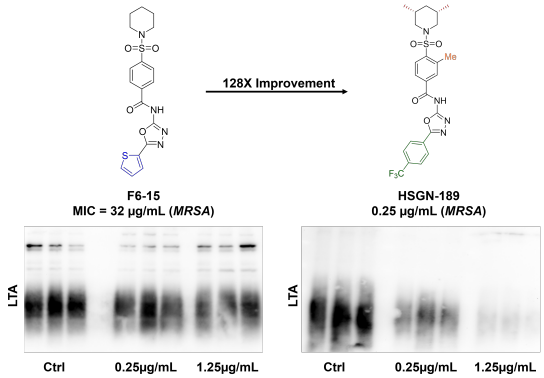

The Gram-positive bacteria cell envelope consists of a membrane and a peptidoglycan cell wall with anionic polymers anchored onto these structures: wall teichoic acids (WTA) and lipoteichoic acids (LTA). Both polymers are vital components of the cell envelope and modulate bacterial growth, replication, colonization and virulence. Thus far potent inhibitors of LTA biosynthesis have been lacking. Here, we reveal new LTA biosynthesis inhibitors that potently inhibit LTA biosynthesis in *S. aureus*. Some of the LTA biosynthesis inhibitors also inhibit bacterial growth at concentrations as low as 0.25 µg/mL.