Published in final edited form as:

Curr Opin Microbiol. 2013 October ; 16(5): . doi:10.1016/j.mib.2013.06.014.

“Teichoic acid biosynthesis as an antibiotic target”

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Abstract

The relentless spread of antibiotic resistant pathogens makes it imperative to develop new chemotherapeutic strategies to overcome infection. The bacterial cell wall has served as a rich source for both validated and unexploited pathways that are essential for virulence and survival. Lipoteichoic acids (LTAs) and wall teichoic acids (WTAs) are cell wall polymers that play fundamental roles in Gram-positive bacterial physiology and pathogenesis, and both have been proposed as novel antibacterial targets. Here we describe recent progress toward the discovery of teichoic acid biosynthesis inhibitors and their potential as antibiotics to combat *Staphylococcus aureus* infections.

Introduction

The bacterial cell envelope performs a variety of essential functions, ranging from providing structure and support against osmotic lysis to sensing changes in the environment and coordinating an adaptive response. The cell wall is the target of many of the most widely used antibiotics, including the β -lactams and the glycopeptides. Resistance to these antibiotics is a growing global problem, and *Staphylococcus aureus* has shown the ability to develop resistance rapidly to novel antibiotics [1, 2]. It is essential to identify new strategies to overcome resistant *S. aureus* infections. Teichoic acids (TAs) are critical abundant anionic glycopolymers found in Gram-positive cell envelopes [3]. They typically possess a backbone of alditol phosphate repeat units tailored with glycosyl and D-alanyl substituents (Figure 1) [3–8, 9*]. In *S. aureus*, the LTA backbone of 1,3-linked glycerol phosphate repeat units is tethered to membrane-anchored gentiobiosyl-diacylglycerol (Glc₂-DAG), while WTAs consist of 1,5-linked ribitol phosphate subunits attached via a disaccharide linkage unit to peptidoglycan (PG).

Despite structural and physicochemical similarities, LTAs and WTAs are synthesized via different pathways. Glc₂-DAG is assembled on the cytoplasmic leaflet of the membrane by the glycosyltransferase YpfP and then flipped to the outer leaflet by LtaA, whereupon LtaS adds glycerol phosphate units onto the Glc₂-DAG anchor using phosphatidyl glycerol as a donor [10–13]. LTAs are glycosylated to some extent, and the donor is thought to be N-acetyl-glucosamine (GlcNAc) -phospho-polyprenyl, but the enzyme responsible for this modification has not been identified [14]. WTAs are assembled on an undecaprenyl phosphate carrier on the cytoplasmic leaflet of the membrane by TarOABFL using CDP-

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glycerol synthesized by TarD and CDP-ribitol synthesized by TarIJ. The WTA polymer is then GlcNAcylated by TarM and TarS, flipped to the outer leaflet of the membrane by the ABC transporter TarGH, and attached to peptidoglycan [15–17, 18*]. LTA D-alanylation occurs extracellularly via the DltABCD machinery, and it is believed that D-alanines are transferred from LTAs to WTAs, but the mechanistic details of D-alanylation have not been fully established [3, 19].

Teichoic Acids as Targets

TAs are promising antibacterial targets, owing to the critical roles they play in bacterial viability *in vitro* [20, 21], during cell division [10, 12, 22], in immune evasion [23, 24], in resistance to host defenses including cationic antimicrobial peptides [25, 26], in resistance to β -lactam antibiotics [9*, 22], and in pathogenesis [27, 28] (Figure 2). Due to their diverse roles, inhibition of different steps of TA biosynthesis can result in different phenotypes, dividing TA inhibitors into various classes depending on the effect of inhibiting their specific target.

LTAs are essential for survival *in vitro* except under the most permissive conditions of salt and temperature or in the presence of suppressor mutations [20, 21]. Among the enzymes in the biosynthetic pathway, LtaA and LtaS appear the most promising as they are exposed to the extracellular milieu and have no human homologs.

In contrast to LTA deletion mutants, WTA null mutants (*tarO* or *tarA*), grow relatively well under laboratory conditions, and growth rates in exponential phase are only modestly reduced [27, 29]. However, these strains reach stationary phase at a significantly lower density than wild type, have plating defects, and are temperature sensitive. They also possess morphological and cell division defects [22], possibly arising from misregulated cell assembly machinery (Figure 2) [22, 30*, 31–33]. These physiological defects may account for the greatly reduced capacity of WTA-deficient *S. aureus* to establish infections in mammalian hosts [27, 28, 34]. In any event, TarO and TarA are possible targets for compounds that strongly attenuate virulence by compromising viability in a host. The existence of natural products that inhibit TarO at very low concentrations supports the potential of these enzymes as targets [22].

Recent work has also shown that deleting or inhibiting WTA biosynthesis resensitizes methicillin-resistant *S. aureus* (MRSA) to β -lactams [22, 32]. Resistance in MRSA is due to the acquisition of a gene encoding a resistant peptidoglycan (PG) transpeptidase, PBP2A, whose function depends on the presence of several factors, including WTAs. It is presumed that these host factors somehow regulate PBP2A activity (Figure 2). The β -lactam sensitivity observed in *tarO* strains can be recapitulated by removing the β -GlcNAc tailoring modification catalyzed by TarS [9*]. Therefore, TarO, TarA, and TarS are potential targets for β -lactam resensitization, and could be useful for restoring the utility of this widely-used class of antibiotics [35]. Since inactivation of TarO or TarA compromises *in vivo* viability, these proteins may have advantages over TarS as targets.

There are also classical antibiotic targets in the WTA pathway since *tarBDFGHIJL* are essential once flux into the pathway has initiated [29, 36, 37]. The essentiality is due to accumulation of toxic intermediates and depletion of the undecaprenol-phosphate lipid carrier, which is also used for PG biosynthesis [36, 38]. Compounds that inhibit these essential targets prevent bacterial growth (see below) [39**, 40].

D-alanine esters are installed on both LTAs and WTAs by the same biosynthetic machinery, DltABCD. The terminal amine is protonated at neutral pH, counteracting the overall negatively-charged poly-phosphate backbone of the teichoic acid. These modifications are

necessary for proper growth as well as virulence and biofilm formation, and may serve to coordinate interactions with cell envelope proteins [41–44]. *S. aureus* deficient for D-alanylation grow at wild type rates at 30°C but are sensitive to high temperatures [30*]. D-alanylation levels are strongly dependent on environmental conditions and increase under conditions of cell wall stress [45]. Regulation of D-alanylation serves to maintain ion homeostasis and limits access of cationic antimicrobial peptides and other toxic compounds (Figure 2) [3, 19]. The pleiotropic effects of removing TA D-alanylation suggest that they are also good targets for novel antimicrobials.

Progress in Compound Discovery

Rapid progress has been made toward developing small molecules to validate TAs as good antimicrobial targets. The first targets that attracted significant attention were in the D-alanylation pathway, but LTA and WTA inhibitors have since been developed, and several natural product derivatives are suggested to disrupt LTAs.

Two DltA inhibitors have been described, both developed using *in vitro* assays involving purified DltA protein. The first, compound **5** (5'-O-[N-(D-alanyl)-sulfamoyl]adenosine, Figure 3), was a rationally designed substrate mimic incorporating D-alanine and adenosine moieties [19]. The inhibitor was determined to have an *in vitro* K_i of 232 nM (0.097 µg/ml). Because D-alanylation is not essential in culture, it was impossible to establish a relevant MIC. However, 1 mM **5** prevented outgrowth of vancomycin-resistant mutants in *B. subtilis* liquid cultures when coadministered with 0.4 nM vancomycin. Since D-alanylation protects cells from glycopeptide antibiotics, this finding was used to support target engagement in cells. In addition to this compound, the biotechnology company Mutabilis has reported DltA inhibitors (Y Bonvin *et al.*, 2010. US Patent 20100130489A1), but these await further characterization. Surface-exposed components of the Dlt pathway (*i.e.*, DltB and possibly DltD) may prove to be better targets than DltA, but assays that report on their activities have not yet been developed.

In order to identify inhibitors of LTA biosynthesis, a cell-based screen for compounds that cause temperature sensitivity in *S. aureus* was carried out [46**]. Compound **1771** (Figure 3) was identified and shown to inhibit the growth of MRSA with a minimal inhibitory concentration (MIC) of 5.3 µg/ml. **1771** also inhibited growth of *B. subtilis*, which contains two *ItaS* homologs, but not *E. coli*. In mouse infection models, the compound was found to be unstable but time to death was significantly delayed with regular intraperitoneal administration. Though no spontaneous resistant mutants were identified, the target is believed to be LtaS based on reduced enzymatic activity in a heterologous expression system.

A specific inhibitor of late-stage WTA synthesis, targocil (Figure 3), was discovered by exploiting the conditional essentiality of late-stage WTA enzymes [39**, 40]. Targocil is a potent, selective, and specific inhibitor of *S. aureus* TarG, the transmembrane component of the ABC transporter that exports WTAs [47]. Two classes of resistant mutants were identified: null mutations in *tarO* or *tarA* and point mutations in the *tarG* target. The null mutants are WTA-deficient and are not expected to contribute to resistance *in vivo*. However, the frequency of target mutations was unacceptably high. A similar screen carried out by Merck yielded additional TarG inhibitors with similarly high frequencies of resistance [28]. Using targocil in combination with β -lactams decreased the frequency of resistance and the combination showed efficacy in an animal model of MRSA infection [22, 28]. If compounds with better resistance profiles can be identified, late-stage WTA inhibitors may hold considerable promise.

In addition to late-stage WTA inhibitors, early-stage inhibitors have also been identified. For example, tunicamycin (Figure 3) is a natural product that inhibits TarO, the first step in WTA synthesis [22]. It also inhibits MraY, which catalyzes a step in PG synthesis, but it is 100-fold more selective for TarO [22]. Tunicamycin concentrations of approximately 0.1 µg/mL completely inhibit TarO, rendering MRSA phenotypically WTA-null. It was shown that inhibiting TarO rendered MRSA strains sensitive to β -lactams; however, tunicamycin exhibits significant eukaryotic toxicity, preventing its clinical use. It may be possible to modify tunicamycin to overcome eukaryotic toxicity, but the chemistry is challenging.

Another possible TarO inhibitor, ticlopidine (Figure 3, sold commercially as the antiplatelet drug Ticlid), was recently identified from a panel of FDA-approved drugs as a compound that resensitized MRSA to β -lactams but had no effect on Gram-negative bacteria [32]. Coadministration of ticlopidine and cefuroxime greatly increased survival in a *Galleria mellonella* MRSA infection model relative to treatment with either drug individually. Ticlopidine also protected against chemical or genetic inhibition of late-stage WTA biosynthesis, likely by inhibiting TarO. Despite the high concentration of ticlopidine required to potentiate β -lactam activity, its FDA-approved dose for antiplatelet treatment is very high, and the compound is potentially useful as an antibiotic.

A compound that acts via polymerized teichoic acids is chitosan (Figure 3), a cationic polymer of glucosamine and GlcNAc derived from chitin. Chitosan prevents growth of wild type *S. aureus* with an MIC of 23.4 µg/mL [48]. Chitosan's mechanism of action is likely mediated by electrostatic interactions with the phosphate backbones of teichoic acids: mutants deficient for teichoic acids are protected from chitosan by up to a factor of 5, and mutants lacking D-alanine esters are sensitized to chitosan by a factor of 100 [49]. The inexpensive supply of chitin and the ability to target any polyanionic polymer make chitosan a promising potential therapeutic.

Another group of molecules that mediate killing via mature teichoic acids are the galloyl catechins such as epicatechin gallate (Figure 3). Epicatechin gallate and related homologs are biologically active compounds found in green tea extracts that inhibit biofilm formation, increase autolysin activity, and at high concentrations cause clumping and cell division defects in MRSA [50]. Though the MIC of epicatechin gallate is well above therapeutically relevant concentrations (256–512 µg/mL), at 12.5 µg/mL it resensitizes MRSA to oxacillin treatment up to 500-fold [51]. This is associated with reduced D-alanylation of teichoic acids and disruption of PBP2A localization, though these two factors may be independent effects of membrane destabilization [52]. Though epicatechin gallate is rapidly hydrolyzed upon entering the body, limiting its therapeutic use to superficial applications, medicinal chemistry efforts continue in order to identify the minimum pharmacophore in hopes of developing a broadly applicable formulation. One dehydroxylated analog was reported to have an 8-fold improved MIC (64 µg/mL) as well as increasing the potentiation of oxacillin up to 2000-fold (0.25 µg/mL) in PBP2A-expressing *S. aureus*, but further effort is needed to improve pharmacokinetic properties [53].

Conclusion

Early results in targeting teichoic acid biosynthesis are encouraging. Both synthetic and modifying enzymes are validated antibiotic targets in a variety of animal models. Several promising compounds have been identified, but many potential targets remain underexplored. More work will be needed to realize the potential of teichoic acids as antibiotic targets and translate these exciting antivirulence compounds into effective therapeutics.

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Papers of particular interest, published within the period of review, have been highlighted as:

*of special interest

**of outstanding interest

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Highlights

- Teichoic acids are conserved glycopolymers found in Gram-positive cell envelopes.
- LTA biosynthetic genes are essential and good targets for traditional antibiotics.
- Some WTA enzymes are essential and good targets for traditional antibiotics.
- Other WTA enzymes are targets for antivirulence agents and/or β -lactam potentiators.
- Proof-of-concept inhibitors have been discovered for TA pathway targets.

Figure 1.

Teichoic acid assembly. Despite structural similarities, lipoteichoic acids (LTAs) and wall teichoic acids (WTAs) are synthesized through different pathways by different mechanisms. LTAs are polymerized by LtaAS on a lipid anchor on the outer leaflet of the plasma membrane using substrates synthesized by PgcA, GtaB, and YpfP, and glycosylated [11, 12]. WTAs are polymerized by TarOABDFIJL on the cytoplasmic leaflet, modified with hexoses by TarM and TarS, flipped to the extracellular leaflet of the plasma membrane by the ABC transporter TarGH, and attached to peptidoglycan, likely by LCP proteins [15, 18*]. *S. aureus* contains a gene duplication of TarIJL named TarI'J'K respectively (not shown). Upon reaching the exterior of the cell, both teichoic acids are further functionalized

with D-alanine esters by DltABCD. D-alanine esters are thought to be installed first on LTAs and then transferred to WTAs.

Figure 2. Teichoic acids are responsible for a wide variety of functions involved in cell division, charge homeostasis, and infection.

Compound	Structure	Target	Effect	Reference
5		DltA	<i>In vitro</i> K _i : 232 nM (0.097 µg/ml)	May <i>et al.</i> , 2005
1771		LtaS	Blocks LTA polymerization MIC: 5.3 µg/ml	Richter <i>et al.</i> , 2013
Targocil		TarG	Blocks WTA flipping MIC: 0.3 - 1 µg/ml	Swoboda <i>et al.</i> , 2009 Lee <i>et al.</i> , 2010
Tunicamycin		TarO	Inhibition of WTA synthesis Sensitizes to β-lactams MSC*: 0.02 µg/ml MIC: 10 - 50 µg/ml	Komatsuzawa <i>et al.</i> , 1994 Campbell <i>et al.</i> , 2011
Ticlopidine		TarO	Reduces cell envelope phosphate Sensitizes to β-lactams MSC*: 60 µg/ml	Farha <i>et al.</i> , 2013
Chitosan	(glucosamine-GlcNAc) _n	Poly-phosphate	Interacts with anionic polymers MIC: 23.4 µg/ml	Raafat <i>et al.</i> , 2008 Raafat <i>et al.</i> , 2009
Epicatechin Gallate		Plasma membrane	Inhibits biofilm formation Promotes LTA release Delocalizes PBP2, PBP2A Decreases D-alanylation MSC*: 12.5 µg/ml	Stapleton <i>et al.</i> , 2007 Bernal <i>et al.</i> , 2010

Figure 3.

Inhibitors of teichoic acid synthesis and modification. For inhibitors of non-essential enzymes, the enzyme inhibition constant is given instead. *MSC: minimum sensitization concentration to β-lactam antibiotics.