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Discovery of Wall Teichoic Acid Inhibitors as Potential Anti-MRSA β-Lactam Combination Agents

Hao Wang,[1] Charles J. Gill,[1] Sang H. Lee,[1] Paul Mann,[1] Paul Zuck,[2] Timothy C. Meredith,[3] Nicholas Murgolo,[1] Xinwei She,[1] Susan Kales,[1] Lianzhu Liang,[1] Jenny Liu,[1] Jin Wu,[1] John Santa Maria,[4] Jing Su,[1] Jianping Pan,[1] Judy Hailey,[1] Debra Mcguinness,[1] Christopher M. Tan,[1] Amy Flattery,[1] Suzanne Walker,[4] Todd Black,[1] and Terry Roemer[1,*]
[1]Infectious Disease Biology, Merck Research Laboratories, Kenilworth, NJ 07033, USA
[2]Screening and Protein Science, Merck Research Laboratories, West Point, PA 19486, USA
[3]Infectious Diseases Area, Novartis Institutes for BioMedical Research, Cambridge, MA 02139, USA
[4]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA
*Correspondence: terry_roemer@merck.com
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SUMMARY

Innovative strategies are needed to combat drug resistance associated with methicillin-resistant *Staphylococcus aureus* (MRSA). Here, we investigate the potential of wall teichoic acid (WTA) biosynthesis inhibitors as combination agents to restore β-lactam efficacy against MRSA. Performing a whole-cell pathway-based screen, we identified a series of WTA inhibitors (WTAIs) targeting the WTA transporter protein, TarG. Whole-genome sequencing of WTAI-resistant isolates across two methicillin-resistant Staphylococci spp. revealed TarG as their common target, as well as a broad assortment of drug-resistant bypass mutants mapping to earlier steps of WTA biosynthesis. Extensive in vitro microbiological analysis and animal infection studies provide strong genetic and pharmacological evidence of the potential effectiveness of WTAIs as anti-MRSA β-lactam combination agents. This work also highlights the emerging role of whole-genome sequencing in antibiotic mode-of-action and resistance studies.

INTRODUCTION

Staphylococcus aureus remains the leading cause of hospital and community-acquired infections by Gram-positive bacteria in much of the developed world (Boucher et al., 2009; Klevens et al., 2007; Johnson, 2011). This is attributed in large part to the emerging resistance of *S. aureus* to the entire armamentarium of β-lactam antibiotics, a broad and historically important class of antibiotics spanning penicillin, methicillin, and the more powerful carbapenems, including imipenem, which kill bacteria by inhibiting synthesis and chemical crosslinking of peptidoglycan (PG), a cell wall polymer, leading to weakening of the cell wall and cell lysis (Walsh, 2003).

The development of antibiotic combination agents has proven to be a highly successful therapeutic strategy to combat drug resistance, particularly against drug-resistant Gram-negative bacteria (Drawz and Bonomo, 2010). Paramount to the rationale of combination agents is the increased potency and efficacy achieved by their combined effects. Ideally, this is achieved by the synergistic bioactivity of both agents affecting two interdependent cellular processes required for cell growth as well as the targeted inactivation of the resistance mechanism to the first agent by the combination agent (Tan et al., 2012). Applying a systems biology approach to discovering synergistic agents with this therapeutic potential is highly warranted; lethal or even growth-crippling chemical genetic interactions highlight a cellular network of interdependent biological processes and potential drug targets from which combination agents may be rationally discovered (Andrusiak et al., 2012; Costanzo et al., 2010; Nichols et al., 2011). We and others have adopted this approach to identify genetic mutations that restore β-lactam activity against MRSA and as such predict that cognate inhibitors of these β-lactam "potentiation" targets may similarly restore the efficacy of the β-lactam (De Lencastre et al., 1999; Berger-Bächi and Rohrer, 2002, Huber et al., 2009; Lee et al., 2011; Tan et al., 2012). Indeed, several cellular processes contribute to buffering MRSA from the effects of β-lactams, including normal synthesis of a second cell wall polymer, wall teichoic acid (WTA) (Campbell et al., 2011; Lee et al., 2011). In support of this notion, target-specific inhibitors of this process, such as tunicamycin (Komatsuzawa et al., 1994; Campbell et al., 2011), an exquisitely selective inhibitor of TarO, responsible for the first step in WTA synthesis (Swoboda et al., 2009), was found to be highly synergistic in combination with β-lactams.

WTA is a Gram-positive-specific anionic glycophosphate cell wall polymer of roughly equal abundance to PG. Unlike PG, however, WTA is not required for cell viability (Weidenmaier et al., 2004; D'Elia et al., 2009b) but plays important roles in cell growth, division, morphology, and as a virulence factor (Schirner et al., 2009; Swoboda et al., 2010; Atilano et al., 2010; Campbell et al., 2011; Dengler et al., 2012, Weidenmaier and Peschel, 2008). WTA polymers are sequentially synthesized on an undecaprenyl phosphate carrier lipid by a series of Tar enzymes localized on the inner face of the cytoplasmic membrane before being exported to the cell surface by a two-component ATP-binding cassette (ABC) transporter system and covalently linked to PG (Brown et al., 2008; Swoboda et al., 2010; see also Figure S1 available online). Interestingly, late steps in WTA

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biosynthesis in either *S. aureus* or *Bacillus subtilis* are essential for cell viability, whereas early steps (encoded by *tarO*/*tagO* and *tarA*/*tagA*, respectively) are not (Weidenmaier et al., 2004; D'Elia et al., 2006a, 2006b, 2009a, 2009b). Further, late-stage WTA genes are in fact conditionally essential because they are dispensable in either a *tarO*/*tagO* or *tarA*/*tagA* deletion background; this is referred to as the "essential gene paradox" (D'Elia et al., 2006a, 2006b, 2009b). Two hypotheses have been given to explain these results: that toxic intermediate WTA precursors accumulate in late-stage WTA mutants and/or sequestration of the essential biosynthetic precursor, bactoprenol, occurs and this leads to depletion of PG because its synthesis also requires bactoprenol as a carrier lipid (D'Elia et al., 2006b, 2009b).

Walker and colleagues have recently exploited this phenomenon by screening for late-stage WTA inhibitors (WTAIs) that phenocopy the genetic characterization of the pathway. Such compounds should display intrinsic bioactivity against wild-type *S. aureus* but lack activity against *S. aureus* strains in which flux into the WTA pathway is abolished either by genetic (e.g., *tarO* deletion) or pharmacological (e.g., tunicamycin) means (Swoboda et al., 2009). One compound they identified, 1835F03, was subsequently optimized for potency and named targocil (Lee et al., 2010; Suzuki et al., 2011). Drug resistance mutant isolation revealed that targocil inhibits TarG, an essential subunit of the WTA ABC transporter (Swoboda et al., 2009; Schirner et al., 2011). As expected, resistance to targocil is also achieved by loss-of-function mutations in *tarO* or *tarA*, which effectively bypass the mechanism of action (MOA) of targocil (Swoboda et al., 2009). Although the frequency of resistance (FOR) to targocil is high (Lee et al., 2010), the contribution of Δ*tarO* and Δ*tarA* mutants to targocil drug resistance could be eliminated in the presence of oxacillin (Campbell et al., 2011, 2012). Collectively, these findings suggest that WTAIs could have significant potential as β-lactam combination agents against MRSA.

Despite extensive genetic and pharmacological studies of WTA biosynthesis in methicillin-sensitive *S. aureus* (MSSA) strains, relatively little is known about this pathway in MRSA strains in the context of a β-lactam potentiation target. Here, we describe an analogous screening approach to identify and characterize three additional classes of late-stage WTAIs that inhibit TarG. We show that multiple TarG inhibitors also display broad antibacterial spectrum and markedly reduced FOR in combination with imipenem. Interestingly, we demonstrate that the clinically acquired MRSA strain COL has a much broader repertoire of bypass mutations than previously identified. We show that these additional mutants are defective in late-stage WTA biosynthesis and are attenuated in their virulence in a murine deep thigh infection model. Further, all mutants displayed striking hypersusceptibility phenotypes to β-lactam antibiotics both in vitro and in an animal model of MRSA infection, reinforcing the rationale for combining WTA inhibitors with β-lactam antibiotics. Coadministration of TarG inhibitors with imipenem displayed compound-specific synergistic or strongly additive effects that approached standard of care antibiotics and pharmacologically demonstrate the potential of WTAIs as effective β-lactam combination agents to treat MRSA.

RESULTS

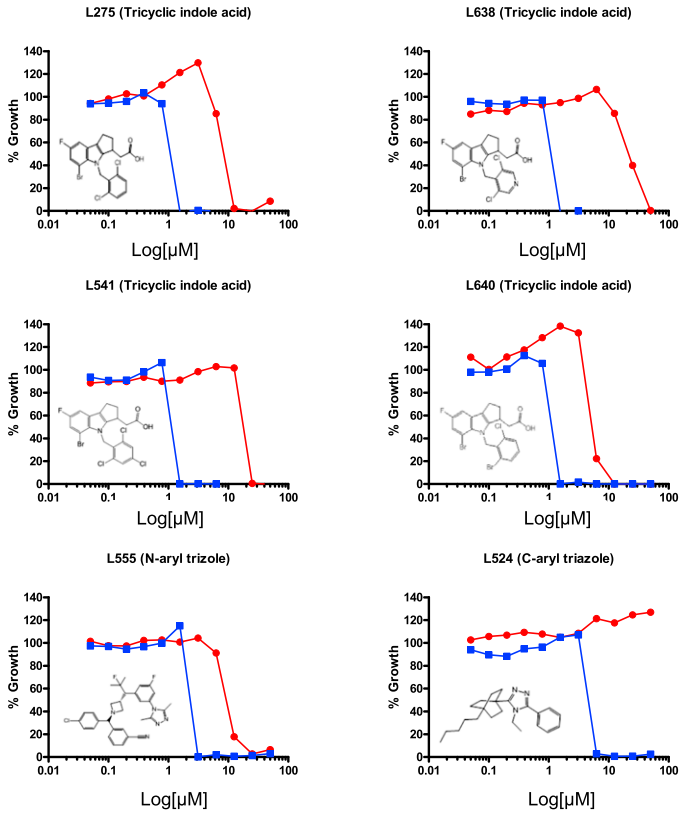
Pathway-Based Whole-Cell Screening for Late-Stage WTAIs

As first demonstrated with targocil, small molecule inhibitors of late-stage WTA enzymes are predicted to display intrinsic growth inhibitory activity against *S. aureus*, which is specifically reversed in a Δ*tarO* strain background (Swoboda et al., 2009). Accordingly, a focused library of ~20,000 *S. aureus* bioactive synthetic compounds (Huber et al., 2009) was screened in triplicate at two drug concentrations (3 and 16 μM) against the Δ*tarO* and wild-type strains in tryptic soy broth (TSB) liquid medium in a 1,536-well plate format with growth measured by optical density. Compounds displaying greater than 80% reduced activity against the Δ*tarO* strain versus wild-type were confirmed provided they displayed distinct dose responses against the two strains and an elevated minimum inhibitory concentration (MIC) against Δ*tarO* strain versus wild-type (Figure 1). The confirmed compounds, their physicochemical properties (see Table S1), and chemical structures are shown (Figure 1A). Four compounds (L275, L638, L541, and L640) are analogs of the tricyclic indole structural class (note L541 and L640 follow-up was limited because of compound availability and structural redundancy with L275 and L638). L555 is a member of a large series of N-aryl triazoles in which each member contains two chiral centers, but only L555 displays bioactive chemotypes consistent with inhibiting WTA biosynthesis (see Figure S2). The sixth compound, L524, is structurally a member of C-aryl triazoles. Interestingly, none of the compounds are structurally related to targocil (see Table S1).

Microbiological Characterization of Late-Stage WTAIs

Based on extensive genetic characterization of WTA biosynthesis in *S. aureus*, inhibitory compounds acting on late-stage WTA assembly are predicted to phenocopy targocil and phenotypes associated with loss-of-function mutations in late-stage essential genes of the pathway (Swoboda et al., 2009). Accordingly, MIC determinations of these compounds were determined in comparison to targocil under a variety of conditions. Like targocil, each of the hit compounds displayed strong antimicrobial activity, with MIC values ranging from 1–8 μg/ml against MSSA strain RN4220 and MRSA strain COL, consistent with targeting an essential cellular process, such as late-stage WTA biogenesis (Figure 1B). Indeed, all compounds also display 4- to 32-fold higher MIC values against Δ*tarO* or Δ*tarA* null strains, analogous to genetic suppression of WTA late-stage essential gene mutations in Δ*tarO* or Δ*tarA* strain backgrounds (D'Elia et al., 2006a, 2009a). *S. aureus* MIC values of each of these compounds were also noticeably antagonized (4- to 16-fold) by the addition of a sub-MIC level of tunicamycin (2 μg/ml) sufficient to specifically inhibit TarO activity (Campbell et al., 2011). Targocil is also reported to have a bacteriostatic mode of action (Swoboda et al., 2009; Campbell et al., 2012), and kill-curve analysis reveals that L275, L638, L524, and L555 all strongly arrest *S. aureus* cell division without compromising viability over an extended 24 hr time course (see Figure S3). Therefore, each of the compounds share striking microbiological characteristics with targocil that are consistent with their role as inhibitors of late-stage WTA biogenesis.

A



B

Strain	Targocil	L275	L640	L638	L555	L524	Conditions
MRSA COL	1	1	1	2	4	8	
COL+ Tuni	>32	8	4	16	64	>32	2 μg/ml Tuni
COL+Imipenem	1	0.5	0.5	1	2	4	4 μg/ml IPM
MSSA (RN4220)	0.5	1	1	1	2	4	
RN4220 + Tuni	>32	4	4	16	8	>32	2 μg/ml Tuni
RN4220 Δ*tarO*	>32	4	4	16	8	>32	deletion mutant
COL 21R/Δ*tarA*	>32	8	4	16	>64	>32	TNP mutant
COL 2L/Δ*tarO*	>32	8	4	16	>64	>32	TNP mutant
		Group1			Singleton	Singleton	

Figure 1. Summary of WTAI Chemotypes

(A) Dose response growth curves of MSSA wild-type strain RN4220 (blue) and Δ*tarO* (red) at indicated drug concentrations for each putative WTAI. Chemical structure and structural class are indicated.

(B) Minimum inhibitory concentrations (MIC; μg/ml) of WTAIs. Targocil bioactivity is included as control and comparator compound. Tunicamycin (Tuni) supplementation (2 μg/ml) is used to phenocopy Δ*tarO* and Δ*tarA* mutants. Imipenem (IPM) supplementation at 4 μg/ml reflects its clinical break point concentration; highly reduced MIC values under such conditions reflect synergy.

See also Figures S2, S3, and S5 and Tables S1 and S2.

Both a MRSA Δ*tarO* strain (Campbell et al., 2011) and an antisense interference-based depletion mutant of *tarL* (Lee et al., 2011) displays highly specific β-lactam hypersusceptibility phenotypes. Accordingly, we tested whether L275, L638, L524, and L555 are synergistic in combination with imipenem by scoring their fractional inhibitory concentration (FIC) index (FICI) using the standard microdilution checkerboard assay, where FIC indices of $\leq$0.5, 1–2, or $\geq$4 indicate synergistic, additive, or antagonistic chemical interactions, respectively (Amsterdam, 2005). Each of the tested compounds tested against MRSA COL produced FIC indices between 0.516 and 0.625, indicating strong additivity in combination with imipenem (see Table S2). Only L524 demonstrated a strong synergistic activity with imipenem against methicillin-resistant *Staphylococcus epidermidis* (MRSE; see Table S2).

To further examine whether L275, L638, L524, and L555 are late-stage WTA inhibitors, L275 was selected as a representative compound to isolate drug-resistant mutants using a recently described *S. aureus* transposon (tsn) system (Wang et al., 2011). Applying this approach in MRSA COL, ten independent tsn insertion mutants were identified, each specifically inactivating *tarO* (n = 8) or *tarA* (n = 2) (see Figure S4). These loss-of-function mutations recapitulate the demonstrated drug resistance compensatory mechanism of targocil (Swoboda et al., 2009). Further, L275^R mutants independently reproduce the initial genetic basis that led to identification of L275 and other compounds.

B. subtilis Expressing TarGH Is Selectively Sensitized to L275, L638, L524, and L555

Based on the observed phenocopy of targocil treatment, we hypothesized that our compounds may target the WTA transporter, TarGH. To address this, we determined compound MICs in both a wild-type strain of *B. subtilis* and strain KS002, which is complemented with *S. aureus* TarGH in place of native TagGH (Schirner et al., 2011).

The complemented strain KS002 displayed 4- $\geq$ 32-fold increased sensitivity to each of the compounds tested in comparison to wild-type *B. subtilis*, suggesting TarGH as the likely target (see Figure S5). Activity against wild-type *B. subtilis* also suggests an extended spectrum for these compounds in comparison to targocil, which is specific for *S. aureus* (Swoboda et al., 2009).

Targocil^R Mutants Are Cross-Resistant to L275, L638, L524, and L555

Fourteen independently derived targocil-resistant (targocil^R; see Table S3) mutants were isolated in MRSA COL, and those genes previously implicated in drug resistance (*tarO*, *tarA*, and *tarG*; Swoboda et al., 2009) were sequenced to identify causal mutations. Such mutations provide an important resource to test

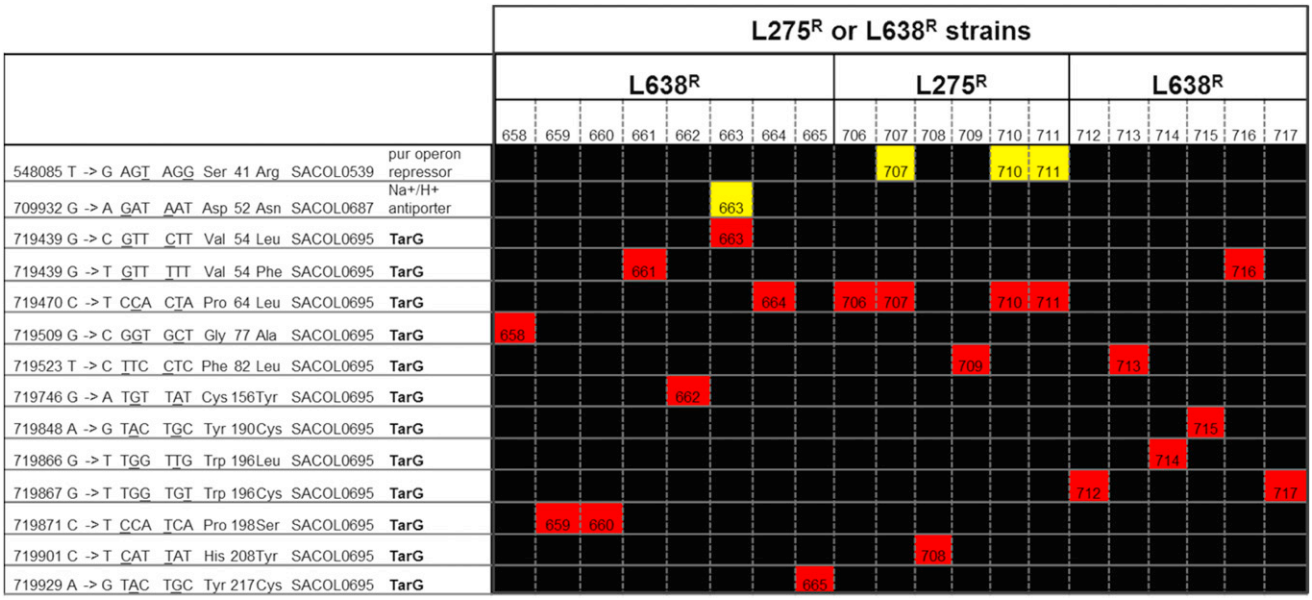


Figure 2. Whole-Genome Sequencing of L275^R and L638^R Mutants

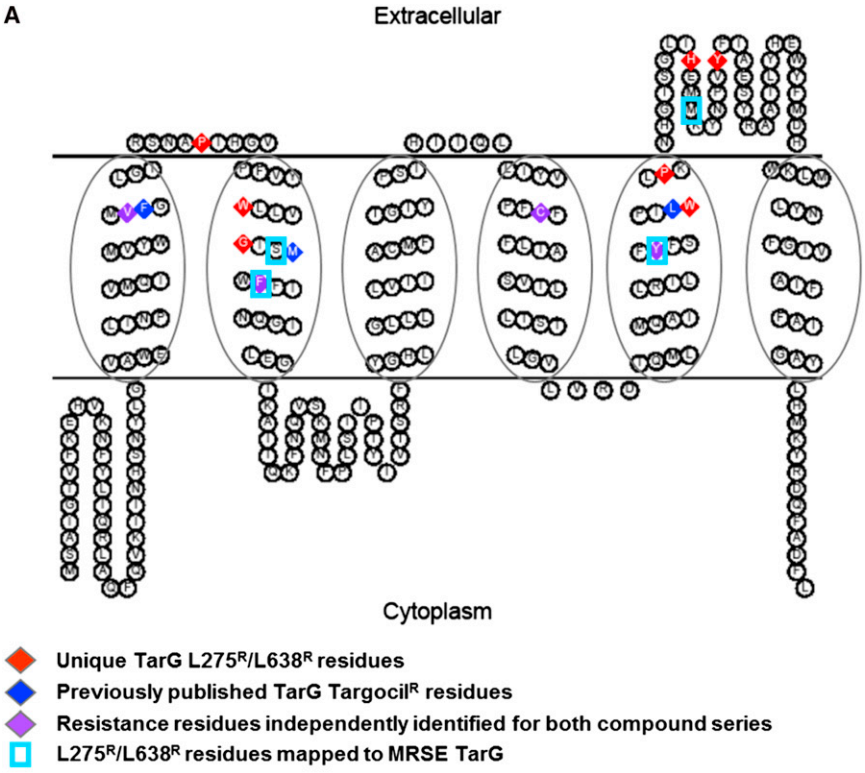
Heatmap summary of all nonsynonymous mutations identified by illumina-based whole-genome sequencing (100X genome coverage) of 20 independently isolated L275^R or L638^R mutants in MRSA COL. Red, nonsynonymous mutation; black, no change versus parental MRSA COL genome sequence. Genome position, base pair change, and resulting amino acid residue substitution are highlighted. Note: additional nonsynonymous mutations (yellow) mapping to SACOL0539 (strains 707, 710, and 711) and SACCOL0687 (strain 663) are presumably unlinked to drug resistance as each strain also possesses a TarG amino acid substitution. See also Figures S1, S4, and S7 and Tables S4 and S5.

whether targocil^R mutants are cross-resistant to the compounds we identified. Expectedly, six targocil^R mutants mapped to either *tarO* or *tarA* and contained either missense, nonsense, or insertional loss-of-function mutations able to buffer the cell from the effects of targocil (see Figure S6A). As expected, all Δ*tarO* and Δ*tarA* mutants also exhibited a striking hypersusceptibility to imipenem with MIC values reduced 64- to 128-fold compared with the wild-type MRSA COL strain. Importantly, eight additional mutants mapped to TarG and correspond to the previously reported targocil^R TarG-W73C or TarG-M80I mutants (Swoboda et al., 2009; Schirner et al., 2011), which despite conferring a 4- to 8-fold level of resistance to targocil, similar to Δ*tarO* and Δ*tarA* bypass mutants, lacked any change in their susceptibility to imipenem, implying that these are not loss-of-function mutations (see Figure S6A). Consistent with this view, TarG-W73C and TarG-M80I mutants lack an obvious growth phenotype and produce WTA as inferred by their full susceptibility to bacteriophage K-mediated cell lysis (see Figure S6B), which is a process dependent on phage recognition of WTA polymers that serve as a cell surface receptor for entry into the cell (Xia et al., 2010).

Employing the above described targocil^R mutant set, we tested whether representative mutants are cross-resistant to L275, L638, L524, or L555. Interestingly, TarG-W73C or TarG-M80I MRSA COL strains are noticeably cross-resistant to each of the four compounds tested (Table S3). Further, the extent of cross-resistance observed was comparable to that of the TarO-G48S loss-of-function mutant. As TarG-W73C and TarG-M80I mutants appear to produce WTA (see Figure S6B), these results strongly suggest that the tested compounds target TarG and that the suppression of their bioactivity is not due to the absence of WTA polymers.

L275 and L638 Drug Resistance Mapping and Mechanism of Action Studies

L275^R and L638^R mutant selection and next-generation whole-genome sequencing (NGS) were performed in MRSA and MRSE strain backgrounds. As expected, fast-growing L275^R and L638^R MRSA COL strains contained an extensive set of independently isolated TarG amino acid substitution mutations (n = 20) (Figure 2). Indeed, several previously described targocil^R mutations, including TarG-F82L, TarG-C156Y, and TarG-Y190C (Swoboda et al., 2009; Schirner et al., 2011), confer resistance to L275 and/or L638. In addition, missense mutations yielding amino acid substitutions at seven additional positions in the TarG protein were identified. Many of these mutations map to the same predicted transmembrane domains as previously published targocil^R mutations (Schirner et al., 2011; Figure 3A). Additional TarG mutations map to the predicted extracellular loop 1 (TarG-P64L) or extracellular loop 3 (TarG-H208Y, TarG-Y217C) of the protein. As NGS analysis failed to identify any additional nonsynonymous mutations beyond *tarG* in 80% of L275^R and L638^R mutants examined, and in the remaining instances in which a second nonsynonymous mutation was detected a TarG amino acid substitution mutation was also faithfully identified, we conclude that *tarG* mutations are causal for the observed drug resistance. In only one instance (two independently derived TarG-V54F mutations) was a growth phenotype observed (see Figure S7). Interestingly, an alternative amino acid substitution at this residue (TarG-V54L) grew



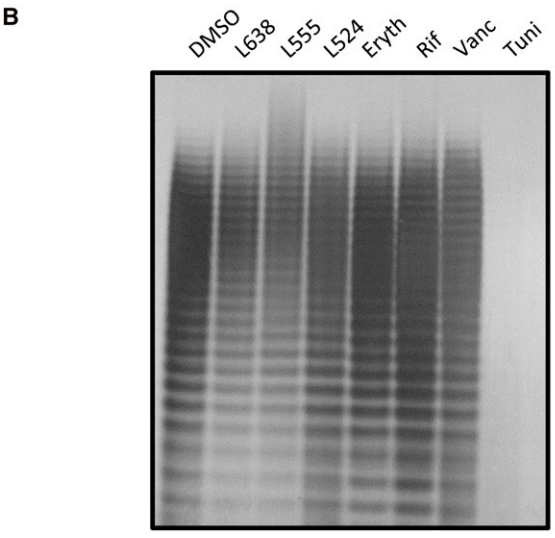


Figure 3. Predicted Structural Topology of TarG and Location of Drug Resistance Amino Acid Substitutions

(A) Predicted topology of *S. aureus* TarG with previously identified targocil^R mutations (blue diamonds), unique point mutations identified here conferring L275^R or L638^R (red diamonds), and resistance residues independently identified for both targocil and L275 or L638 (purple diamonds). Note: *S. aureus* and *S. epidermidis* TarG are 96% homologous and share identical length (see Figure S8). Accordingly, *S. epidermidis* TarG L275^R or L638^R mutations are also highlighted (boxed in blue). TarG transmembrane topology was predicted using the SOSUI program and diagram generated with TOPO2 software.

(B) WTA extraction and analysis of WTA levels in MRSA COL challenged with 10X MIC of L638, L555, L524 or control compounds Erythromycin (Eryth), rifamicin (Rif), or Vancomycin (Vanc). Tunicamycin (4 μg/ml; Tuni) treatment at subMIC serves as a positive control for complete WTA depletion.

See also Figure S1 and Tables S4 and S5.

indistinguishably from wild-type, demonstrating the specificity of this mutation in effecting TarG function. Remarkably, all L275^R and L638^R TarG mutations displayed a common cross-resistance to L524, L555, and targocil but not to other antibiotic classes tested (see Table S4).

Similarly, L275^R and L638^R mutant selection and NGS analysis performed in MRSE identified single nonsynonymous missense mutations mapping to the *S. epidermidis* TarG protein. Indeed, two of the drug-resistant mutations correspond to the same residues identified in *S. aureus* TarG (TarG-F82L and TarG-Y190H). Additional mutations, TarG-S79I and four independently derived mutations mapping to residue M211 (TarG-M211L and TarG-M211I), are predicted to localize within transmembrane 2 or extracellular loop 3 domains, respectively (Figure 3A; see also Figure S8). Collectively, these studies provide strong genetic evidence that TarG is the molecular target of L275 and L638 across methicillin-resistant *Staphylococci* and suggest that they may interfere with TarG function by binding either to the extracellular surface and/or substrate channel of the WTA transporter.

To further investigate inhibitory effects on WTA biogenesis by our compounds, WTA polymers were extracted from drug-treated MRSA COL, normalized according to cell biomass, and examined by PAGE analysis (Meredith et al., 2008). As L638, L524, and L555 are highly potent and sufficient biomass is required to isolate WTA from drug-treated cells, high initial innocula (1 × 10^9 cells/drug treatment) were required. WTA extracted from mock-treated control cells appears as a ladder of discrete size polymers (Figure 3B). As expected, WTA biogenesis was completely abolished by tunicamycin treatment and unaffected by antibiotic controls. Conversely, L638 and L555-treated cells displayed modest but reproducible changes in WTA polymer levels and/or size (Figure 3B). L524-treated cells, however, exhibited only a very minor decrease in WTA (as observed by reduced straining of higher molecular weight polymers), similar to repeated attempts with targocil under identical conditions (data not

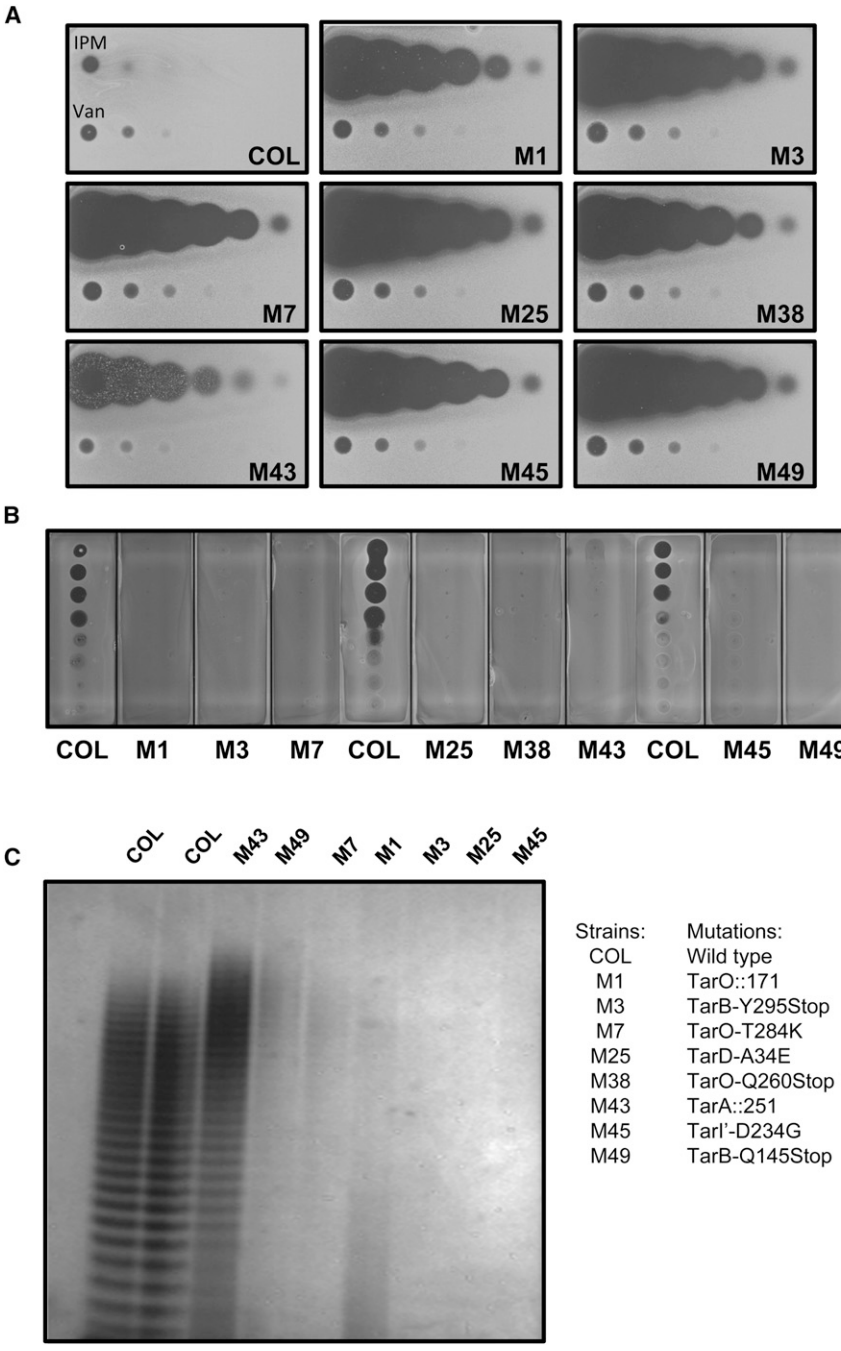


Figure 4. L638^R *tar* Mutants Share β-Lactam Hypersusceptibility and WTA Depletion Phenotypes

(A) Altered imipenem (IPM) susceptibility of MRSA COL *tar* mutants by agar susceptibility assay (see inset for description of specific mutations). Threefold serial dilutions of IPM (starting with 8 μg material) spotted left to right across plate. Vancomycin (Van; 2 μg starting material) serves as control for specificity of β-lactam susceptibility phenotype.

(B) Bacteriophage K resistance phenotype of MRSA COL *tar* mutants.

(C) WTA extraction and PAGE analysis from L638^R MRSA COL *tar* mutants. Note: WTA material normalized to cell biomass prior to loading.

See also Figure S9 and Tables S4, S5, and S11.

shown). Although no in vitro biochemical assay is available to test whether these compounds inhibit TarG directly, the incomplete depletion of WTA polymer levels by TarG inhibitors likely reflects the fact that such agents effectively inhibit growth. Consequently, pre-existing WTA comprising cells prior to TarG inhibitor treatment is not diluted, as minimal cell doublings occur postdrug treatment despite inhibition of new WTA biosynthesis, whereas under tunicamycin subMIC conditions in which TarO-mediated WTA biosynthesis of new material is specifically inhibited, cells robustly grow and dilute pre-existing WTA through successive cell divisions.

Extended NGS Analysis of L638^R Mutations in MRSA COL

NGS analysis was also performed with a large number of slower growing MRSA COL L638^R mutants (n = 55) arising from this study to confirm expected presence of Δ*tarO* or Δ*tarA* mutations and examine whether additional L638^R compensatory drug-resistance mechanisms exist. Indeed, several L638^R mutants mapped to *tarO* or *tarA* and are presumed to oblate gene function (Figure 4). However, several additional mutants also mapped to late-stage WTA genes, including *tarB*, *tarD*, and *tarI'* (Figure 4). Moreover, all isolated *tarB*, *tarD*, and *tarI'* mutants displayed similar phenotypes to Δ*tarO* and Δ*tarA* mutants. In addition to their L638^R phenotype, *tarB*, *tarD*, and *tarI'* mutants displayed a striking cross-resistance to targocil and other WTAIs (see Table S5), as well as restoring susceptibility to imipenem and prominent resistance to phage K-mediated cell lysis, implying they are also loss-of-function mutations in these late-stage WTA genes (Figure 4). Further, in numerous instances no additional nonsynonymous mutations beyond those associated with *tar* genes were identified by NGS analysis of L638^R mutants, thus ruling out the possibility that compensatory mutations are responsible for the observed phenotypes. To directly determine the severity of WTA phenotypes associated with *tarO*, *tarA*, *tarB*, *tarD*, and *tarI'* mutants, their WTA was extracted and analyzed by PAGE. All mutants examined displayed dramatic alterations in WTA abundance and/or polymer size (Figure 4C). Interestingly, these results demonstrate that MRSA COL is able to tolerate profound loss-of-function mutations in late-stage WTA biosynthesis genes and that such mutations expand its repertoire of resistance to TarG inhibitors.

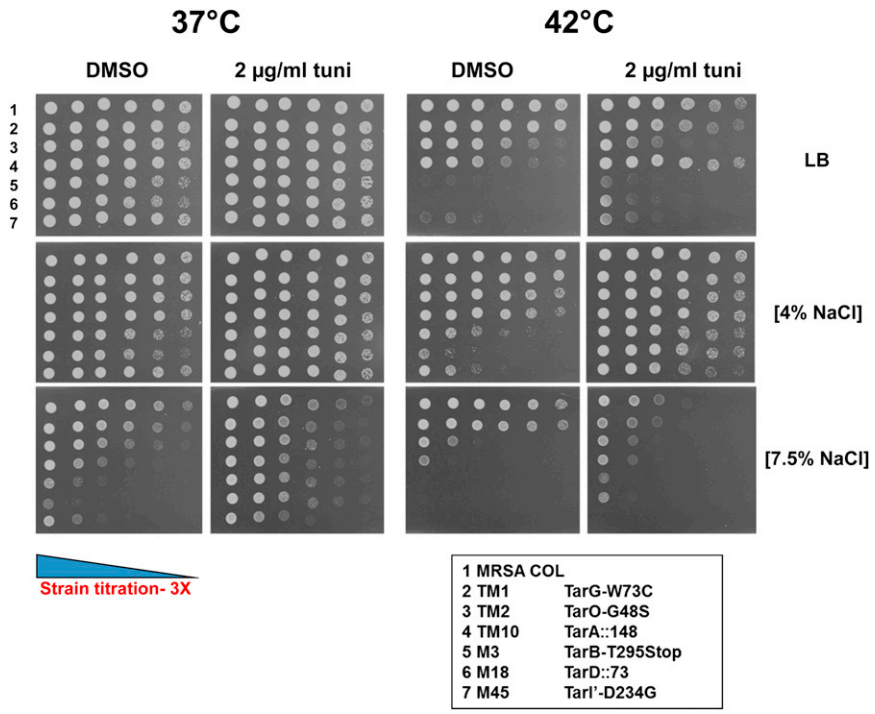


Figure 5. Conditional Essentiality of MRSA COL *tar* Mutants

Growth phenotype of MRSA COL *tar* mutants spotted as 3-fold serial dilutions starting at 5×10^4 cells/spot (left to right) on LB medium, LB + 4% NaCl, LB + 7.5% NaCl, and +/− tunicamycin (2 μg/ml) supplementation grown at 37°C or 42°C for 18 hr. See also Figure S6 and Tables S3 and S11.

tarB, *tarD*, and *tarI'* Mutants Possess Temperature and Osmotic-Sensitive Growth Phenotypes

Identifying viable mutations in late-stage WTA genes with dramatic loss-of-function phenotypes was not expected because they were shown to be essential in both *S. aureus* and *B. subtilis* (D'Elia et al., 2006a, 2006b). To reconcile these findings, *tarB*, *tarD*, and *tarI'* mutant phenotypes were compared to Δ*tarO* and Δ*tarA* mutants under conditions of elevated temperature and osmotic stress. *tarB* and *tarD* mutants grown on LB agar displayed modest growth phenotypes at 37°C, and all *tar* loss-of-function mutants tested exhibited marked growth phenotypes under high osmotic pressure with 7.5% NaCl (Figure 5). Consistent with the expectation that these phenotypes are linked to the *tar* mutation, supplementing the medium with 2 μg/ml tunicamycin partially reversed their observed phenotypes. *tarB*, *tarD*, and *tarI'* mutants also displayed striking temperature-sensitive growth phenotypes at 42°C, whereas *tarA* and *tarO* mutants shared only mild growth phenotypes, as previously reported (D'Elia et al., 2006a, 2009a). Thus, the relative severity of *tar* mutant growth phenotypes mirror that determined in a MSSA RN4220 strain background but is observed at higher temperature in MRSA COL. All temperature-sensitive growth phenotypes were noticeably suppressed by tunicamycin or 4% NaCl supplementation and fully suppressed by adding both tunicamycin and 4% NaCl (Figure 5). Further, at 37°C under hypotonic growth conditions (no NaCl in Luria broth [LB] medium), *tarB* and *tarD* mutants are also growth impaired and fully suppressed by cosupplementing tunicamycin and 4% NaCl (see Figure S9). At 42°C under hypotonic conditions, all *tar* mutants (including *tarO* and *tarA* mutants) displayed extreme growth phenotypes fully reversed by tunicamycin and 4% NaCl (see Figure S9). These results corroborate the temperature-sensitive and osmotic remedial growth phenotypes of Δ*tarO* mutants (Hoover and Gray, 1977; Vergara-Irigaray et al., 2008) and extend these finding to *tarA* as well as *tarB*, *tarD*, and *tarI'* loss-of-function mutants. Therefore, relatively minor differences in growth conditions can profoundly impact the viability of *S. aureus* strains defective in late-stage WTA biosynthesis.

Attenuated Virulence Phenotypes of MRSA COL Strains Defective in Early- or Late-Stage WTA Biogenesis

Identifying $L275^R$ and $L638^R$ loss-of-function mutants that map to early and late-stage WTA biosynthetic genes enabled us to examine their possible virulence phenotypes in MRSA COL using a previously described murine thigh infection model (Gill et al., 2007). We reasoned that in vivo phenotypes may well be expected based on results obtained from extensive in vivo studies using MSSA-derived *tarO* mutants (Weidenmaier et al., 2004, 2005; Weidenmaier and Peschel, 2008). Moreover, temperature and osmotic stress growth phenotypes of MRSA COL *tarB*, *tarD*, and *tarI'* mutants we observed under laboratory growth conditions may decrease *S. aureus* pathogenicity. As expected, *tarO* and *tarA* mutants typically displayed an approximate 1 log reduction in bacterial burden after 24 hr versus the wild-type parental strain over a broad range of infection doses in this animal model (Figure 6A; Table S6). Interestingly, at the lowest infection dose used (1×10^6 cells), the *tarO* mutant strain displayed an even more pronounced attenuated virulence, nearing as much as a 4 log reduction in bacterial burden compared to wild-type MRSA COL. Similarly, *tarD* and *tarI'* mutants also displayed dramatic attenuated virulence phenotypes, particularly across lower infection doses, where as much as 3 log reductions in bacterial burden were observed, whereas the *tarB* mutant more closely mirrored the *tarA* with bacterial burden after 24 hr reproducibly reduced ~1 log across all infection doses tested. These data demonstrate the relevance of WTA as a virulence determinant in *S. aureus* and suggest that their attenuated virulence phenotypes may further reduce the threat of drug resistance in a therapeutic context.

Imipenem Efficacy against MRSA *tar* Mutants Resistant to TarG Inhibitors

As *tarO*, *tarA*, *tarB*, *tarD*, and *tarI'* loss-of-function mutations enhance MRSA COL susceptibility to β-lactam antibiotics in vitro, we tested whether this phenotype extends to the deep thigh

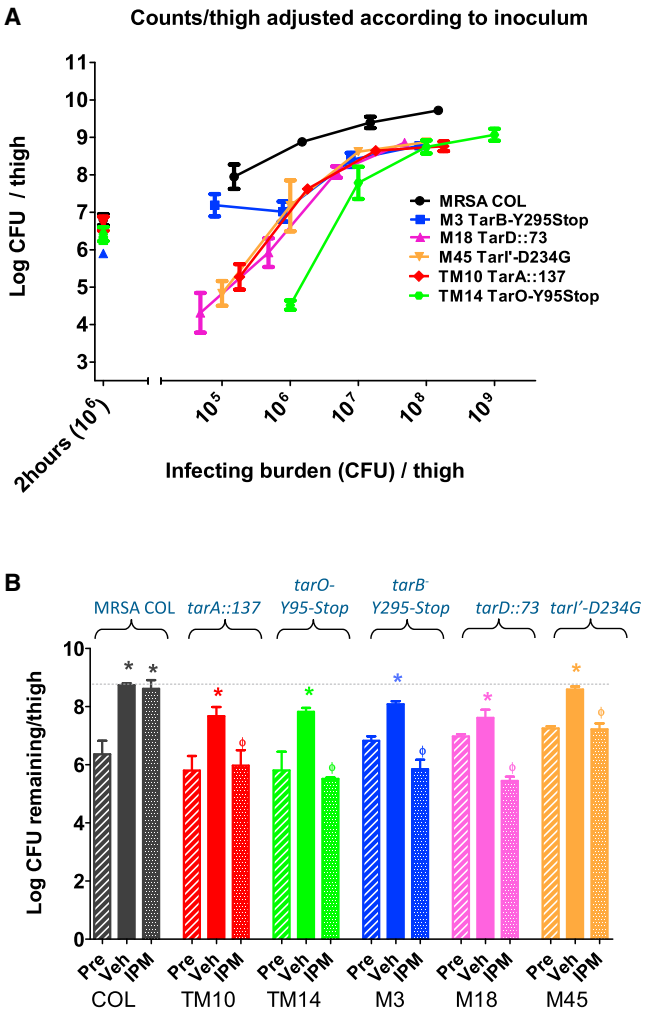


Figure 6. Attenuated Virulence and Imipenem Hypersusceptibility of MRSA COL *tar* Mutants in a Murine Deep Thigh Infection Model

(A) Attenuated virulence of *tar* mutants versus MRSA COL wild-type (black line) across 4 log escalation in infection dose. See inset for description and color-coding of specific mutants. Infection inocula of M14 (TarO-Y95stop) strain was one log great than other strains tested. Two hour (10^6) bacterial burden control confirms infection dose (10^6/thigh) when enumerated from animals 2 hr postinfection. See also Table S6.

(B) Restored efficacy of Imipenem against MRSA COL *tar* mutants. Immune-suppressed CD-1 mice (five mice per group) were challenged intramuscularly with 1×10^6 cfu/thigh of MRSA COL or the indicated MRSA COL *tar* mutant. Imipenem (IPM; 10 mg/kg) or vehicle (Veh; 10 mM MOPS) were dosed subcutaneously (s.c.) three times over a 24 hr period. Thigh homogenates were obtained 2 hr postinfection and prior to treatment (Pre), or post-24-hr treatment with Veh or IPM and serially plated to determine cfu/thigh remaining. Bacterial burden was enumerated and compared among three groups. *$p < 0.05$ versus respective 2 hr control; ϕ $p < 0.05$ versus respective 24 hr control. See also Table S7.

murine infection model. Imipenem is ineffective in treating MRSA-COL-infected animals in this model when dosed at 10 mg/kg subcutaneously three times a day (tid) for 24 hr versus the mock-treated control group administered 10 mM MOPS as vehicle (Figure 6B). However, imipenem efficacy was restored in mice infected with *tarO*, *tarA*, *tarB*, or *tarD* mutant strains, producing an ~3 log reduction in bacterial burden versus the parental COL strain identically treated with imipenem (Figure 6B; Table S7). Similarly, imipenem treatment achieved an ~2 log reduction in bacterial burden with mice infected with a *tarI'*-deficient strain versus wild-type COL. Therefore, we expect that the drug resistance to TarG inhibitors, mediated by the selection for these compensatory bypass mutants will be reduced in an MRSA infection setting by combining such agents with a β-lactam antibiotic. These data also provide clear genetic demonstration that in addition to TarO and TarA, late-stage WTA enzymes, including TarB, TarD, or TarI', are also therapeutically relevant MRSA β-lactam potentiation targets.

TarG Inhibitors ± Imipenem Frequency of Resistance

Targocil displays a high FOR of $\sim 7 \times 10^{-7}$ cells at 8X MIC (Lee et al., 2010), which is substantially reduced in the presence of the β-lactam, oxacillin (Campbell et al., 2011). Accordingly, we examined the spontaneous rate of drug-resistant mutants isolated among this extended set of TarG inhibitors. L524 and L555 displayed markedly higher or marginally lower FOR than targocil, respectively, over a range of MIC levels tested (Table 1). Whereas L638 tested at 2X MIC or 4X MIC only minimally reduced FOR ($\sim 2 \times 10^{-7}$) compared to targocil, a borderline-acceptable FOR of 1.9×10^{-8} was achieved at 8X MIC. Interestingly, L275 displayed a highly favorable dose-dependent FOR, with spontaneous drug-resistant isolates recovered at a frequency of $\sim 4 \times 10^{-8}$ at 2–4 X MIC, and $\leq 3.6 \times 10^{-10}$ at 6–8X MIC levels (Table 1). Further, in combination with imipenem at a subMIC level of 4 μg/ml (its clinical break point concentration against MRSA; Tan et al., 2012), similar L275 FOR levels could be achieved at as low as 2X its MIC. Remarkably, resistance associated with L275 in combination with a subinhibitory concentration of imipenem was therefore reduced ~10^4-fold from the original FOR of targocil.

TarG Inhibitors ± Imipenem In Vivo Efficacy

TarG inhibitors display favorable in vitro potency but possess high plasma protein binding, which could diminish their efficacy in a treatment model of MRSA infection (see Table S8). As this issue may be particularly problematic if the site of infection is muscle tissue, such as the deep thigh infection model, we turned to an alternative animal efficacy model involving an intraperitoneal challenge and subsequent kidney colonization by MRSA COL (Gill et al., 2007). Targocil, L275, L638, or L555 administered subcutaneously at 200 mg/kg tid did not significantly reduce bacterial burden after 24 hr treatment, as measured by colony-forming units per gram kidney tissues (cfu/g) versus vehicle-control-treated animals (Figure 7; see also Table S9). Further, imipenem singly administered subcutaneously at 10 mg/kg tid in this animal model only modestly reduced bacterial burden by ~1.5 log cfu/g versus vehicle control group. However, a strongly additive or synergistic efficacy was achieved by coadministering TarG inhibitors with a subefficacious treatment of imipenem. For example, coadministering TarG inhibitors subcutaneous at 200 mg/kg tid and imipenem administration markedly reduced bacterial burden among treatment groups ranging from 2-3 log cfu/g versus vehicle control group. Although only L275 displayed significant synergy in combination with imipenem versus imipenem alone (t test $p < 0.05$), coadministering these

Table 1. Spontaneous Resistance Frequency of TarG Inhibitors ± Imipenem

	Drug Alone					With 4 μg/ml IPM				
	2X	4X	6X	8X	Solubility Issue	2X	4X	6X	8X	MIC
L275	6.2×10^{-8}	1.9×10^{-8}	3.6×10^{-10}	$<3.6 \times 10^{-10}$	No	$<3.6 \times 10^{-10}$	$<3.6 \times 10^{-10}$	$<3.6 \times 10^{-10}$	$<3.6 \times 10^{-10}$	1
L638	2.1×10^{-7}	1.3×10^{-7}	4.2×10^{-8}	1.9×10^{-8}	No	4.6×10^{-8}	1.5×10^{-9}	2.7×10^{-10}	$<2.7 \times 10^{-10}$	2
L524	N/A	N/A	N/A	N/A	from 4X to 8X	N/A	N/A	N/A	N/A	5
L555	3.8×10^{-7}	2.1×10^{7}	2.2×10^{-7}	2.4×10^{-7}	8x	6.1×10^{-7}	4.2×10^{-8}	1.3×10^{-8}	1.8×10^{-8}	4
Targocil	1.7×10^{-6}	9.0×10^{-7}	N/A	N/A	from 4X to 8X	1.8×10^{-7}	1.9×10^{-7}	1.7×10^{-7}	1.6×10^{-7}	1

N/A, not applicable (too high to count). See also Table S10.

agents (albeit at relatively high drug concentrations) reduced bacterial burden to levels achieved by linezolid or ciprofloxacin treatment.

DISCUSSION

Applying a chemical biology screen designed to mimic the well-characterized WTA gene dispensability phenotypes in *S. aureus*, we identify a series of synthetic compounds each chemically distinct from targocil. Extensive drug resistance mapping studies performed in MRSA and MRSE provide genetic evidence that like targocil, small molecules we report here inhibit TarG as their primary drug target. Corroborating this conclusion, WTA levels are partially depleted in TarG inhibitor-treated cells, particularly L638 and L555 structural classes, and bypass mutations to these WTAIs correspond to loss-of-function mutations in *tar* genes that function upstream of TarG. Depending on their structural class, TarG inhibitors described here display either the selectivity and propensity for resistance as targocil or an improved antibacterial spectrum and markedly reduced frequency of drug resistance. As drug-resistant bypass mutations isolated in MRSA COL are dramatically hypersensitive to β-lactam antibiotics, the effective FOR of these compounds can be dramatically reduced in combination with subinhibitory concentrations of imipenem. These studies suggest TarG to be a highly druggable antibacterial target and demonstrate that cognate inhibitors display in vivo efficacy when paired with imipenem in treating an animal MRSA infection.

Essential versus Conditional Essential Phenotypes of Late-Stage WTA Biosynthetic Genes

Two hypotheses for the reported essentiality of late-stage WTA biosynthetic genes and nonessentiality of early-stage WTA genes have been widely considered (D'Elia et al., 2006a, 2006b, 2009c). Specifically, loss-of-function mutations in late-stage WTA genes may accumulate toxic WTA intermediates and/or decrease available bactoprenol necessary for peptidoglycan synthesis to growth impairing levels. So, why do multiple late-stage WTA genes appear nonessential in MRSA COL when they have been demonstrated to be essential in MSSA strain RN4220? MRSA COL may simply produce more bactoprenol or possess greater flexibility in recycling bactoprenol from the WTA intermediates than *S. aureus* RN4220 or *B. subtilis* strains in which the terminal phenotype of WTA mutations have been examined (D'Elia et al., 2006a, 2006b). Alternatively, *tarB*, *tarD*, and *tarI'* mutants isolated here may be viable because they retain some minor level of residual activity that is required to reverse bactoprenol flux and such an activity would be absent among null mutations, rendering the latter inviable. Accordingly, the fact that such mutants make little to no WTA does not impair viability but some minimal level of WTA biosynthesis may be necessary to recycle bactoprenol. We also note that our *tar* mutant phenotypes are derived from amino acid substitution or C-terminal truncation mutations, all of which in the context of a postulated multimeric WTA enzyme complex (Formstone et al., 2008) may produce less severe phenotypes than complete gene deletion mutations. Importantly, MRSA COL mutations in late-stage WTA biosynthesis are in fact conditionally essential at 42°C. Moreover, consistent with *S. aureus* WTA

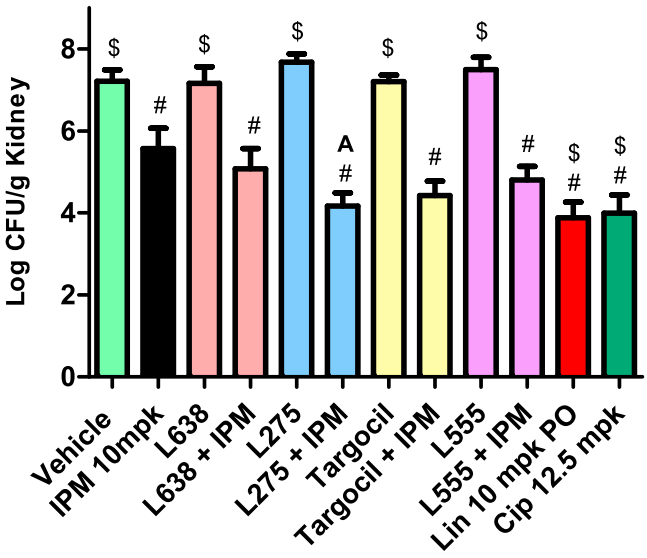


Figure 7. TarG inhibitors Augment Imipenem Efficacy

Immune-suppressed Balb/c mice (five mice per group) were challenged intraperitoneally with ~2 × 10^4 cfu of MRSA COL and dosed s.c. with the WTA inhibitor (200 mg/kg), imipenem (IPM; 10 mg/kg) coformulated with 50 mg/kg cilastatin, or coadministration of the TarG inhibitor and imipenem/cilistatin at 2, 5, and 8 hr postinfection challenge. Kidneys were aseptically collected, homogenized, and plated 24 hr after challenge to determine cfu remaining per gram kidney tissue. Linezolid (Lin; 10 mg/kg dosed orally) and ciprofloxacin (Cip; 12.5 mg/kg dosed s.c.) serve as positive control antibiotics for demonstrating efficacy. #, significant ($p < 0.05$) versus vehicle; $, significant ($p < 0.05$) versus IPM alone; A, significant ($p < 0.05$) versus IPM alone. See also Tables S8 and S9.

gene dispensability phenotypes (D'Elia et al., 2006a), MRSA COL *tarB*, *tarD*, and *tarI'* mutant phenotypes were noticeably suppressed by supplementing tunicamycin at levels that phenocopy deletion of *tarO*. Therefore, we conclude that the paradoxical severity of growth phenotypes among mutants in early versus late-stage WTA synthesis is conserved in MRSA COL. However, the absolute strength of these phenotypes can vary contextually in different *S. aureus* genetic backgrounds as might be expected considering the extensive genomic diversity identified among multiple fully sequenced *S. aureus* strains (Feng et al., 2008).

Off-Target Activity of L275, L638, L640, and L555

Unlike targocil, which is a highly specific inhibitor of *S. aureus* TarG activity (Swoboda et al., 2009; Schirner et al., 2011), several of the compounds we have identified likely possess an off-target activity. First, bioactivity of each of the new TarG inhibitors (with the exception of L524) is not fully suppressed by genetic or chemical inhibition of TarO. Second, these TarG inhibitors display a notable antibacterial activity that exceeds the spectrum of bacteria possessing a TarG ortholog or WTA biosynthetic pathway, including *Streptococcus pyogenes* as well as Gram-negative bacteria, including *Moraxella catarrhalis* and *Haemophilus influenzae* (see Table S10). Finally, L275, L638, and L555 display dramatically lower FOR against MRSA COL compared to targocil over a range of drug concentrations assessed either singly or in combination with imipenem. As all resistance mutants to these compounds isolated in both MRSA and MRSE shared either a TarG or the WTA bypass-mediated mechanism of resistance and these compounds lack anti-*Candida albicans* activity, we favor that this off-target activity reflects a secondary target rather than nonspecific/general cellular toxicity of the compounds. Studies to identify a potential secondary target to these compounds are ongoing.

TarG Is a Highly Druggable Target

Despite the opportunity for this pathway-based screen to identify inhibitors to any of the late-stage WTA enzymes, only TarG WTAIs have been reported by us and prior work (Swoboda et al., 2009). Interestingly, these inhibitors represent structurally distinct chemical series. We speculate that TarG is a particularly "druggable" target when compared to other Tar enzymes. The *S. aureus* WTA transporter is predicted to be a heteromeric complex consisting of dimers of the cytoplasmic ATPase subunit TarH and a WTA substrate channel forming subunit, TarG (Cuthbertson et al., 2010; Schirner et al., 2011). Therefore, TarG is uniquely exposed to the extracellular milieu and potentially susceptible to inhibitors that need not enter the cytoplasm. Consistent with this, all drug-resistant mutations to WTAIs we describe map to the presumed substrate channel or extracellular domains of TarG. Considering the substantial size of the substrate channel required to translocate WTA across the plasma membrane, structurally diverse compounds may have access to the channel and sterically interfere with WTA precursor export. In such a manner, TarG inhibitors would be mechanistically analogous to diverse protein synthesis inhibitors, which bind to the ribosome polypeptide export tunnel to block release of nascent polypeptides (Walsh, 2003).

WTAIs as β-Lactam Combination Agents

Significant therapeutic advantages are anticipated by combining a WTAI with existing β-lactam antibiotics to treat MRSA infections. These include improved antibacterial potency by the strong additivity or synergistic activity of the two agents and reduced drug resistance in the context as dual agents. Unexpectedly, the degree of observed synergy between imipenem and TarG inhibitors is generally lower than genetic analysis would predict. Campbell et al. (2012) have suggested that this may reflect the fact that β-lactams are only effective against actively growing cells and the bacteriostatic nature of TarG inhibitors might antagonize β-lactam activity. Conflicting with this view, however, is the fact that β-lactams suppress the FOR of TarG inhibitors. Alternatively, the lack of striking in vitro synergy between these agents may reflect a limitation in the sensitivity of the standard microdilution checkerboard assay (Amsterdam, 2005), particularly when testing potent bioactive agents. Whereas the checkerboard assay effectively scores synergy between nonbioactive agents, synergistic effects between bioactive agents may occur rapidly and possibly over only a few cell division cycles, obscuring such chemical-chemical interactions. Regardless, we demonstrate that their combined effects are pronounced in an animal model of MRSA infection. This exaggerated efficacy in a host environment reflects the dual manner in which these agents impair normal WTA synthesis, hence depleting an important *S. aureus* virulence determinant (Weidenmaier et al., 2004; Weidenmaier and Peschel, 2008) and therefore disrupting the integrity of the cell wall to potentially

expose otherwise masked cell surface immune recognition determinants required to activate an innate immune response (Guan and Mariuzza, 2007; Pietrocola et al., 2011).

Importantly, reduced drug resistance is achieved by combining TarG inhibitors with imipenem, as recently demonstrated with targocil and oxicillin (Campbell et al., 2012) as well as a second β-lactam synergistic combination involving the FtsZ inhibitor, PC190723, and imipenem (Tan et al., 2012). Remarkably, L275 at 2X MIC combined with the break point concentration of imipenem reduces the FOR of the combined agents to extremely low levels. This can likely be attributed to strong counterselection of bypass resistance mutants in the presence of the β-lactam due to their dramatic hypersensitivity to imipenem and the impact of L275 secondary target activity, particularly at higher drug concentrations.

Chemical Biology, Drug Resistance Analysis, and Whole-Genome Sequencing

We demonstrate that combining a robust whole-cell-based screen to identify pathway-specific bioactive compounds with drug resistance selection and NGS technology provides a powerful strategy to successfully identify target-specific inhibitors, their MOA, and drug resistance mechanisms and to genetically predict the pharmacological effects of such agents. Drug-resistant mutants identified in this study also provided a valuable resource to genetically validate other genes in the WTA pathway as new β-lactam potentiation targets. Further, the resulting mutations were valuable reagents to verify that genetic depletion of *tarO*, *tarA*, *tarB*, *tarD*, or *tarI'* in MRSA produce markedly attenuated virulence and β-lactam hypersusceptibility phenotypes in a host setting. Accordingly, the strategy can be used to draw strong target validation conclusions. Chemical biology studies on the effects of these compounds themselves provided a "surrogate" genetic strategy to chemically select for a broad set of WTA pathway mutants, therefore bypassing molecular genetic methodologies necessary to evaluate such targets. We believe that combining resistance, suppression, or other selection studies with NGS-based mapping of causal mutations will have an extraordinary impact, not only in antimicrobial discovery but broadly in the fundamental pursuit of linking chemistry and biology.

SIGNIFICANCE

Innovative strategies to combat drug resistance associated with methicillin-resistant *Staphylococcus aureus* (MRSA) and other β-lactam-resistant *Staphylococci* are needed. Here, we have investigated the potential of wall teichoic acid (WTA) inhibitors as combination agents to effectively restore β-lactam susceptibility and reduce drug resistance among pre-existing drug-resistant *Staphylococci*. Performing a whole-cell pathway-based screen for WTA inhibitors (WTAIs), we identified three potent and structurally distinct classes of bacteriostatic agents with extended Gram-positive spectrum targeting TarG, the channel forming subunit of the WTA transporter. We use whole-genome sequencing analysis in MRSA as well as in methicillin-resistant *Staphylococcus epidermidis* (MRSE) to map the drug resistance landscape. These analyses cross-validate a TarG-mediated mechanism of action for each of these WTAIs. Multiple drug-resistant loss-of-function bypass mutations in earlier steps of WTA biosynthesis were also recovered, including *tarO* and *tarA*, as well as *tarB*, *tarD*, and *tarI'*, the latter of which are essential for growth in the methicillin-sensitive *Staphylococcus aureus* (MSSA) strain background RN4220 but not MRSA COL. Phenotypic characterization of *tar* mutants reveals their attenuated virulence and profound hypersusceptibility to β-lactam antibiotics in a murine infection model. Pharmacological evidence of their β-lactam potentiation activity is also achieved in an in vivo infection setting. Thus, genetic and pharmacological studies demonstrate the potential of WTAIs as effective β-lactam combination agents to treat MRSA. This work also highlights the incredible robustness and efficiency of combining classical drug resistance selection and whole-genome sequencing to identify drug targets and impact small molecule MOA and resistance studies.

EXPERIMENTAL PROCEDURES

Microbiological Studies

MRSA COL is a hospital-acquired penicillinase-negative strain extensively used in *Staphylococcus aureus* methicillin resistance and virulence studies (De Lencastre et al., 1999; Tan et al., 2012) and from which its genome has been fully sequenced and annotated (Gill et al., 2005). MRSE strain (MB6255) is a previously described methicillin-resistant *S. epidermidis* clinical isolate (CLB26329; Huber et al., 2009) isolated from a New York intensive care unit in 2004. MICs were determined by the broth microdilution method in accordance with the recommendations of the Clinical and Laboratory Standards Institute. The standard checkerboard technique was used to quantify synergy between antibiotic agents (Amsterdam, 2005). MRSA COL and MRSE MB6255 were grown in cation-adjusted Mueller Hinton broth (CAMHB) medium and assayed in a 96-well format using 2-fold dilutions of imipenem and TarG inhibitors. MIC determinations were assessed visually. FICI values were determined by adding the FIC value of each compound required to achieve a MIC when paired with the second agent. Bacteriophage K cell lysis assays (Swoboda et al., 2009; Xia et al., 2010), transposon-mediated selection of L275 drug-resistant mutants (Wang et al., 2011), WTA extraction analyses (Meredith et al., 2008), and time kill studies (Tan et al., 2012) were performed as previously described. An agar susceptibility assay was performed as previously described (Lee et al., 2011).

Isolation of MRSA COL Targocil^R, L275^R, and L638^R Mutants

MRSA and MRSE strains were grown to late-exponential phase (OD600 ~1.0; approximately 10^9 cfu/ml) and spread on BHIA plates containing 2-fold escalating agar MIC levels of TarG inhibitors. To establish the number of viable cells in the starting inoculum, the culture was serially diluted and plated on BHIA plates lacking the TarG inhibitor. Resistant isolates were restreaked on plates containing the same TarG inhibitor concentration. The frequency of resistance (FOR) either in the absence or presence of 4 μg/ml imipenem was determined, dividing the number of resistant isolates by the viable cfu in the late-exponential inoculum. See Table S11 for detailed description of mutants.

TarG Inhibitor Efficacy Studies

Efficacy studies were performed using a previously described murine septicemia model of *S. aureus* infection, where immune-suppressed Balb/c mice (five mice per group) were challenged with 1.8×10^4 cfu MRSA COL in 3% hog gastic mucin (Gill et al., 2007). Mice were dosed subcutaneously (s.c.) with the WTA inhibitor (200 mg/kg), imipenem (IPM; 10 mg/kg) coformulated with 50 mg/kg cilastatin, or coadministration of the TarG inhibitor and imipenem/cilistatin at 2, 5, and 8 hr postinfection challenge. Kidney homogenates were serially plated 24 hr after initiation of therapy to determine cfu/kidney remaining. Pharmacokinetic and plasma protein binding properties of TarG inhibitors are provided in Table S8. All animal experiments were performed

in accordance with Merck and AAALAC guidelines for the ethical treatment of animals.

In Vivo Virulence Studies of MRSA COL *tar* Mutants

Virulence phenotypes were assessed using a previously described murine deep thigh infection model (Gill et al., 2007; Tan et al., 2012). Bacterial cultures of MRSA COL (MB 5393), *tarO*, *tarA*, *tarB*, *tarD*, and *tarI'* were grown in TSB medium overnight to late-exponential phase (OD600 ~1.0; approximately 10^9 cfu/ml). Five groups of mice were thigh inoculated (0.1 ml/injection) with an isolate, each at increasing inoculum concentrations ranging from ~10^5–10^9 cfu/ml. Thigh homogenates were serially plated 24 hr after infection to determine cfu/thigh remaining.

Restored Efficacy of Imipenem against MRSA COL *tar* Mutants

Imipenem efficacy studies were performed using a previously described murine deep thigh infection model (Gill et al., 2007; Tan et al., 2012). Female CD-1 mice inoculated into the thighs with overnight cultures (~10^6 cfu/thigh) of MRSA COL, or representative *tar* mutants were treated with vehicle or imipenem (10 mg/kg per dose × 3 over 24 hr). Thigh homogenates were serially plated after 2 or 24 hr (vehicle, imipenem) to determine cfu/thigh remaining.

SUPPLEMENTAL INFORMATION

Supplemental Information includes nine figures and eleven tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.11.013.

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