Dual Targeting of Cell Wall Precursors by Teixobactin Leads to Cell Lysis

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Teixobactin represents the first member of a newly discovered class of antibiotics that act through inhibition of cell wall synthesis. Teixobactin binds multiple bactoprenol-coupled cell wall precursors, inhibiting both peptidoglycan and teichoic acid synthesis. Here, we show that the impressive bactericidal activity of teixobactin is due to the synergistic inhibition of both targets, resulting in cell wall damage, delocalization of autolysins, and subsequent cell lysis. We also find that teixobactin does not bind mature peptidoglycan, further increasing its activity at high cell densities and against vancomycin-intermediate Staphylococcus aureus (VISA) isolates with thickened peptidoglycan layers. These findings add to the attractiveness of teixobactin as a potential therapeutic agent for the treatment of infection caused by antibiotic-resistant Gram-positive pathogens.

Antibiotic resistance development is a major threat to human health. Constant development of novel antibiotics is required to keep pace with the emergence and spread of antibiotic resistance in bacterial pathogens (1). The majority of antibiotics in use today are derivatives of molecules discovered in the early to mid-twentieth century. The lack of novel compounds, coupled with the emergence and spread of antibiotic resistance, has resulted in an increasingly dangerous situation (2–4).

One approach to discover novel antibiotics is to improve our ability to cultivate microorganisms that produce them. Traditional culturing methods allow access to an estimated 1% of the biodiversity in soil. A novel cultivation technique, using an isolation chip or iChip, provides access to an untapped reservoir of biodiversity in soil. A novel cultivation technique, using an isolation chip or iChip, provides access to an untapped reservoir of biodiversity in soil. An improved bactericidal activity of teixobactin is due to the synergistic inhibition of both targets, resulting in cell wall damage, delocalization of autolysins, and subsequent cell lysis. We also find that teixobactin does not bind mature peptidoglycan, further increasing its activity at high cell densities and against vancomycin-intermediate S. aureus (VISA) strains that have increased cell wall density.

MATERIALS AND METHODS

Antimicrobial agents, bacterial strains, and primers. Teixobactin was purified according to the procedure described previously (5). Vancomycin and tunicamycin were purchased from Sigma-Aldrich. The bacterial strains and primers are listed in Table S2 in the supplemental material. JE2, HG003, and SA113 were used throughout the study. HG003 and SA113 are closely related laboratory methicillin-susceptible S. aureus (MSSA) strains. JE2 is a MRSA USA300 isolate. All strains displayed the same MIC to teixobactin. Strains were selected for specific assays due to preexisting characterized mutants in those strain backgrounds.

MIC. The MIC was determined by the broth microdilution method according to CLSI guidelines. Muller-Hinton broth (MHB) was supplemented with 0.1% Tween 80 to prevent the absorption of compounds to plastic surfaces. Cell concentration was adjusted to about 5 × 10^8 CFU/ml, and cells were incubated for 20 h at 37°C. The MIC was defined as the lowest concentration of antimicrobial agents that resulted in no visible

Received 16 May 2016 Returned for modification 9 June 2016
Accepted 6 August 2016
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01050-16.
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growth. The MICs of teixobactin and vancomycin in the presence of purified cell wall components were measured with the method described above. Purified peptidoglycan suspension was added to MHB to an optical density at 600 nm (OD$_{600}$) of 0.1. The medium containing antibiotics and peptidoglycan was preincubated at 37°C for 1 h before adding bacterial cells.

**Scanning electron microscopy.** Overnight cultures of JE2 in MHB were diluted 1:100 in 100 ml TSB and incubated at 37°C and 225 rpm to an OD$_{600}$ of 0.4 to 0.6. Antibiotics were added at 10× MIC, and cultures were incubated for an additional 4 h. Cultures were filtered through a cellulose membrane. The cells on the membrane were fixed with 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h and treated in a graded series of 1% OsO₄ with 0.1 M sodium cacodylate buffer. Cells were then dehydrated in ethanol and critical-point dried using CO₂. The membrane was washed, blocked with nonspecific antibody, and treated with anti-Atl antibody (12) for 1 h. Finally, the membrane was washed and incubated with anti-rabbit IgG secondary antibody. After incubation of the membrane with substrate, the blot was analyzed by a gel imaging system (Bio-Rad).

**Isolation of peptidoglycan.** Isolation of peptidoglycan for competition assay was described previously (15). Exponential-phase HG003 cells were harvested by centrifugation, washed with ice-cold saline, and resuspended in 2 ml of saline. The cells were boiled for 20 min. After centrifugation, the cells were resuspended with saline, mixed with glass beads, and lysed by bead beater (2 rounds, with 20 s at the highest speed and cooling on ice for 5 min). After harvesting the suspensions, the cells were collected by centrifugation, suspended in 1 ml 2% SDS, and boiled for 30 min. After cooling to room temperature, the cells were washed with distilled water at least 5 times. The pellets were dissolved in 0.1 M Tris-HCl (pH 6.8) with trypsin (0.5 mg/ml; Sigma) and incubated at 37°C overnight. Finally, the pellets were washed with distilled water 3 times. This suspension was stored at −80°C until used for competition assay.

**Isolation and detection of cell wall teichoic acid.** Isolation of teichoic acid was conducted as follows (16). Antimicrobial agents (teixobactin and vancomycin [10× MIC] as well as tunicamycin [0.025× MIC]) were added to mid-exponential-phase HG003 cells in MHB, and the cells were cultivated for an additional 4 h. Cell pellets were collected by centrifugation, washed with 50 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 6.5), resuspended in 0.5 ml 50 mM MES (pH 6.5) with 4% (wt/vol) SDS, and boiled for 1 h. The cells then were collected by centrifugation and washed with 50 mM MES (pH 6.5) with 4% SDS twice, washed with 50 mM MES (pH 6.5) with 2% (wt/vol) NaCl, and finally washed with 50 mM MES (pH 6.5). The cells were resuspended with 20 mM Tris-HCl (pH 8.0) with trypsin (0.5 mg/ml) and incubated at 37°C overnight. Following this, the pellets were washed with distilled water at least 3 times. Pellets were then resuspended with 0.1 ml of 0.1 M NaOH and incubated at room temperature overnight. Supernatants were used for competition assay or detection of WTA by SDS-PAGE. Samples were run in SDS-PAGE gel (16% Tris-Tricine gel; Novex), and the gel was stained by a Pierce silver stain kit (Thermo Fisher) by following the manufacturer’s procedure.

**Purification of Atl R1–3 and conjugation with Cy3 dye.** The three-Atl-repeat domain, R1–3 (17, 18), was expressed with a N-terminal His$_6$ tag in Escherichia coli M15 using the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid pEIQ30Atl R1–3 (19, 20). Cells were cultivated in 2% yeast extract–tryptone broth (YT) to an OD of 0.5 at 37°C and then induced with 0.5 mM IPTG for 4 h at 20°C. The cells were harvested by centrifugation and washed twice in PBS containing complete protease inhibitor cocktail (Roche); the cell pellet was lysed using a French press. The crude extract was centrifuged (5,000 × g), and R1–3–His was affinity purified with nickel-nitrilotriacetic acid (Ni-NTA) superflow (Qiagen) as described by the manufacturer. The imidazole elution buffer was then exchanged by PBS using a Vivaspin 2 column with a 10-kDa cutoff (Sartorius). For protein conjugation with Cy3, the Amersham Cy3 antibody labeling kit (GE) was used. Conjugation of His6R1–3 and the separation of protein from free dye were performed as described by the manufacturer.

**Epifluorescence microscopy.** Localization of Atl on the cell surface exposed to antibiotics was determined similarly to a previous study (21). Antimicrobial agents at 10× MIC were added to mid-exponential-phase HG003 cells in MHB, and the cells were cultivated for an additional 30
The cells were washed twice with PBS and incubated with Cy3-R1–3 for 5 min at room temperature. After washing twice with PBS, a 2-μl cell suspension was applied to glass slides and distribution of fluorescence was confirmed with a Leica fluorescence microscopy system.

RESULTS

Teixobactin-induced lysis is dependent on the Atl autolysin.

Teixobactin has been shown to have excellent bactericidal activity against S. aureus (5). We examined the effect on cell morphology of teixobactin and vancomycin after 4 h of exposure at 10× MIC by electron microscopy (Fig. 1A). Exposure to teixobactin results in collapse of the cell wall, while vancomycin damage to the cell wall was less severe.

β-Lactam-induced lysis is known to be mediated by Atl, the major cell wall autolysin of S. aureus (17, 22). To investigate the contribution of Atl to the activity of teixobactin, the antibacterial activity against an Δatl mutant from the Nebraska transposon insertion library (available through BEI [https://www.beiresources.org/]) was examined and compared to wild-type strain JE2. Although the MIC of teixobactin was not affected by mutation of atl (see Table S1 in the supplemental material), the bactericidal activity was markedly reduced. Teixobactin did not significantly reduce the viable cell count of the Δatl mutant after 24 h of exposure, compared to a 3-log reduction in CFU per milliliter in the wild-type strain (Fig. 1B). Also, 24 h of teixobactin exposure did not cause lysis in an atl mutant, confirming the lysis phenotype is Atl dependent (Fig. 1C).

Teixobactin causes a decrease in atl expression. Teixobactin’s bactericidal activity is dependent on Atl; hence, we decided to examine the lytic capacity of the supernatant of a culture treated with teixobactin. Atl undergoes maturation by proteolytic processing, resulting in generation of two extracellular lytic enzymes, an amidase and a glucosaminidase (12, 14, 23). If teixobactin treatment resulted in increased lytic enzyme production, we would expect the supernatant to have increased lysis capacity. To test this, we performed bacteriolytic assays with supernatants of treated and untreated cultures (Fig. 2A). The untreated control supernatant had marked lytic activity and strongly reduced the turbidity of a cell suspension over time. On the other hand, the supernatant of a teixobactin-treated culture did not have any visible lytic capacity. To further investigate this, we examined the lysis profile of the extracellular and cell wall-anchored Atl with zymography (Fig. 2B). Both supernatant and cell wall-associated protein isolated from cells treated with 10× MIC of teixobactin for 4 h showed reduced murein hydrolase activity compared to those from untreated samples (see Fig. S1A in the supplemental material). Furthermore, real-time PCR revealed that expression of...
In teixobactin-treated cells was about 25-fold lower than that in untreated control cells (Fig. 2C). Finally, the reduced Atl levels were confirmed by Western blot analysis (see Fig. S1B). Vancomycin also reduced the murein hydrolase activity (Fig. 2A to C), which has been reported previously (24, 25). These results suggest that lipid II inhibition results in the activation of a signal transduction pathway that results in reduced autolysin expression, presumably to reduce cell death in the presence of this extreme cell wall stress. Importantly, the reduction of autolysins in the supernatant in response to antibiotic occurs after 2 to 4 h of exposure (see Fig. S1A).

Teixobactin causes Atl-dependent cell lysis but paradoxically causes inhibition of autolysin expression. Hence, the teixobactin-treated cell wall must be more susceptible to autolysins present at the time of addition of the antibiotic.

**Teixobactin-induced lysis is enabled by inhibition of cell wall teichoic acid biosynthesis.** The localization of Atl at the septum to mediate cell division has been proposed to occur via exclusion from the rest of the cell wall by the presence of teichoic acids (21). In the absence of WTA, Atl binding on the cell surface is delocalized, causing lysis (21). Furthermore, WTA are required for β-lactam resistance in methicillin-resistant *S. aureus*, and the cells which lack WTA are sensitized to β-lactam-induced cell lysis (26–28). The genes involved in teichoic acid biosynthesis are called the *tar* genes (for teichoic acid ribitol). TarO (previously referred to as TagO), an N-acetylglucosamine-1-phosphate transferase, catalyzes the first step in this biosynthetic pathway, and mutation of *tarO* results in a teichoic acid-deficient strain (29). We examined cell lysis of wild-type and *tarO* mutant strains in the presence of vancomycin and teixobactin. As previously reported, teixobactin causes increased lysis of the wild-type strain compared to vancomycin. Both antibiotics caused lysis of the *tarO* mutant, demonstrating that lipid II inhibition results in cell lysis, but only in a teichoic acid null background (Fig. 3A and B). As teixobactin blocks lipid II and lipid III, this suggests that the lytic activity of teixobactin is due to the combined inhibition of both targets. To

**FIG 2** Teixobactin causes downregulation of *atl* expression. (A) Bacteriolytic assay using supernatant from JE2 incubated with teixobactin (10× MIC) or vancomycin (10× MIC) for 4 h. (B) Zymography of teixobactin-treated samples on SDS-PAGE gel containing heat-killed *S. aureus* RN4220 as a substrate. The dark bands indicate the clear zone in the SDS-PAGE gel, which is caused by the lysis of substrate. Left and right lanes were supernatant (Sup) and cell wall-associated samples (CW), respectively. (C) Comparison of *atl* transcription by quantitative real-time PCR. The bars indicate the relative values compared to the no-antibiotic-treatment sample. These data represents the means and SD from 3 independent experiments.

*atl* in teixobactin-treated cells was about 25-fold lower than that in untreated control cells (Fig. 2C). Finally, the reduced Atl levels were confirmed by Western blot analysis (see Fig. S1B). Vancomycin also reduced the murein hydrolase activity (Fig. 2A to C), which has been reported previously (24, 25). These results suggest that lipid II inhibition results in the activation of a signal transduction pathway that results in reduced autolysin expression, presumably to reduce cell death in the presence of this extreme cell wall stress. Importantly, the reduction of autolysins in the supernatant in response to antibiotic occurs after 2 to 4 h of exposure (see Fig. S1A).

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found that vancomycin displayed bactericidal killing against a MIC to vancomycin. We then performed killing experiments and inhibition in MIC to teixobactin but only a modest 2-fold decreased activity of teixobactin, oxacillin, and vancomycin against 12 mutants of two-component regulators, and capsule biosynthesis components (30–32), and the saeR mutant shows high susceptibility to β-lactams in Staphylococcus epidermidis (33). This increased susceptibility to cell wall-acting antibiotics may be due to decreased teichoic acids in the cell wall. This would further corroborate the role of teichoic acids in prevention of lysis mediated by lipid II inhibition. Again, teixobactin’s ability to inhibit both peptidoglycan and teichoic acid biosynthesis explains its ability to kill and lyse S. aureus so efficiently. More work is required to more precisely measure the impact of saeS mutation on teichoic acid content of the cell wall and elucidate how the SaeRS two-component system could influence WTA levels in S. aureus.

Teixobactin treatment results in delocalization of autolysins. It has been proposed that teichoic acids control autolysin binding to peptidoglycan by an exclusion principle, whereby cell wall teichoic acid interaction with peptidoglycan inhibits autolysin binding (21, 34). Consequently, an absence of teichoic acids at the septum facilitates appropriate localization of autolysins during cell division. It was also shown that mutation of tarO results in delocalization of amidase (21). Atl possesses three repeat sequences, each about 150 amino acids long (R1–3), which bind to peptidoglycan (19, 35). We examined the localization of Atl using a fluorescently labeled R1–3 repeat domain (Cy3-R1–3) (21). We found that Cy3-R1–3 delocalization occurs in cells treated with teixobactin for 30 min, similar to that previously seen in a tarO mutant. These experiments were performed against the model laboratory strain HG003 (Fig. 5; see also Fig. S2 in the supplemental material). This suggested that teixobactin treatment resulted in delocalization of Atl due to inhibition of WTA biosynthesis of the cell. This delocalization explains the intense lytic capacity of teixobactin. Vancomycin did not cause delocalization of the amidase over a similar time period. A longer 4-h treatment with vancomycin did result in delocalization of the fluorescent amidase. Delocalization by teixobactin is rapid and does not allow the cell time to reduce autolysin production and limit Atl-mediated damage. Collectively, these results suggest that coinhibition of lipid II and lipid III by teixobactin causes a pronounced weakening of the cell wall compared to lipid II inhibition alone. This results in increased delocalization of autolysins, leading to cell lysis and death.

Teixobactin does not bind cell wall peptidoglycan. We have previously shown that teixobactin has killing activity superior to that of vancomycin against dense populations of S. aureus (5). This is due in part to teixobactin’s ability to inhibit teichoic acid production. However, a further important limitation of vancomycin activity, particularly at high cell densities, is the binding of vancomycin to mature peptidoglycan. Vancomycin has no antibacterial activity when bound to mature peptidoglycan. This explains the reduced bactericidal activity of vancomycin against dense populations of cells. Furthermore, this circumstance can lead to vancomycin-intermediate resistance. Many VISA strains have thicker cell walls with altered cross-linking (36). This leads to increased binding of vancomycin to mature peptidoglycan at the D-Ala-D-Ala pentapeptide and sequestration of the antibiotic. Teixobactin does not bind the pentapeptide; hence, it may not
bind the mature cell wall peptidoglycan. To test this, we purified peptidoglycan from *S. aureus* HG003 and performed MIC testing after a preincubation of vancomycin or teixobactin with purified peptidoglycan (Table 1). Interestingly, the MIC to vancomycin increased 16-fold after this preincubation due to binding with peptidoglycan decreasing the concentration of active antibiotic in the medium. Incubation with teixobactin, on the other hand, resulted in only a modest 2-fold increase in MIC, and this did not increase with higher concentrations of peptidoglycan (note that the intrinsic variability in MIC determination is also 2-fold). This showed that teixobactin does not bind mature peptidoglycan, which likely contributes to its activity against dense populations of *S. aureus* and its activity against all VISA strains examined.

### DISCUSSION

Teixobactin represents a recently discovered class of antibiotics, and it exhibits a number of unique and desirable characteristics. These include an apparent absence of resistance development and an improved lytic capacity compared to the cell wall-acting antibiotics oxacillin and vancomycin. In this study, we sought to further explore the mechanism of action of teixobactin and how it yields the intense lytic and bactericidal activity against *S. aureus*, including VISA isolates. We find that teixobactin exhibits excellent bacterial killing, likely due to the synergistic inhibition of both peptidoglycan and WTA biosynthesis. In spite of downregulation of Atl, inhibition of teichoic acid biosynthesis and peptidoglycan biosynthesis results in significant Atl-mediated lysis and cell death. We also find that teixobactin does not bind to and hence is not antagonized by mature peptidoglycan, explaining activity against VISA isolates. We find that sub-MIC levels of tunicamycin enhanced the bactericidal activity of vancomycin without affecting the MIC. At this concentration (0.4 μg/ml), tunicamycin inhibits the biosynthesis of WTA without affecting cell growth. This showed that lack of WTA enhanced the killing by lipid II inhibition. Bactericidal activity of teixobactin plus tunicamycin was stronger than that of vancomycin plus tunicamycin. This may be due to the different stages of teichoic acid biosynthesis that are inhibited by tunicamycin and teixobactin. In the WTA biosynthesis pathway, the first two enzymes, TarO and TarA, are not essential under laboratory conditions, while most of the downstream factors are essential (37). The mechanism of this lethality may be due to accumulation of toxic intermediates or to depletion of cellular pools of cell wall precursors (37). Teixobactin is thought to inhibit the later steps of WTA biosynthesis by binding to lipid III outside the cell membrane (5). Interestingly, the teichoic acid profile from cells treated with tunicamycin or teixobactin is markedly different, presumably due to the different stages of biosynthesis inhibited by each compound.

Teixobactin causes the downregulation of *atl* gene expression.

It has already been reported that inhibition of peptidoglycan biosynthesis elicits various secondary responses and downregulation of *atl* expression (24, 38). In prokaryotes, sensing and signal transduction is primarily conducted by two-component systems (TCs), consisting of a histidine kinase sensor and a cognate response regulator. There are 16 pairs of TCs in *S. aureus*, and some of them are involved in autolysis control, biofilm formation, cell wall synthesis, or drug resistance (39). Among these systems, WalKR is essential for viability and is involved in cell wall metabolism. A strain expressing a constitutively active form of WalR displays upregulated *atl* transcription (40). GraRS is a well-studied system which regulates the resistance to cationic antimicrobial peptides. A Δ*graRS* mutant displays reduced expression of *atl* (41). These findings suggest that *atl* transcription is inactivated via WalKR and/or GraRS systems under cell wall stress conditions. Here, we find that mutation of *saeS* of the SaeRS two-component system resulted in increased sensitivity to killing by vancomycin independent of an effect on *atl* expression. Mutation of *saeS* results in loss of teichoic acid in the cell wall, resulting in sensitivity to lysis by lipid II inhibition. With this in mind, inhibitors of two-component systems, particularly WalKR, GraRS, and SaeRS, have the potential to further sensitize cells to cell wall-acting antibiotics and may result in improved bactericidal activity.

Intriguingly, teixobactin exhibits potent activity against *M. tuberculosis* (5). Mycobacterium has a unique cell wall structure composed of arabinogalactan, peptidoglycan, and mycolic acid (42). The mechanism of ethambutol, an antituberculosis agent, is inhibition of arabinogalactan biosynthesis (43). Recently, it was reported that the inhibitor of WeaA, which is the ortholog of TarO and is involved in arabinogalactan biosynthesis, has good antitubercular activity (44). It will be interesting to examine teixobactin’s proposed ability to inhibit arabinogalactan and how the dual inhibition of peptidoglycan and arabinogalactan synthesis may result in enhanced bactericidal activity against this important human pathogen.

### ACKNOWLEDGMENTS

We thank Dallas Hughes (NovoBiotic Pharmaceuticals) for providing teixobactin and Jeffrey L. Bose (The University of Kansas) for providing anti-Atl antibody. We also thank William Fowle (Northeastern University) for conducting scanning electron microscope experiments.

This work was partially supported by NIH grant R01AI110578 to K.L. and by a Charles A. King fellowship to B.P.C.

The study was designed by F.G., T.S., K.L., and B.P.C. Experiments were designed by T.H., A.N., A.B.G., P.E., and B.P.C. Experiments were conducted by T.H., A.B.G., and P.E. The paper was written by T.H., A.N., and B.P.C.

All authors except for T.H. have no conflicts of interest to declare. T.H. is an employee of Shionogi & Co. Ltd.

### FUNDING INFORMATION

This work, including the efforts of Kim Lewis, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (R01AI110578). This work, including the efforts of Brian P. Conlon, was funded by Charles A. King Trust.

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