Telavancin Disrupts the Functional Integrity of the Bacterial Membrane through Targeted Interaction with the Cell Wall Precursor Lipid II

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ABSTRACT

Telavancin is an investigational lipoglycopeptide antibiotic currently being developed for the treatment of serious infections caused by Gram-positive bacteria. The bactericidal action of telavancin results from a mechanism that combines inhibition of cell wall synthesis and disruption of membrane barrier function. The purpose of this study was to further elucidate the mechanism by which telavancin interacts with the bacterial membrane. A flow cytometry assay employing the fluorescent dye DiOC$_2$(3) was used to probe the membrane potential of actively growing Staphylococcus aureus cultures. Telavancin caused pronounced membrane depolarization that was both time- and concentration-dependent. Membrane depolarization was demonstrated against a reference S. aureus strain as well as phenotypically diverse isolates expressing clinically important methicillin-resistant (MRSA), vancomycin-intermediate (VISA) and heterogeneous VISA (hVISA) phenotypes. The cell wall precursor Lipid II was shown to play an essential role in telavancin-induced depolarization. This was demonstrated both in competition binding experiments with exogenous D-Ala-D-Ala-containing ligand as well as in experiments using cells expressing altered levels of Lipid II. Finally, monitoring the optical density of S. aureus cultures exposed to telavancin showed that cell lysis does not occur during the time course in which membrane depolarization and bactericidal activity are observed. Taken together, these data indicate that telavancin’s membrane mechanism requires interaction with Lipid II, a high affinity target that mediates binding to the bacterial membrane. Targeted interaction with Lipid II and the consequent disruption of both peptidoglycan synthesis and membrane barrier function provides a mechanistic basis for the improved antibacterial properties of telavancin relative to vancomycin.
INTRODUCTION

The increasing prevalence of serious Gram-positive infections, including those caused by methicillin-resistant Staphylococcus aureus (MRSA), highlights the need for new agents with enhanced antimicrobial properties (2, 10, 21, 26, 35). One promising approach has been the development of lipoglycopeptide antibiotics, semi-synthetic derivatives of glycopeptides that contain hydrophobic substituents and possess improved antimicrobial properties (1, 4, 13, 32, 39). Telavancin, a lipoglycopeptide derivative of vancomycin, exhibits enhanced in vitro potency, concentration-dependent bactericidal activity, and activity both in vitro and in vivo against organisms that display reduced susceptibility to vancomycin (17, 18, 23, 24, 28, 31, 33, 36, 42). Telavancin has been evaluated in Phase 3 clinical trials for the treatment of complicated skin and skin structure infections and hospital-acquired pneumonia (46, 53).

The bactericidal action of telavancin results from a mechanism that includes inhibition of cell wall synthesis and disruption of essential membrane barrier functions (25). Telavancin possesses the glycopeptide core of vancomycin, which binds with high affinity to the acyl-D-alanyl-D-alanine (D-Ala-D-Ala) terminus of cell wall precursors through a network of hydrogen bonds and hydrophobic packing interactions (3, 45). Inhibition of cell wall synthesis by telavancin therefore involves binding to late-stage peptidoglycan precursors, including membrane-embedded Lipid II. These interactions prevent both polymerization of precursor into peptidoglycan and subsequent cross-linking events. Telavancin also binds to bacterial membranes and causes membrane depolarization and increased membrane permeability. The mechanism by which telavancin binds to and disrupts the function of the bacterial membrane has not been determined.
The present study was undertaken to further explore the interaction of telavancin with the bacterial membrane. Using a flow cytometry assay optimized for accurate measurement of membrane potential in bacteria, we demonstrate that telavancin causes pronounced, concentration-dependent depolarization in *S. aureus* cells. Isolates of *S. aureus* expressing important and emerging resistance phenotypes, such as MRSA, hVISA, VISA, and daptomycin-nonsusceptible MRSA are equally susceptible to depolarization by telavancin. We provide evidence, through multiple lines of investigation, that membrane disruption by telavancin requires binding to the bacterial-specific target, Lipid II. Finally, we demonstrate that telavancin does not lyse bacteria during the time course that membrane effects are assayed. Importantly, this latter observation indicates that telavancin-induced membrane depolarization is not a consequence of a weakened cell wall. The studies reported here enhance our understanding of telavancin’s mechanism of action and, in assays designed to be representative of physiological conditions, demonstrate that therapeutically relevant concentrations of telavancin inhibit essential functions of the bacterial membrane.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. aureus* strains used in this study include ATCC 33591 (MRSA), ATCC 29213 (MSSA), ATCC 700698 (hVISA Mu3), and ATCC 700699 (VISA Mu50); all obtained from the American Type Culture Collection (ATCC, Manassas, VA). Daptomycin nonsusceptible MRSA (MED2034) was obtained from the telavancin clinical program.

**Antibacterials, media and reagents.** Telavancin and THRX-881620 were manufactured by Theravance, Inc (South San Francisco, CA). $[^{14}C]$-Telavancin was prepared by ViTrax (Placentia, CA) with the radiolabel on the aminomethyl carbon substitution of the resorcinol position. Vancomycin, penicillin G, nisin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP),
N,N’-diacetyl-L-Lys-D-Ala-D-Ala (dKAA), acetyl-L-Lys-D-Ala-D-Ala, fosfomycin, D-cycloserine, bacitracin, 1-decanol, lysostaphin, and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO), and 3,3'-diethyloxacarbocyanine iodide [DiOC\(_2(3)\)] was obtained from Invitrogen (Carlsbad, CA). Telavancin stock solutions (4 mg/mL) were prepared by solubilizing powder in a 1:1 (vol:vol) mixture of DMSO and water that was acidified by adding 28 µL of 1N HCl per 10 mL of solution. Serial dilutions of telavancin were also performed in acidified 50% DMSO. Polypropylene labware was used for preparation of stock solutions and subsequent dilution procedures. Nisin stock solutions were prepared in deionized water, which was warmed, vortexed, and briefly bath sonicated prior to 0.2 µm syringe filtration (polyethersulfone). Cation-adjusted Mueller Hinton II broth (MHB) (Difco, Detroit, MI) was used for bacterial growth. Viable counts were determined by plating on tryptic-soy agar (TSA) plates (Hardy Diagnostics, Santa Monica, CA). Cultures subjected to the non-growing condition were incubated in Dulbecco’s Phosphate Buffered Saline with cations (PBS) (Invitrogen, Carlsbad, CA).

Flow cytometry. Membrane potential was assayed by flow cytometry using the diethyloxacarbocyanine dye DiOC\(_2(3)\), which allows for variation in cell size to be normalized by red to green fluorescence ratio analysis (40, 48). Metabolically active S. aureus generate a membrane potential of approximately -120 mV; DiOC\(_2(3)\) fluorescence can report changes across the entire range of -30 to -130 mV. Depolarization experiments were conducted with S. aureus ATCC 29213, unless otherwise noted. Cultures were grown to early-exponential phase (A\(_{625}\) nm = 0.3) in MHB at 37°C. Cell concentrations were adjusted to 10\(^6\) CFU/mL by dilution in MHB, compounds were added to 5 mL aliquots (where indicated, dKAA preceded the addition of telavancin), and samples were incubated at 37°C in a shaking water bath. At selected intervals, 250 µL samples were aspirated and mixed 1:1 with DiOC\(_2(3)\) in MHB, with DiOC\(_2(3)\) final concentration at 30 µM. Samples were stained for 5 min prior to evaluation.
An Epics XL flow cytometer (Beckman Coulter Inc., Fullerton, CA) was used to collect 5000 events for each sample at medium flow rate and signal was acquired with log amplification. Collecting fluorescence from a fixed number of individual cells ensures the culture is uniformly sampled irrespective of cell density. Green fluorescence emission was detected through a 525 nm (40 nm bandpass) emission filter (FL1) and red fluorescence was detected through a 620 nm (30 nm bandpass) emission filter (FL3). Analysis was performed with FCS Express (De Novo Software, Los Angeles, CA). In order to distinguish normal and depolarized populations of cells, gates were applied to plots of red versus green fluorescence, with percent depolarization representing the cell count within the depolarized region divided by the total cell count. The depolarized region was defined by gating a region only slightly exceeding the boundary of a fully depolarized population induced by CCCP. IC$_{50}$ values were determined from percent depolarization by fitting the concentration response curve by nonlinear regression to a sigmoidal 4 parameter equation using SigmaPlot (Systat, San Jose, CA).

In our analyses of membrane potential, we sought to ensure that only perturbations caused by direct interaction of compound with the membrane be recorded as depolarization. Accordingly, our assay was optimized to exclude secondary effects, such as those resulting from a weakened cell wall, that can induce stress to the membrane and cause depolarization. Experiments using penicillin G to inhibit cell wall synthesis either partially (sub-MIC) or completely (supra-MIC) showed that membrane potential could be accurately measured in cells treated for up to 90 min. Therefore, 90 min was used as the endpoint for the depolarization assay, unless otherwise noted.

Three different inhibitors of cell wall synthesis, fosfomycin, D-cycloserine, and bacitracin, were used to modulate Lipid II levels in S. aureus cells. Cultures were pretreated with these...
compounds at 37°C with shaking for 10 min to suppress Lipid II production. After pretreatment with fosfomycin (250 µg/mL), D-cycloserine (64 µg/mL), or bacitracin (250 µg/mL) cells were exposed to 32 µg/mL telavancin for 15 min or 2 µg/mL nisin for 5 min. Percent depolarization was calculated relative to control samples without pretreatment. Modified culture conditions were also used to modulate Lipid II levels. Cells were incubated in the respective condition (PBS at 37°C or MHB at 23°C) for 10 min before exposure to 32 µg/mL telavancin.

8 **Binding of $^{14}$C-telavancin to bacteria.** *S. aureus* cultures ($10^8$ CFU/mL) were incubated at 37°C in either MHB or PBS in a deep-well polypropylene microplate. Cultures were exposed to $^{14}$C-telavancin (8 µg/mL), and at each time point samples were transferred to a 0.2 µm filter plate (MultiScreen-HTS-GV, Millipore). The filter plate was pre-washed with PBS containing 0.002% Tween-20 to reduce non-specific binding. Samples were filtered and then washed three times with PBS, 0.002% Tween-20, a process that was verified to effectively wash unbound $^{14}$C-telavancin through the filter so that only bacteria remained. The filter plate was dried overnight at 37°C. Scintillant (MicroScint-20, Perkin Elmer) was applied to dried samples, which were then read on a Wallac MicroBeta scintillation counter (Perkin Elmer).

18 **Antimicrobial activity.** Minimum inhibitory concentrations (MICs) were determined using dry-form panels manufactured by Trek Diagnostic Systems (Cleveland, Ohio). Time-kill assays were performed according to CLSI-defined methods (12).

22 **Cell lysis.** Turbidity was evaluated using a PharmaSpec UV-1700 spectrophotometer (Shimadzu, Columbia, MD). *S. aureus* cultures (ATCC 33591) were grown to early-exponential phase ($A_{625}$ nm = 0.3) in MHB, 5 mL was aliquoted into 50 mL conical tubes, compounds were
added to the desired final concentration, and samples were incubated in a shaking 37°C water bath. At each time point, a 100 µL sample was aspirated and transferred to a quartz microcuvette and the optical density at 625 nm was recorded. Lysostaphin, which cleaves the pentaglycine cross-link in peptidoglycan, was used for assay validation and reduced the optical density to background levels consistent with complete cell lysis.

**Microscopy.** Phase contrast images of bacteria were captured on a Zeiss Axioskop with 100x Plan-Neofluar objective (1.3NA, Ph3, oil) with a Photometrics CoolsnapFx CCD camera. Cells were deposited on a slide, a coverslip was applied, and the edges were sealed. Bacteria were analyzed for lysis by the degree of cellular contrast; intact cells appeared dark whereas lysed cells were transparent. Bacteria were imaged at 0, 2, and 6 hours post treatment. Populations of at least 30 cells were evaluated for each experimental condition.

**RESULTS**

**Effect of telavancin on bacterial membrane potential.** To further investigate telavancin’s mechanism of action, we employed a flow cytometry-based depolarization assay with the fluorescent dye, DiOC\(_2\)(3), which has been demonstrated to accurately measure membrane potential in bacteria (40, 41, 48). Monitoring changes to membrane potential is a useful method to assess the significance of a membrane effect, as it reports the functional integrity of the bacterial membrane. Membrane potential is sensitive to modest disruptions of barrier function, those that would allow small solutes such as protons to cross the membrane, and requires sustained effect to maintain the depolarized state. Dissipation of membrane potential (i.e. depolarization) leads directly to loss of function of essential aspects of cell physiology such as ATP generation and nutrient uptake (22).
The effect of telavancin on bacterial membrane potential was assessed under test conditions (cation-adjusted Mueller-Hinton broth, physiological temperature, and low cell density) that are routinely employed for antibiotic susceptibility testing. Histogram displays of cellular red/green fluorescence ratios show distinctions in the magnitude of depolarization induced in antibiotic-treated cultures. In Figure 1, an untreated, fully polarized (normal metabolic state) population of *S. aureus* (ATCC 29213) is depicted in the uniform distribution furthest right in the graph. Upon depolarization, populations were observed to move leftward. Nisin, a pore-forming lantibiotic used as a control (8), fully depolarized *S. aureus* cells at 15 min (Fig. 1A). When treated with the proton ionophore CCCP, *S. aureus* populations were similarly completely depolarized (data not shown). Telavancin demonstrated time-dependent depolarization activity against *S. aureus* cells. Cultures exposed to 8 µg/mL telavancin became increasingly depolarized as the duration of exposure increased; a broad depolarization response observed at 45 min changed to a near uniform distribution of maximally depolarized cells by 90 min (Fig. 1B).

In order to analyze a wider variety of conditions, cell population histograms were converted to percent depolarization. A definition of depolarization was applied by gating a region only slightly exceeding the boundaries of a fully depolarized population induced by CCCP or nisin. Percent depolarization was the cell count within the depolarized region divided by the total cell count.

Concentration response curves were obtained for telavancin and control compounds, and nonlinear regression was applied to establish IC$_{50}$ values. Telavancin (MIC, 0.25 µg/mL) demonstrated concentration-dependent depolarization activity against *S. aureus*, with an IC$_{50}$ value of 3.6 µg/mL. The IC$_{50}$ value determined for nisin (MIC, 4 µg/mL) was 2.9 µg/mL. In
contrast, no IC<sub>50</sub> value could be calculated for vancomycin (MIC, 1 µg/mL), even at concentrations up to 96 µg/mL.

Membrane depolarization in phenotypically diverse <em>S. aureus</em> strains. Telavancin depolarization activity was investigated in strains that exhibit important and emerging resistance phenotypes. Since telavancin shares the core structure of vancomycin, it was important to test isolates with reduced susceptibility to vancomycin in order to determine whether these phenotypes might impact telavancin activity. Telavancin maintained potent antimicrobial activity against and dissipated the membrane potential of <em>S. aureus</em> cells expressing clinically important phenotypes such as MRSA, VISA, hVISA, and daptomycin non-susceptibility (Table 1). In all strains, the percent depolarization increased over time resulting in <em>S. aureus</em> populations in which a majority of cells were fully depolarized.

The strain exhibiting the lowest level of sensitivity to vancomycin, Mu50 (MIC, 8 µg/mL), was susceptible to telavancin with an MIC of 0.5 µg/mL, and against this strain telavancin induced 61% depolarization. Against the other isolates tested, telavancin maintained comparable antimicrobial potency (MIC, ≤ 0.5 µg/mL) and depolarization activity (≥ 77%). A daptomycin nonsusceptible <em>S. aureus</em> isolate (MIC, 4 µg/mL) was among the isolates evaluated. Considering that the antimicrobial mode of action of daptomycin occurs via membrane depolarization, it was important to determine whether telavancin retained its membrane mechanism against this important phenotype. Telavancin exerted a strong depolarization effect (77%) against the daptomycin non-susceptible <em>S. aureus</em> isolate.

Antagonism of telavancin-induced membrane depolarization by D-Ala-D-Ala ligand. The mechanism by which telavancin binds to and disrupts the function of the bacterial membrane has
not yet been determined. Telavancin may bind to the membrane through interaction with membrane-embedded Lipid II, or alternatively by direct association with the membrane independent of Lipid II. Telavancin has been shown to bind with high affinity to the D-Ala-D-Ala residues of cell wall precursors (25). Previous binding studies with radiolabeled telavancin showed reduced binding to the membrane in the presence of the tripeptide, N,N'-diacetyl-L-Lys-D-Ala-D-Ala (dKAA), which mimics the structure of cell wall precursors, including Lipid II. We conducted depolarization studies to determine whether Lipid II, which is embedded in the bacterial membrane, plays a role in the membrane mechanism of telavancin.

When depolarization assays were conducted in the presence of dKAA, significant antagonism of telavancin activity at the *S. aureus* membrane was observed (Table 2). Upon pretreatment with 0.05, 0.5 and 5 mM dKAA, a pattern of graded antagonism was observed with near maximal antagonism achieved by 5 mM dKAA at 60 min with both 8 and 32 µg/mL telavancin. Control experiments demonstrated that dKAA had no effect on the assay. As expected, a molar excess of dKAA also antagonized antibacterial activity. The telavancin MIC shifted 16-fold (from 0.25 to 4 µg/mL) in the presence of 5 mM dKAA, which links depolarization activity to susceptibility.

Telavancin-induced depolarization is Lipid II dependent. The antagonism data presented above suggests that telavancin-induced depolarization may require binding to Lipid II. To test this hypothesis directly, depolarization assays were conducted using cells expressing altered levels of Lipid II. Three different inhibitors of cell wall synthesis; fosfomycin, D-cycloserine, and bacitracin, were used to reduce Lipid II levels in *S. aureus* cells. Fosfomycin is an inhibitor of MurA, the enzyme responsible for the first step in PG synthesis, and thus blocks the formation of UDP-MurNAc. D-Cycloserine inhibits both alanine racemase and D-Ala-D-Ala ligase, two enzymes required for the synthesis of the D-Ala-D-Ala dipeptide of Lipid II. Bacitracin binds
directly to undecaprenyl pyrophosphate, the portion of Lipid II that remains in the membrane once GlcNAc-MurNAc is polymerized, and prevents its use in subsequent cycles of Lipid II synthesis. All three inhibitors thus block synthesis of Lipid II.

S. aureus cells were preincubated with each of the Lipid II synthesis inhibitors and then exposed to telavancin or nisin. Time and concentration of exposure to telavancin and nisin were selected to achieve 50-70% depolarization for each compound alone (telavancin 32 µg/mL, 15 min; nisin 2 µg/mL, 5 min). As has been described by other investigators (8), we observed that the membrane depolarization activity of nisin was Lipid II dependent (Table 3). Fosfomycin and D-cycloserine reduced nisin activity to 26% and 36%, respectively, of the levels observed in control cultures. Telavancin-induced depolarization was suppressed to a similar extent (29% to 38%) by these agents and by bacitracin (27%) (Table 3). Interestingly, bacitracin treatment suppressed nisin activity to 2% of control levels. This observation suggests that the target shared by nisin and bacitracin, the pyrophosphate moiety of undecaprenyl pyrophosphate, may play a role in nisin-mediated depolarization. All three inhibitors of Lipid II synthesis suppressed telavancin-induced depolarization by approximately two-thirds compared to cells without pretreatment.

Cellular levels of Lipid II were also manipulated by shifting cultures to either nutrient-free media or reduced temperature. The amount of Lipid II available to deliver new MurNAc-(pentapeptide)-GlcNAc precursors for peptidoglycan synthesis is dependent on cellular metabolism (56). A slowly growing or non-growing cell will have a reduced rate of Lipid II production, which can be induced by lower temperatures or minimal media (9, 11).
Cultures of *S. aureus* were grown in MHB and then washed and resuspended in PBS, and allowed to equilibrate for 10 min at 37°C prior to treatment with telavancin. At all concentrations across the dose range of 2 to 32 µg/mL telavancin, depolarization was completely suppressed. Figure 2A shows the time course of depolarization for 32 µg/mL telavancin in PBS versus MHB. The membrane potential of untreated cells observed under the conditions and times explored in these assays was not different from the standard condition of MHB at 37°C.

In another experiment, cells were grown normally and shifted to 23°C for 10 min prior to treatment with 32 µg/mL telavancin (Fig. 2B). Depolarization was suppressed but seen to slowly rise to 30% by 60 min versus >90% depolarization at 37°C, a culture temperature that supports optimal cell growth and division. Additionally, samples treated with 32 µg/mL telavancin were shifted from 23°C to 37°C at 15 min and 30 min time points (Fig. 2B). The shift from sub-optimal to optimal growth temperature results in a dramatic increase in depolarization seen at the next sampled time point. In fact, irrespective of when the sample is temperature shifted, 15 min at 37°C results in a similar percentage of depolarized cells (approx. 50%), which also matches the percentage at the 15 min time point under the standard assay conditions.

We also explored a telavancin analog with substantially reduced binding affinity to D-Ala-D-Ala residues. THRX-881620 is the hexapeptide (des-N-methyl-leucyl) derivative of telavancin lacking the N-terminal amino acid of the carboxylate binding pocket (25). THRX-881620 (MIC, 8 µg/mL) induced only 35% depolarization relative to telavancin. Like telavancin, however, THRX-881620 did not depolarize cells incubated in PBS.

Control experiments were performed to confirm that modified culture conditions, such as temperature and nutrient content, did not alter overall membrane properties. 1-Decanol, a model
surfactant, partitions into the membrane due to its amphipathic properties (20, 54) and induces depolarization independent of Lipid II. We reasoned that if the modified culture conditions did not influence membrane properties, then 1-decanol would maintain its depolarization activity across all conditions. 1-Decanol caused immediate and maximal depolarization of *S. aureus* at concentrations similar to its MIC (128 µg/mL), and this activity was consistent when compared between 37°C and 23°C as well as between MHB and PBS.

**Binding of [14C]-telavancin to bacteria.** A radiolabeled binding assay was used to quantitate the amount of telavancin bound to *S. aureus*. Cells were treated with [14C]-telavancin and monitored over a 2 hour time period (Fig. 3). Association of [14C]-telavancin with metabolically-active cells (MHB) increased over time. In contrast, association of [14C]-telavancin with cells incubated in nutrient-free media (PBS) occurred only during the initial binding period (15 min), after which no further binding was observed. After 2 hours, 2.8-fold more telavancin was bound to cells in MHB than those in PBS.

**Bactericidal activity in the absence of cell lysis.** A series of experiments were conducted to determine whether the direct action of telavancin on the membrane results in cell lysis. The degree of cellular lysis was determined spectroscopically by measuring culture turbidity at 625 nm. *S. aureus* cultures exposed to 8 and 32 µg/mL telavancin for 6 hr showed no significant change in optical density, indicating lack of cell lysis (Fig. 4). Similarly, the turbidity of cultures exposed to vancomycin did not significantly change (data not shown). In contrast, lysostaphin reduced the optical density of control cultures to background levels consistent with complete cell lysis. Monitored in parallel with turbidity, viable counts showed that telavancin effectively reduced the inoculum by more than 3-log_{10} CFU/mL at each concentration (Fig. 4).
Lysis can also be considered to occur through irreversible membrane dissolution, which may be independent of whether the cell maintains an intact wall. Since turbidity may not fully detect this type of lysis, phase contrast microscopy was used to allow direct observation of cellular integrity. No change in cell integrity was observed in cells following exposure to telavancin (up to 32 µg/mL) that were monitored for up to 6 hours.

DISCUSSION

The essential function of a cell membrane is to separate the cell from its environment. The membrane permits a cell to maintain a unique intracellular composition as well as ionic gradients across the membrane, both of which are essential for cellular viability. In bacteria, the proton gradient is required for both ATP synthesis and nutrient import, and is the primary contributor to membrane potential (22). Collapse of the proton gradient (i.e. depolarization) inhibits these functions and thus results in loss of bacterial viability (16, 30).

Telavancin effectively depolarized *S. aureus* as seen in cell population histograms (Fig. 1). Concentration response curves were used to establish an IC$_{50}$ value of 3.6 µg/mL for telavancin-induced membrane depolarization. This activity was observed at concentrations that are approximately 10-fold higher than the telavancin MIC, but well below clinically achievable plasma levels ($C_{\text{max}}$ 82 µg/mL) (52). In contrast, no IC$_{50}$ value could be calculated for vancomycin, even at concentrations as high as 96 µg/mL, indicating that vancomycin has no direct activity against the bacterial membrane. At all concentrations the degree of depolarization increased with time. *S. aureus* cells exposed to telavancin exhibited a broad depolarization response at 45 min and were fully depolarized by 90 min at 8 µg/mL (Fig. 1). The time dependence and broad depolarization response may be explained by the heterogeneity of Lipid II
present in bacterial populations; cells undergoing active division should produce more Lipid II than those that have just completed a division cycle or have yet to begin another division cycle. Further, the path to reach Lipid II will be shorter at stages of growth where the septum is beginning to form than towards the end of formation (43). The time at which a majority of the population was completely depolarized correlated to the loss of bacterial viability (Fig. 4) and is best explained by the need to accumulate a sufficient concentration of telavancin in the membrane.

Binding studies with $^{14}$C-telavancin were conducted to determine the amount of antibiotic that binds to the cell. The concentration of telavancin bound to the cell continued to increase over time and correlated to the onset of depolarization, resulting in 2.8-fold more telavancin bound to cells cultured in MHB than those in PBS. Since cells cannot grow or divide when suspended in PBS, binding in this condition represents the static number of D-Ala-D-Ala binding sites in the cell, consisting of uncrosslinked cell wall, nacent peptidoglycan, and Lipid II. The differential between MHB and PBS likely represents new Lipid II synthesis. While no further cell division can occur once exposed to telavancin, the cell wall machinery can generate new Lipid II molecules to which telavancin can bind. This result is similar to another Lipid II-dependent antibiotic, mersacidin (9).

Telavancin binds specifically to terminal D-Ala-D-Ala residues in cell wall precursors. Cell fractionation studies with radiolabeled telavancin showed that the majority of telavancin bound to the cell was localized to the membrane (25). These observations prompted us to investigate the role of Lipid II in telavancin-induced membrane depolarization. In our depolarization assay, telavancin activity was suppressed by the substrate mimicking peptide, dKAA (Table 2). The competitive binding assay suggests that membrane disruption is caused when telavancin binds to
Lipid II, and lacks disruptive activity when binding is blocked. Antagonism by dKAA elevated the telavancin MIC by 16-fold, which is in agreement with depolarization results. This concept is further supported by results obtained using THRX-881620, the hexapeptide analog of telavancin with impaired binding affinity for D-Ala-D-Ala. Relative to telavancin, THRX-881620 induced only one-third the depolarization and also exhibited weaker antimicrobial activity.

In order to more rigorously test the dependence of telavancin on Lipid II, depolarization assays were conducted using cells expressing altered levels of Lipid II. Depolarization was suppressed by all conditions that reduced Lipid II in *S. aureus*, including pretreatment with Lipid II synthesis inhibitors, suspension in nutrient-free media, and incubation at low temperature, demonstrating the requirement of telavancin to bind Lipid II in order to disrupt membrane function.

First, we demonstrate the dependence of telavancin on Lipid II by blocking synthesis of Lipid II with specific cell wall inhibitors. *S. aureus* cells were pretreated with either fosfomycin, D-cycloserine, or bacitracin and then exposed to telavancin or nisin. All three inhibitors of Lipid II synthesis suppressed telavancin-induced depolarization by approximately two-thirds compared to cells without pretreatment (Table 3). Nisin-induced depolarization was similarly suppressed by the three inhibitors, except that bacitracin pretreatment caused even stronger suppression (2% of control levels). This observation suggests that the target shared by nisin and bacitracin, the pyrophosphate moiety of undecaprenyl pyrophosphate, may play a role in nisin-mediated depolarization.

Depolarization by telavancin was completely suppressed when cells were suspended in nutrient-free media (Fig. 2A). Thus, an orthogonal approach to modulating Lipid II levels gives a similar
result as with the antibiotic pretreatment described above. Suspending bacteria in isotonic PBS with cations permits physiological gradients across the membrane but deprives the cell of nutrients required for cell wall synthesis and growth. In the absence of cell wall synthesis and Lipid II generation, cells lack the specific cellular target so that telavancin is unable to bind the membrane and cause depolarization. Further support for this concept is derived from $[^{14}\text{C}]-\text{telavancin}$ binding experiments with bacteria. The concentration of telavancin bound to bacteria increased over time when grown in MHB, but was flat when suspended in PBS. Similarly, the binding of another Lipid II-dependent antibiotic, mersacidin, to bacteria was substantially reduced in PBS compared to growth media (9). Under conditions that support metabolic activity and thus the continuous synthesis of Lipid II, telavancin is presented with additional target molecules thereby increasing the concentration of telavancin in the membrane to levels that result in disruption of normal function.

An intermediate growth condition was induced by incubation at reduced temperature in broth. Bacteria cultured at 23ºC in MHB sustained a degree of cell wall synthesis, with corresponding Lipid II generation, which permitted telavancin to depolarize approximately one third of the cell population by 60 min (Fig. 2B). Cultures shifted from 23ºC to 37ºC post-telavancin treatment showed a dramatic increase in depolarization at the next sampled time point, which matches the magnitude of depolarization seen under standard conditions. This slow growing 23ºC culture might be considered representative of S. aureus at an infection site that are near the low limit of growth. Therefore, even very slow growing S. aureus produce sufficient levels of Lipid II to be targeted by telavancin. Any increase in bacterial growth rate is effectively countered by increased telavancin activity. By varying cellular Lipid II content with defined conditions, we observe the dynamic nature of telavancin-induced depolarization as a function of the presence or absence of its target, Lipid II.
A number of antibiotics target Lipid II but they all act differently, both in the required binding contacts and the ability to impact cell wall synthesis or membrane function (5, 56). For example, the antibacterial action of nisin results from its high affinity for Lipid II combined with the ability to assemble into nisin-Lipid II complexes that form pores in the membrane (7, 8, 57). In contrast, telavancin lacks the structural characteristics required to form discrete pores. It therefore must exert its membrane disruptive activity through other mechanisms, one such possibility being the induction of positive membrane curvature (see (34)) via the insertion of the decylaminoethyl sidechain into the membrane. In a series of experiments using model membranes, telavancin-induced proton permeability was shown to be anionic phospholipid-dependent but Lipid II-independent (6), suggesting that Lipid II is not involved in the biophysical mechanism of membrane disruption. Therefore, in bacteria, membrane-embedded Lipid II appears to act exclusively as a high affinity target that mediates binding of telavancin to the membrane.

As described in recent surveillance of clinical isolates (17), telavancin retains antibacterial potency against MRSA, VISA, hVISA, and daptomycin non-susceptible staphylococci. In good agreement, isolates of S. aureus expressing these resistance phenotypes are equally susceptible to depolarization by telavancin (Table 1). Exposure to telavancin therefore results in significant disruption of membrane function regardless of phenotype. The strong depolarization activity against VISA and daptomycin non-susceptible strains highlights that telavancin retains its membrane mechanism against these emerging, clinically-important phenotypes. VISA and hVISA are increasingly identified as a possible cause of vancomycin treatment failure in the clinic (27, 37, 38, 47).
In VISA, resistance to glycopeptides is mediated by alterations in the structural organization of the cell wall, most notably by thickened cell walls containing an increased number of cell wall D-Ala-D-Ala residues (14, 15, 19, 29, 49, 50). These binding sites act as decoy targets for vancomycin. The overproduction of uncrosslinked D-Ala-D-Ala residues creates a reservoir in the mature cell wall that effectively sequesters vancomycin and prevents it from reaching the lethal target sites of membrane-embedded Lipid II and nascent peptidoglycan. Binding experiments show that the affinity of vancomycin for soluble D-Ala-D-Ala residues is four- to six-fold higher than that of telavancin (25). Affinities for soluble D-Ala-D-Ala can be considered a surrogate measure for uncrosslinked cell wall residues. In contrast, the binding affinity of telavancin for the D-Ala-D-Ala moiety of Lipid II is 160-fold higher than for soluble D-Ala-D-Ala (6). The strong affinity of telavancin for Lipid II, combined with weaker cell wall affinity, enables it to more readily pass through the wall to sites of peptidoglycan biosynthesis.

Cell lysis is a common terminal event for bacteria killed by cell wall-active antibiotics, particularly those of the beta-lactam class (55). However, the substrate-dependent mechanism of glycopeptides results in a different impact on the cell wall, and it has been demonstrated that vancomycin suppresses autolysis, presumably by blocking access to murein hydrolases (51). Based on the turbidity assay and microscopic analysis presented above, and further corroborated by transmission electron microscopy (44), telavancin does not trigger autolysis nor does it cause lysis by direct action on the membrane. Therefore, membrane disruption does not result from a weakened cell wall. In addition, these data support that telavancin does not act as a non-specific surfactant. Further, when turbidity was evaluated in parallel with viable counts, telavancin is bactericidal without causing cell lysis. We conclude from these findings that telavancin disrupts the functional integrity of the bacterial membrane as a primary component of its mode of action.
Telavancin exerts its antibacterial effects through a mechanism of action that combines inhibition of cell wall synthesis and disruption of essential membrane barrier functions. In this manuscript, we have further elucidated the membrane mechanism of telavancin. Telavancin selectively associates with the bacterial membrane by binding to Lipid II, conferred by the intramolecular cooperativity of D-Ala-D-Ala binding and membrane anchoring. Accumulation of telavancin in the membrane leads to impaired barrier function. We propose that Lipid II binding positions the lipophilic sidechain of telavancin into the membrane, which enables it to perturb the lipid bilayer. The exact mechanism of biophysical disruption remains under investigation. In summary, through interaction with the membrane-embedded target Lipid II, telavancin both inhibits peptidoglycan synthesis and disrupts membrane barrier function. Our findings provide a rational mechanistic basis for the potent antibacterial activity of telavancin.

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TABLE 1. Telavancin depolarization activity against phenotypically diverse *S. aureus*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>Depolarization (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 33591</td>
<td>MRSA</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>ATCC 700699</td>
<td>VISA (Mu50)</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>ATCC 700698</td>
<td>hVISA (Mu3)</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>MED2034</td>
<td>Daptomycin non-susceptible</td>
<td>77 ± 5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent depolarization is reported for treatment with 32 µg/mL telavancin, and represents the mean ± standard deviation from at least three independent experiments.
TABLE 2. Antagonism of telavancin-induced depolarization by D-Ala-D-Ala ligand

<table>
<thead>
<tr>
<th>dKAA (mM)</th>
<th>MIC (µg/mL)</th>
<th>Depolarization (%)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8 µg/mL</td>
</tr>
<tr>
<td>0</td>
<td>0.25</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>0.05</td>
<td>0.5</td>
<td>33 ± 14</td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>7 ± 6</td>
</tr>
</tbody>
</table>

\(^a\)S. aureus cells were pretreated with N,N'-diacetyl-L-Lys-D-Ala-D-Ala (dKAA) prior to telavancin exposure for 60 min.

\(^b\)Values represent the mean ± standard deviation from at least three independent experiments.
TABLE 3. Effect of Lipid II synthesis inhibitors on depolarization of *S. aureus* by telavancin

<table>
<thead>
<tr>
<th>Antibiotic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Depolarization (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Telavancin</td>
<td>Nisin</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>29 ± 6</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>D-Cycloserine</td>
<td>38 ± 8</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>27 ± 4</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

<sup>a</sup>*S. aureus* cells were pretreated 10 min prior to the addition of telavancin or nisin.

<sup>b</sup>Depolarization was calculated relative to control sample without pretreatment. Values represent the mean ± standard deviation from at least two independent experiments.
Figure Legends

FIG. 1.
Dissipation of *S. aureus* membrane potential measured by flow cytometry with DiOC$_2$(3). Cell populations exhibiting depolarization appear towards the left on the x-axis. (A) Fully polarized (untreated) and depolarized (nisin, 8 µg/mL) cells. (B) Depolarization of *S. aureus* by telavancin (TLV). Cultures were incubated for 15, 45, and 90 min in the presence of 8 µg/mL telavancin.

FIG. 2.
Effect of reduced cellular levels of Lipid II on depolarization of *S. aureus* by telavancin. (A) Bacteria treated at 37°C in MHB (closed circles) or PBS (open circles). (B) Bacteria treated at 23°C in MHB (closed triangles), followed by temperature shift to 37°C at 15 min (open triangles) and 30 min (upside-down triangles). All samples were treated with 32 µg/mL telavancin. Values represent the mean ± standard deviation from three independent experiments.

FIG. 3.
Binding of [$^{14}$C]-telavancin to *S. aureus* cells. Bacteria were cultured at 37°C in MHB (closed circles) or PBS (open circles). Values represent the mean ± standard deviation of three independent experiments.

FIG. 4.
Comparison of culture turbidity to viable counts in *S. aureus* exposed to telavancin. Curves represent turbidity (A, circles) and viable counts (B, triangles) for cultures exposed to 8 µg/mL (grey) and 32 µg/mL (black) telavancin. Lysostaphin (open circles) was used as a positive
control for cell lysis. Values represent the mean ± standard deviation from three independent experiments.