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

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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 the inhibition of cell wall synthesis and the disruption of membrane barrier function. The purpose of the present study was to further elucidate the mechanism by which telavancin interacts with the bacterial membrane. A flow cytometry assay with the diethyloxacarbocyanine 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 and in experiments with cells expressing altered levels of lipid II. Finally, monitoring of 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. The targeted interaction with lipid II and the consequent disruption of both peptidoglycan synthesis and membrane barrier function provide a mechanistic basis for the improved antibacterial properties of telavancin relative to those of vancomycin.

The increasing prevalence of serious infections caused by gram-positive bacteria, including those caused by methicillin (meticillin)-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, semisynthetic derivatives of glycopeptides that contain hydrophobic substituents and that possess improved antimicrobial properties (1, 4, 13, 32, 39). Telavancin, a lipoglycopeptide derivative of vancomycin, exhibits enhanced potency in vitro, 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 III 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 the inhibition of cell wall synthesis and the disruption of essential membrane barrier functions (25). Telavancin possesses the glycopeptide core of vancomycin, which binds with a 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 the polymerization of the 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 the 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, heterogeneous vancomycin-intermediate *S. aureus* (hVISA), vancomycin-intermediate *S. aureus* (VISA), and daptomycin-nonsusceptibile 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, the latter observation indicates that telavancin-induced membrane depolarization is not a consequence of a weakened

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cell wall. The findings of 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. The *S. aureus* strains used in this study included ATCC 33591 (MRSA), ATCC 29213 (methicillin-susceptible *S. aureus*), ATCC 700698 (hVISA Mu3), and ATCC 700699 (VISA Mu50); all strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA). A daptomycin-nonsusceptible MRSA strain (strain MED 2034) 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 dimethyl sulfoxide (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 telavancin powder in a 1:1 (vol/vol) mixture of DMSO and water that was acidified by adding 28 μl of 1 N HCl per 10 ml of solution. Serial dilutions of telavancin were also performed in acidified 50% DMSO. Polypropylene laboratory ware was used for the preparation of stock solutions and the subsequent dilution procedures. Nisin stock solutions were prepared in deionized water, which was warmed, vortexed, and briefly bath sonicated, prior to filtration through a 0.2-μm-pore-size syringe (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 plates (Hardy Diagnostics, Santa Monica, CA). Cultures subjected to the nongrowing condition were incubated in Dulbecco's phosphate-buffered saline with cations (PBS; Invitrogen, Carlsbad, CA).

Flow cytometry. The membrane potential was assayed by flow cytometry with the diethyloxacarbocyanine dye $DiOC_2(3)$, which allows the variation in cell size to be normalized by analysis of the ratio of red fluorescence to green fluorescence (40, 48). Metabolically active *S. aureus* generates 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} = 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 was added before the addition of telavancin), and the 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 the final concentration of $DiOC_2(3)$ being 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 5,000 events for each sample at the medium flow rate, and the signal was acquired with logarithmic amplification. The collection of fluorescence from a fixed number of individual cells ensures that the culture is uniformly sampled, irrespective of cell density. Green fluorescence emission was detected through a 525-nm (40-nm band-pass) emission filter (FL1), and red fluorescence was detected through a 620-nm (30-nm band-pass) emission filter (FL3). Analysis was performed with FCS Express software (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 the 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. The 50% inhibitory concentrations IC_{50}s were determined from the percent depolarization by fitting the concentration-response curve by nonlinear regression to a sigmoidal four-parameter equation by the use of SigmaPlot software (Systat, San Jose, CA).

In our analyses of membrane potential, we sought to ensure that only perturbations caused by the direct interaction of compound with the membrane were 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 with penicillin G to inhibit cell wall synthesis either partially (sub-MIC) or completely (supra-MIC) showed that the 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 the 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. The percent depolarization was calculated relative to the level of depolarization of control samples without pretreatment. Modified culture conditions were also used to modulate the lipid II levels. Cells were incubated under each condition (PBS at 37°C or MHB at 23°C) for 10 min before exposure to 32 μg/ml telavancin.

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-pore-size filter plate (MultiScreen-HTS-GV; Millipore). The filter plate was prewashed with PBS containing 0.002% Tween 20 to reduce nonspecific binding. The 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 the dried samples, which were then read on a Wallac MicroBeta scintillation counter (Perkin-Elmer).

Antimicrobial activity. MICs were determined by the use of dry-form panels manufactured by Trek Diagnostic Systems (Cleveland, OH). Time-kill assays were performed according to methods defined by the CLSI (12).

Cell lysis. Turbidity was evaluated with a PharmaSpec UV-1700 spectrophotometer (Shimadzu, Columbia, MD). *S. aureus* (ATCC 33591) cultures were grown to early exponential phase (A_{625} = 0.3) in MHB, 5 ml was aliquoted into 50-ml conical tubes, compounds were added to the desired final concentration, and the 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 the bacteria were captured on a Zeiss Axioskop equipped with a Plan-Neofluar ×100/1.3 objective and with a Photometrics CoolsnapFx charge-couple-device camera. Cells were deposited on a slide, a coverslip was applied, and the edges were sealed. The 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 h posttreatment. 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 the membrane potential in bacteria (40, 41, 48). Monitoring changes to membrane potential is a useful method for assessment of the significance of a membrane effect, as it reports the functional integrity of the bacterial membrane. The membrane potential is sensitive to modest disruptions of barrier function, like those that would allow small solutes such as protons to cross the membrane, and requires a sustained effect to maintain the depolarized state. Dissipation of the membrane potential (i.e., depolarization) leads directly to the 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 the test conditions (cation-adjusted MHB, physiological temperature, and low cell density) that are routinely employed for antibiotic susceptibility testing. Histogram displays of cellular red fluorescence/green fluorescence ratios

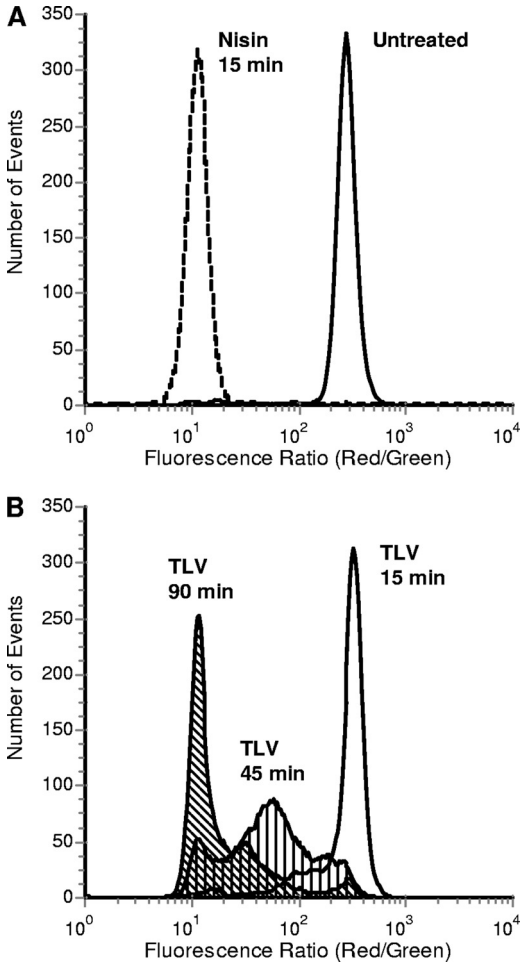

FIG. 1. Dissipation of *S. aureus* membrane potential measured by flow cytometry with $DiOC_2(3)$. Cell populations exhibiting depolarization appear toward the left on the *x* axis. (A) Fully polarized (untreated) and depolarized (nisin, 8 μg/ml) cells; (B) depolarization of *S. aureus* by telavancin (TLV). The cultures were incubated for 15, 45, and 90 min in the presence of 8 μg/ml telavancin.

show distinctions in the magnitude of depolarization induced in antibiotic-treated cultures. In Fig. 1, the results for an untreated, fully polarized (normal metabolic state) population of *S. aureus* (ATCC 29213) cells are depicted in the uniform distribution on the farthest right in the graph. Upon depolarization, the 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 the *S. aureus* cells were treated with the proton ionophore CCCP, they 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 nearly 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}s. Telavancin (MIC, 0.25 μg/ml) demonstrated concentration-dependent depolarization activity against *S. aureus*, with an IC_{50} of 3.6 μg/ml. The IC_{50} determined for nisin (MIC, 4 μg/ml) was 2.9 μg/ml. In contrast, no IC_{50} could be calculated for vancomycin (MIC, 1 μg/ml), even at concentrations up to 96 μg/ml.

Membrane depolarization in phenotypically diverse *S. aureus* 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 affect the activity of telavancin. Telavancin maintained potent activity against and dissipated the membrane potential of *S. aureus* cells expressing clinically important phenotypes such as methicillin resistance, vancomycin intermediate, heterogeneous vancomycin intermediate and daptomycin nonsusceptibility (Table 1). In all strains, the percent depolarization increased over time, resulting in *S. aureus* populations in which a majority of the 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 *S. aureus* 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-related mechanism of action against a strain with this important phenotype. Telavancin exerted a strong depolarization effect (77%) against the daptomycin-nonsusceptible *S. aureus* 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 an interaction with membrane-embedded lipid II or, alternatively, by direct association with the mem-

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

Strain	Relevant phenotype	% Depolarization[a]
ATCC 33591	Methicillin resistant	77 ± 7
ATCC 700699	Vancomycin intermediate (Mu50)	61 ± 3
ATCC 700698	Heterogeneous vancomycin intermediate (Mu3)	78 ± 4
MED 2034	Daptomycin nonsusceptible	77 ± 5

[a] Percent depolarization is reported for treatment with 32 μg/ml telavancin, and the values represent the means ± standard deviations from at least three independent experiments.

TABLE 2. Antagonism of telavancin-induced depolarization by D-Ala–D-Ala ligand

dKAA concn (mM)[a]	MIC (μg/ml)	% Depolarization with telavancin at[b]:	
		8 μg/ml	32 μg/ml
0	0.25	49 ± 3	94 ± 8
0.05	0.5	33 ± 14	68 ± 13
0.5	1	17 ± 4	19 ± 1
5	4	7 ± 6	14 ± 2

[a] *S. aureus* cells were pretreated with dKAA prior to exposure to telavancin for 60 min.
[b] Values represent the means ± standard deviations from at least three independent experiments.

TABLE 3. Effect of lipid II synthesis inhibitors on depolarization of *S. aureus* by telavancin

Antibiotic[a]	% Depolarization[b]	
	Telavancin	Nisin
None	100	100
Fosfomycin	29 ± 6	26 ± 4
D-Cycloserine	38 ± 8	36 ± 1
Bacitracin	27 ± 4	2 ± 1

[a] *S. aureus* cells were pretreated for 10 min prior to the addition of telavancin or nisin.
[b] Depolarization was calculated relative to that for the control sample without pretreatment. Values represent the means ± standard deviations from at least two independent experiments.

brane independent of lipid II. Telavancin has been shown to bind with a 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 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-related mechanism of action 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 nearly maximal antagonism being 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 the 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 suggest that telavancin-induced depolarization may require binding to lipid II. To test this hypothesis directly, depolarization assays were conducted with cells expressing altered levels of lipid II. Three different inhibitors of cell wall synthesis, fosfomycin, D-cycloserine, and bacitracin, were used to reduce the lipid II levels in *S. aureus* cells. Fosfomycin is an inhibitor of MurA, the enzyme responsible for the first step in peptidoglycan 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 the synthesis of lipid II.

S. aureus cells were preincubated with each of the lipid II synthesis inhibitors and were then exposed to telavancin or nisin. The time and concentration of exposure to telavancin and nisin were selected to achieve 50 to 70% depolarization for each compound alone (for telavancin, 32 μg/ml and 15 min; for nisin, 2 μg/ml and 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 the activity of nisin to 26% and 36% of the levels observed in control cultures, respectively. 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 the activity of nisin to 2% of the 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 that for cells that were not pretreated.

The cellular levels of lipid II were also manipulated by shifting the cultures to either nutrient-free medium or a 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 nongrowing cell will have a reduced rate of lipid II production, which can be induced by lower temperatures or minimal medium (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 that for telavancin in MHB. The membrane potential of untreated cells observed under the conditions and times explored in these assays was not different from that observed under 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 was seen to slowly rise to 30% by 60 min, whereas there was >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 time points of 15 min and 30 min (Fig. 2B). The shift from a suboptimal growth temperature to the optimal growth temperature resulted in a dramatic increase in the level of depolarization seen at the next time point that a sample was obtained. In fact, irrespective of when the temperature is shifted for the sample, 15 min at 37°C results in a similar percentage of depolarized cells (approximately 50%), which also matches the

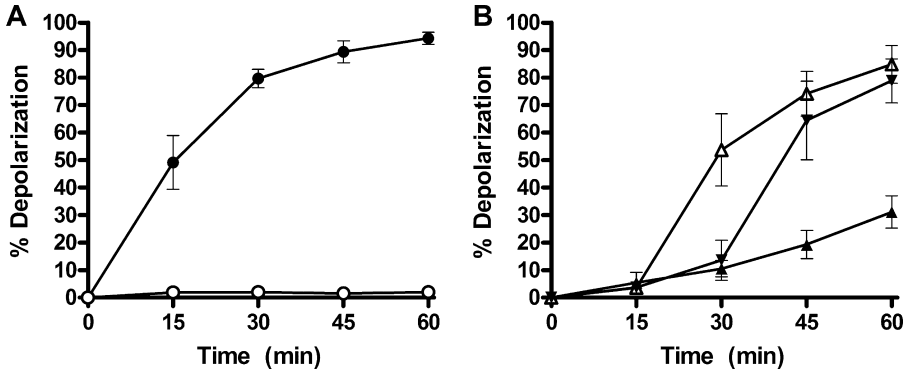

FIG. 2. Effect of reduced cellular levels of lipid II on the depolarization of *S. aureus* by telavancin. (A) Bacteria treated at 37°C in MHB (closed circles) or PBS (open circles); (B) bacteria treated at 23°C in MHB (closed triangles), followed by a temperature shift to 37°C at 15 min (open triangles)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

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 that lacks the N-terminal amino acid of the carboxylate binding pocket (25). THRX-881620 (MIC, 8 μg/ml) induced only 35% depolarization relative to the level induced by 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 the overall properties of the membrane. 1-Decanol, a model surfactant, partitions into the membrane due to its amphipathic properties (20, 54) and induces depolarization independently 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 the immediate and maximal depolarization of *S. aureus* at concentrations similar to its MIC (128 μg/ml), and this activity was consistent when it was compared between 37°C and 23°C as well as between MHB and PBS.

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

Bactericidal activity in the absence of cell lysis. A series of experiments was 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 the turbidity of the culture at 625 nm. *S. aureus* cultures exposed to 8 and 32 μg/ml telavancin for 6 h showed no significant change in optical density, indicating a lack of cell lysis (Fig. 4). Similarly, the turbidity of cultures exposed to vancomycin did not change significantly (data not shown). In contrast, lysostaphin reduced the optical density of control cultures to background levels, consistent with complete cell lysis. When viable counts were monitored in parallel with turbidity, the 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 the direct observation of cellular integrity. No change in cellular integrity was observed in cells following exposure to telavancin (up to 32 μg/ml) that were monitored for up to 6 h.

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

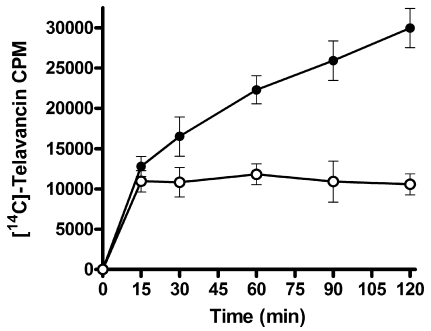

FIG. 3. Binding of [^{14}C]telavancin to *S. aureus* cells. Bacteria were cultured at 37°C in MHB (closed circles) or PBS (open circles). Values represent the means ± standard deviations of three independent experiments.

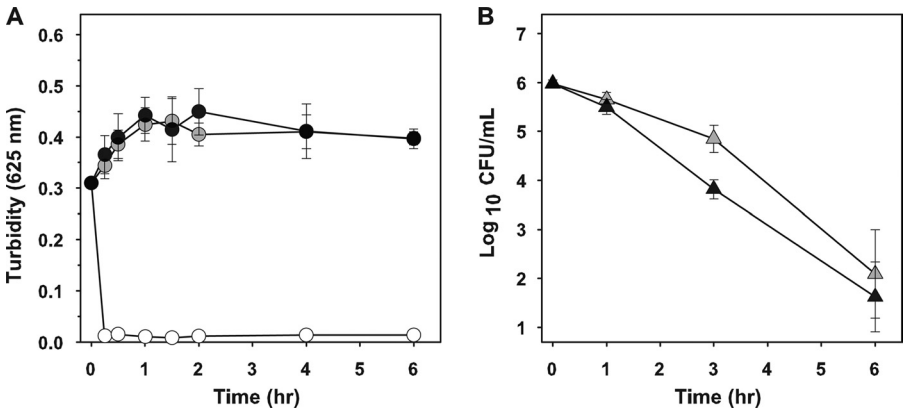

FIG. 4. Comparison of culture turbidity to viable counts in *S. aureus* exposed to telavancin. Curves represent turbidity (A, circles) and viable counts (B, triangles) for cultures exposed to 8 μg/ml (gray symbols) and 32 μg/ml (black symbols) telavancin. Lysostaphin (open circles) was used as a positive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

contributor to membrane potential (22). Collapse of the proton gradient (i.e., depolarization) inhibits these functions and thus results in the 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} 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 levels in plasma (maximum concentration in plasma, 82 μg/ml) (52). In contrast, no IC_{50} 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 tested, the degree of depolarization increased with time. *S. aureus* cells exposed to telavancin at 8 μg/ml exhibited a broad depolarization response at 45 min and were fully depolarized by 90 min (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 that have yet to begin another division cycle. Furthermore, the path to reach lipid II will be shorter at stages of growth where the septum is beginning to form than at stages toward the end of septum 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 cultured in PBS. Since cells cannot grow or divide when they are suspended in PBS, binding under this condition represents the static number of D-Ala–D-Ala binding sites in the cell, consisting of uncrosslinked cell wall, nascent peptidoglycan, and lipid II. The differential between MHB and PBS likely represents new lipid II synthesis. While no further cell division can occur once the cell is exposed to telavancin, the cell wall machinery can generate new lipid II molecules to which telavancin can bind. This result is similar to that for another lipid II-dependent antibiotic, mersacidin (9).

Telavancin specifically binds to terminal D-Ala–D-Ala residues in cell wall precursors. Cell fractionation studies with radiolabeled telavancin showed that the majority of the 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, the activity of telavancin was suppressed by the substrate-mimicking peptide dKAA (Table 2). The results of the competitive binding assay suggest that membrane disruption is caused when telavancin binds to lipid II and that telavancin lacks disruptive activity when binding is blocked. Antagonism by dKAA elevated the telavancin MIC by 16-fold, which is in agreement with the depolarization assay results. This concept is further supported by the results obtained with THRX-881620, the hexapeptide analog of telavancin with an 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 with cells expressing altered levels of lipid II. Depolarization was suppressed under all conditions that reduced lipid II levels in *S. aureus*, including pretreatment with lipid II synthesis inhibitors, suspension in nutrient-free medium, and incubation at low temperature, demonstrating the requirement of telavancin to bind to lipid II in order to disrupt membrane function.

We demonstrate the dependence of telavancin on lipid II by blocking the synthesis of lipid II with specific cell wall inhibitors. *S. aureus* cells were pretreated with either fosfomycin, D-cycloserine, or bacitracin and were then exposed to telavancin or nisin. All three inhibitors of lipid II synthesis suppressed telavancin-induced depolarization by approximately two-thirds compared to the level of depolarization of cells that were not pretreated (Table 3). Nisin-induced depolarization was similarly suppressed by the three inhibitors, except that bacitracin pretreatment caused even stronger suppression (to 2% of the control levels). This observation suggests that the target shared by nisin and bacitracin, the pyrophosphate moiety of undeca-

prenyl pyrophosphate, may play a role in nisin-mediated depolarization.

Depolarization by telavancin was completely suppressed when cells were suspended in nutrient-free medium (Fig. 2A). Thus, an orthogonal approach to the modulation of lipid II levels gives a result similar to that achieved with the antibiotic pretreatment described above. The suspension of bacteria in isotonic PBS with cations permits physiological gradients across the membrane but deprives the cell of the 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 to the membrane and cause depolarization. Further support for this concept is derived from the results of [^{14}C]telavancin binding experiments with bacteria. The concentration of telavancin that bound to bacteria increased over time when the bacteria were grown in MHB but remained constant when the bacteria were suspended in PBS. Similarly, the binding of another lipid II-dependent antibiotic, mersacidin, to bacteria was substantially reduced when the bacteria were in PBS than when they were in growth medium (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 the disruption of normal function.

An intermediate growth condition was induced by incubation of the bacteria at a reduced temperature in broth. Bacteria cultured at 23°C 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 that were shifted from 23°C to 37°C after telavancin treatment showed a dramatic increase in depolarization at the next time point that matched the magnitude of depolarization seen under standard conditions. This slowly growing culture at 23°C might be considered representative of *S. aureus* cells at an infection site that are near their low limit of growth. Therefore, even very slowly growing *S. aureus* cells produce sufficient levels of lipid II to be targeted by telavancin. Any increase in the bacterial growth rate is effectively countered by increased telavancin activity. By varying the cellular lipid II content with defined conditions, we observed 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 binding contacts required and in the ability to affect 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 its 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 must therefore exert its membrane-disruptive activity through other mechanisms, with one such possibility being the induction of positive membrane curvature (34) via the insertion of the decylaminoethyl side chain into the membrane. In a series of experiments with model membranes, telavancin-induced proton permeation 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 the binding of telavancin to the membrane.

As described in a recent surveillance of clinical isolates (17), telavancin retains potency against MRSA, VISA, hVISA, and daptomycin-nonsusceptible 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 the significant disruption of membrane function, regardless of the phenotype. The strong depolarization activity against VISA and daptomycin-nonsusceptible strains highlights the fact that telavancin retains its membrane-related mechanism of action against these emerging, clinically important phenotypes. VISA and hVISA strains are increasingly identified as possible causes of vancomycin treatment failure in the clinic (27, 37, 38, 47).

In VISA isolates, 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 sixfold 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 that for soluble D-Ala–D-Ala (6). The strong affinity of telavancin for lipid II, combined with a 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). On the basis of the results of the turbidity assay and microscopic analysis presented above and as 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 the fact that telavancin does not act as a nonspecific surfactant. Furthermore, 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 the inhibition of cell wall synthesis and the disruption of essential membrane barrier functions. In this report, we have further elucidated the membrane-related mechanism of action 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. The accumulation of telavancin in the membrane leads to impaired barrier function. We propose that lipid II binding positions the lipophilic side chain 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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