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Telavancin, a Multifunctional Lipoglycopeptide, Disrupts both Cell Wall Synthesis and Cell Membrane Integrity in Methicillin-Resistant *Staphylococcus aureus*

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The emergence and spread of multidrug-resistant gram-positive bacteria represent a serious clinical problem. Telavancin is a novel lipoglycopeptide antibiotic that possesses rapid in vitro bactericidal activity against a broad spectrum of clinically relevant gram-positive pathogens. Here we demonstrate that telavancin's antibacterial activity derives from at least two mechanisms. As observed with vancomycin, telavancin inhibited late-stage peptidoglycan biosynthesis in a substrate-dependent fashion and bound the cell wall, as it did the lipid II surrogate tripeptide *N*,*N*′-diacetyl-L-lysinyl-D-alanyl-D-alanine, with high affinity. Telavancin also perturbed bacterial cell membrane potential and permeability. In methicillin-resistant *Staphylococcus aureus*, telavancin caused rapid, concentration-dependent depolarization of the plasma membrane, increases in permeability, and leakage of cellular ATP and K^+. The timing of these changes correlated with rapid, concentration-dependent loss of bacterial viability, suggesting that the early bactericidal activity of telavancin results from dissipation of cell membrane potential and an increase in membrane permeability. Binding and cell fractionation studies provided direct evidence for an interaction of telavancin with the bacterial cell membrane; stronger binding interactions were observed with the bacterial cell wall and cell membrane relative to vancomycin. We suggest that this multifunctional mechanism of action confers advantageous antibacterial properties.

The emergence and spread of bacterial resistance to vancomycin, an important antibiotic used to treat serious infections caused by gram-positive bacteria, has prompted active research to discover new glycopeptides and semisynthetic analogs with improved antimicrobial properties. Vancomycin and related glycopeptide antibiotics inhibit cell wall synthesis in susceptible bacteria by binding with high specificity to peptidoglycan precursors containing the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) motif (8). The peptide portion of glycopeptide antibiotics forms a carboxylate binding pocket that imparts, through a combination of five hydrogen bonds plus favorable hydrophobic interactions, strong affinity for the D-Ala-D-Ala-containing terminus of lipid II (8, 46, 54). Rational approaches toward the design of glycopeptides with improved antimicrobial activities have been described previously (for reviews, see references 35 and 36). One promising approach has been the discovery of lipoglycopeptides, analogs containing hydrophobic groups substituted at the amine position of the disaccharide moiety (20, 39, 40, 45).

Telavancin, a semisynthetic derivative of vancomycin possessing a hydrophobic (decylaminoethyl) side chain appended to the vancosamine sugar and a hydrophilic [(phosphonomethyl)aminomethyl] group on the resorcinol-like 4′ position of amino acid 7 (33), is in late-stage clinical development for the treatment of serious gram-positive infections. Telavancin and other lipoglycopeptides exhibit superior in vitro activity compared to vancomycin (28, 30, 31, 37, 39, 48), including rapid, concentration-dependent bactericidal activity against glycopeptide-susceptible organisms as well as glycopeptide-intermediate susceptible *Staphylococcus aureus* and vancomycin-resistant *S. aureus* (5, 15, 43; K. D. Leuthner, C. M. Cheung, and M. J. Rybak, Abstr. 44th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1266, 2004). Two explanations have been offered for the enhanced properties of lipoglycopeptides, both of which involve effects on late steps of bacterial cell wall peptidoglycan biosynthesis that occur at the exterior surface of the bacterial cell membrane. One proposed mechanism is that hydrophobic substituents of lipoglycopeptides confer strong dimerization and membrane-anchoring properties that allow for increased binding affinities for peptidoglycan intermediates at the target site in bacteria (1, 3, 4, 9). The second proposed mechanism is that the liposaccharide elements of lipoglycopeptides interact directly with and inhibit transglycosylase enzymes that mediate the polymerization of precursors into immature, un-cross-linked peptidoglycan (16, 23, 24, 30, 50).

The aim of the present study was to investigate the molecular mechanism of antibacterial action of telavancin. We provide evidence that telavancin possesses at least two mechanisms of action in methicillin-resistant *S. aureus* (MRSA), including a previously unidentified mechanism. As observed with vancomycin, telavancin inhibited peptidoglycan synthesis by binding to D-Ala-D-Ala-containing residues of peptidoglycan intermediates. We also observed that telavancin perturbed bacterial membrane function, a mechanism that has not been previously described for a lipoglycopeptide. Exposure to telavancin resulted in increased membrane permeability, leakage of intracellular ATP and K^+, and dissipation of cell membrane potential. These events correlated temporally with the rapid

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onset of bactericidal activity. This multifunctional activity may provide advantages to telavancin that are absent in antibacterials such as vancomycin that possess only a single antibacterial mechanism (46, 53). These studies significantly enhance our understanding of the mechanism of action of telavancin and demonstrate the potential advantages of a multivalent antibiotic.

MATERIALS AND METHODS

Antibacterials, media, and reagents. Telavancin and THRX-881620 were prepared by Theravance, Inc. (South San Francisco, Calif.). Telavancin is a semisynthetic vancomycin derivative containing a decylaminoethyl chain appended to the vancosamine residue and a phosphonic acid-containing substituent at the resorcinol position (33). THRX-881620 is the hexapeptide (des-*N*-methylleucyl) derivative of telavancin lacking the N-terminal amino acid of the carboxylate binding pocket. Vancomycin, *N*,*N*′-diacetyl-L-Lys-D-Ala-D-Ala (dKAA), and lysostaphin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Moenomycin (flavomycin) was obtained from Hoechst (Frankfurt, Germany). Mueller-Hinton Broth (Difco, Detroit, Mich.) supplemented with 25 mg of Ca^{2+}/liter and 12.5 mg of Mg^{2+}/liter (CA-MHB) was used for bacterial growth. Minimal medium was prepared essentially as described previously (1). Tryptic soy agar (Hardy Diagnostics, Santa Monica, Calif.) was used for plate counts. [^{3}H]vancomycin (7.0 Ci/mmol) and [^{3}H]telavancin (8.9 Ci/mmol) were prepared by ViTrax (Placentia, Calif.). Radiolabeled substrates for macromolecular synthesis and transglycosylase activity assays were from Amersham (Little Chalfont, United Kingdom). 3,3′-Dipropylthiadicarbocyanine iodide [DiSC3(5)], SYTO-9, and propidium iodide dyes were obtained from Molecular Probes (Eugene, Oreg.).

Bacterial strains. *S. aureus* ATCC 33591 (MRSA) was purchased from the American Type Culture Collection (Manassas, Va.). *Escherichia coli* BAS849/pUG18 was constructed at Theravance by transforming the pUG18 plasmid (11) for *murG* overexpression into *E. coli* BAS849, which has an outer membrane permeability defect (47).

Antibacterial activity. MICs were determined by broth microdilution, utilizing CA-MHB as described by the NCCLS (38).

Macromolecular synthesis. Macromolecular synthesis was assayed by measuring the incorporation of radioactive precursors into 10% trichloroacetic acid (TCA)-precipitable material for peptidoglycan, RNA, and protein assays or into methanol-chloroform extractable material for fatty acid assays. Mid-exponential-phase (A_{600} = 0.3; ~10^8 CFU/ml) MRSA growing in CA-MHB for fatty acid, RNA, and peptidoglycan assays or resuspended in minimal medium to an A_{600} of 0.3 for protein assay were preincubated for 5 min with inhibitors and, where indicated, dKAA, prior to the addition of radiolabeled substrates. The following radiolabeled substrates were added to cells for the indicated assays: peptidoglycan assay, 0.1 μCi of [^{14}C]*N*-acetylglucosamine/ml; RNA assay, 0.1 μCi/ml of [^{14}C]uridine; fatty acid assay, 1 μCi/ml of [^{14}C]acetate; protein assay, 2 μCi/ml of [^{14}C]leucine. Mixtures were incubated 10 min at 37°C. Cell viability was determined at this point by enumerating colonies formed on agar plates. For peptidoglycan, RNA, and protein assays the reactions were stopped by adding equal volumes of 10% TCA. After 10 min incubation at room temperature samples were transferred into 96-well filter plates and filter-washed five times with 5% TCA. After drying overnight, radioactivity was determined by scintillation counting. For the fatty acid assay, lipids were extracted with chloroform-methanol (10) and the amount of radioactivity in the lipid fraction determined by scintillation counting.

Polymerization of lipid II into nascent peptidoglycan. The transglycosylase (polymerization) reaction was assayed by the method of Branstrom et al. (11) by using membranes prepared from *E. coli* BAS849/pUG18, UDP-MurNAc-pentapeptide, or UDP-MurNAc-tetrapeptide substrate purified from enterococci by the method of Kohlrausch and Holtje (32) and [^{14}C]UDP-*N*-acetylglucosamine (1 μCi/ml). Reaction mixtures were incubated for 2 h to allow accumulation of lipid II. Inhibitors and, where indicated dKAA, were preincubated with this mixture for 10 min. Reactions were initiated by the addition of Detergent-OUT resin (Genotech, St. Louis, Mo.) and incubated for 2 h at room temperature to allow conversion of lipid II into peptidoglycan. Nascent polymerized peptidoglycan was detected by chromatography on Whatman 3MM paper in isobutyric acid: 1 N NH_4OH (5:3 [vol/vol]). Spots corresponding to peptidoglycan were quantified by utilizing a STORM PhosphorImager (Amersham Biosciences, Piscataway, N.J.).

Solution-phase binding to a D-Ala-D-Ala-containing ligand. Binding of the tripeptide ligand dKAA by telavancin, vancomycin, and THRX-881620 was determined by affinity capillary electrophoresis (ACE) and electrospray ionization mass spectrometry (ESI-MS). The ACE binding determinations were performed essentially as described previously (6, 17) with the following modifications. Electrophoretic mobilities were measured by using an Agilent HPCE 3D CE system (Palo Alto, Calif.) equipped with a fused silica capillary (Polymicro Technology, Phoenix, Ariz.) measuring 60 cm by 75 μm (inside diameter) (effective length of 52 cm) operated at 25°C with an applied voltage of 25 kV. The capillary was rinsed with 0.1 N NaOH, followed by deionized water and running buffer, before each run. Running buffers consisted of 25 mM sodium phosphate, pH 6.5, and various concentrations of ligand ranging from 0 to 500 μM. Solutions (0.05 mg/ml) of telavancin, vancomycin, and THRX-881620 were prepared in 25 mM sodium phosphate, pH 6.5. Sample injections were conducted by applying a pressure of 0.5 lb/in^2 for 5 s. Analytes were detected at 214 nm. Dimethyl sulfoxide (0.02% vol/vol) was used as the electro-osmotic flow marker, and data were analyzed with Agilent ChemStation software. Association constants were determined by Scatchard analysis, as described previously (6, 18).

The ESI mass spectra measurements were recorded on an ABI/MDS Sciex API 4000 triple quadrupole mass spectrometer (Foster City, Calif.) optimized on each glycopeptide-ligand complex so as to reduce in-source dissociation. Glycopeptides and ligand were prepared in 5 mM ammonium acetate, pH 6.0, to desired concentrations. Quadruplicate measurements were taken for each equimolar combination of glycopeptide and ligand (concentrations ranged from 2.5 to 10 μM). Twenty mass spectra were summed and averaged for each determination. Mass spectrometer conditions were as follows: ion spray voltage, +5,000 V; declustering potential, +86 V; exit potential, +10 V; prefilter, −16 V; curtain gas setting, 10; gas 1 setting, 50; step size, 0.1 atomic mass units; scan range, 850 to 1,100 atomic mass units; scan time, 5 s. Solution-phase association constants were determined by measuring peak heights of the bound versus unbound glycopeptides by using assumptions and calculations as described previously (27).

Membrane potential and permeability. The depolarization of plasma membrane potential was determined essentially as described previously (55) by using the membrane potential-sensitive fluorescent dye DiSC3(5) with the following modifications. Cells were grown in CA-MHB to an A_{600} of 0.3, harvested, washed once in buffer containing 5 mM HEPES and 5 mM glucose (pH 7.2) (buffer A), resuspended in the same buffer with 100 mM KCl and 2 μM of DiSC3(5), and incubated for 15 min at room temperature to allow for dye uptake and fluorescence quenching. The fluorescence change (excitation = 622 nm, emission = 670 nm) was measured immediately after the addition of antibiotic compounds on a SpectraMax fluorescence microplate reader (Molecular Devices, Sunnyvale, Calif.).

Membrane permeability was assayed in real time by FLIPR (Molecular Devices) by using SYTO9 and propidium iodide stains provided in the LIVE/DEAD *Bac*Light kit (L-7012; Molecular Probes). Cells were grown in CA-MHB to an A_{600} of 0.3, harvested, washed once in buffer A, resuspended in the same buffer containing 5 μM SYTO-9 and 30 μM propidium iodide, and equilibrated for 10 min at room temperature. The fluorescence emission (excitation = 488 nm, emission = 510 nm) was measured immediately prior to and following the addition of antibiotic compounds. Cell viability was measured by enumerating colonies formed on agar plates.

Release of cellular ATP and K^+. For the measurement of ATP efflux, telavancin and vancomycin were applied at various concentrations and incubated with mid-exponential-phase MRSA cells in buffer A (~10^8 CFU/ml) for 15, 30, and 60 min. Cells were removed by centrifugation, and the supernatant was assayed for ATP with the CellTiter-Glo luminescent kit (Promega, Madison, Wis.). Data were collected on a α-FP HT microplate luminometer (Packard, Meriden, Conn.). K^+ efflux was measured by using the K^+-sensitive fluorescent dye 1,3-benzenedicarboxylic acid, 4,4′-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl)]bis (PBFI; Molecular Probes). MRSA cells were grown to the exponential phase in CA-MHB (A_{600} = 0.3), washed twice with buffer A, and resuspended in buffer A containing 1 mM PBFI. Sample fluorescence (excitation = 346 nm, emission = 505 nm) background data were collected for 50 s (equilibration) in an F-2000 fluorescence spectrophotometer (Hitachi, San Jose, Calif.) before the addition of antibacterial compounds, after which data were collected for an additional 2.5 min.

Binding and cell fractionation. Mid-exponential-phase (A_{600} = 0.3) cultures (100 ml) of MRSA grown in CA-MHB were incubated with ^{3}H-labeled compounds (5 ng/ml) and, where indicated, dKAA (400 μM) for 30 min at 37°C with shaking. Bacteria were collected by centrifugation and resuspended in protoplast buffer (25% sucrose, 50 mM Tris [pH 7.0], 10 mM $MgCl_2$, 15 μg of freshly prepared lysostaphin/ml) and, depending on the sample, 0.4 mM dKAA. Following incubation with shaking for 40 min at 37°C, cells were again pelleted and the supernatant (peptidoglycan fraction) was reserved. The cell pellet was washed once in protoplast buffer with or without 0.4 mM dKAA. The resulting

pellet (protoplast fraction) was dried prior to scintillation counting. The tritiated compounds were verified to be intact by high-performance liquid chromatography with radiometric detection.

RESULTS

Inhibition of macromolecular biosynthesis in intact cells. In the search for an additional mechanism of action that may explain the enhanced pharmacodynamic properties of telavancin, it was previously reported that inhibition of bacterial fatty acid synthesis occurred at concentrations where vancomycin had no such inhibitory activity (28). Here we studied the concentration dependence of inhibition of synthesis of fatty acids, RNA, protein, and peptidoglycan in intact MRSA cells under conditions where minimal cell killing occurred. The control compounds, triclosan for fatty acid synthesis, rifampin for RNA synthesis, linezolid for protein synthesis, and vancomycin for peptidoglycan synthesis, each demonstrated specific inhibition of the expected target at low concentrations compared to effects on other biosynthetic pathways (Fig. 1 and data not shown). Telavancin inhibited peptidoglycan synthesis potently and specifically with a 50% inhibitory concentration (IC_{50}) of 0.14 μM, approximately 14-fold more potent than vancomycin (IC_{50} = 2.0 μM) (Fig. 1). At the lowest concentration where telavancin maximally inhibited peptidoglycan synthesis (~0.25 μM), less than 25% inhibition of synthesis was observed for all other macromolecules, including fatty acids.

To assess the contribution of an intact carboxylate binding pocket toward the activity of telavancin, we prepared THRX-881620, the des-*N*-methylleucyl (hexapeptide) derivative of telavancin containing a disrupted peptide binding pocket. Hexapeptide derivatives of vancomycin and related glycopeptides exhibit reduced binding affinities for D-Ala-D-Ala-containing peptide substrates and concomitant reduced antibacterial activities (4, 21). THRX-881620 was approximately 40-fold less potent than telavancin at inhibiting peptidoglycan biosynthesis (IC_{50} = 5.8 μM) and exhibited reduced selectivity toward other macromolecular synthesis pathways (Fig. 1).

Substrate-dependent inhibition of late-stage peptidoglycan biosynthesis. The ability of telavancin to inhibit the transglycosylase reaction was monitored by measuring the conversion of radiolabeled lipid II synthesized in situ from UDP-[^{14}C] GlcNAc and UDP-MurNAc-pentapeptide (UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) or UDP-MurNAc-tetrapeptide (UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala) substrates into polymerized peptidoglycan. In reactions with the UDP-MurNAc-pentapeptide substrate, the potent transglycosylase inhibitor moenomycin inhibited peptidoglycan polymerization with an IC_{50} of 0.02 μM, consistent with published values (11). Under these assay conditions, telavancin inhibited transglycosylase activity with a potency (IC_{50} = 0.6 μM) comparable to that observed for inhibition of peptidoglycan biosynthesis in intact cells (Fig. 1 and Table 1) and, on a molar basis, was 12-fold more active than vancomycin (IC_{50} = 7 μM). The activity differential for inhibition of transglycosylase activity by telavancin and vancomycin mirrored the differential observed for inhibition of peptidoglycan synthesis in intact cells (14-fold). The activity of THRX-881620 in the transglycosylase assay (IC_{50} = 45 μM) was approximately 75-fold lower than that of telavancin (Table 1). When UDP-MurNAc-tetrapeptide substrate, possessing terminal L-Lys-D-Ala rather than the terminal D-Ala-D-Ala found in the natural lipid II pentapeptide-containing substrate, was used in this assay, no inhibition was seen with telavancin, vancomycin, or THRX-881620, whereas the activity of moenomycin, which interacts directly with the enzyme, remained unaffected (data not shown). Taken together, these results suggest that telavancin inhibits late-stage peptidoglycan synthesis in a substrate-dependent manner.

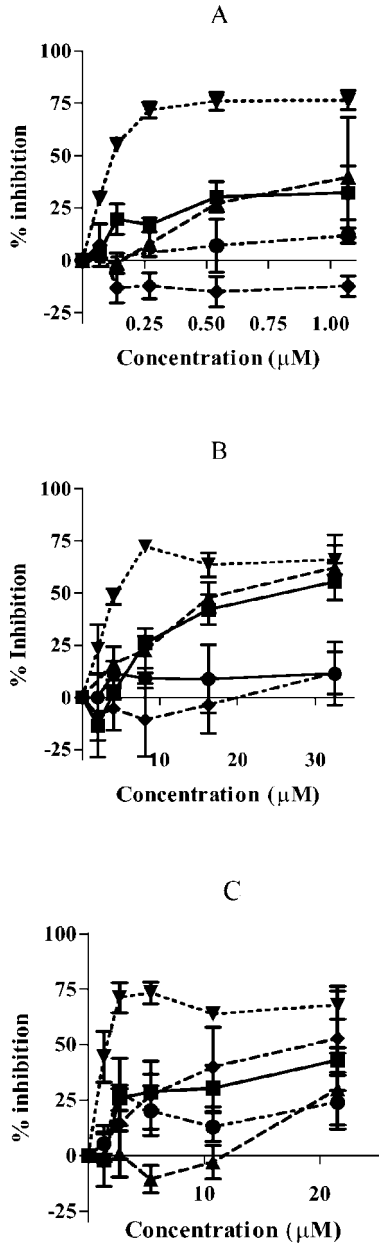

FIG. 1. Inhibition of macromolecular synthesis in MRSA 33591 by telavancin, THRX-881620, and vancomycin. The percentage of inhibition of RNA (■), fatty acid (▲), peptidoglycan (▼), and protein (◆) and the percentage of killing (●) after incubation for 10 min are plotted versus drug concentration. Each point is the mean ± standard deviation of 3 estimates. The MICs of telavancin (A), THRX-881620 (B), and vancomycin (C) were 0.3, 8, and 0.7 μM, respectively. The IC_{50}s for peptidoglycan synthesis inhibition determined from these concentration-inhibition curves were 0.14, 5.8, and 2.0 μM for telavancin, THRX-881620, and vancomycin, respectively.

TABLE 1. Effect of dKAA on antibacterial activity and transglycosylase inhibition by telavancin, THRX-881620, and vancomycin[a]

dKAA concn (mM)	Telavancin		THRX-881620		Vancomycin	
	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}
0	0.5 (0.3)	1.2 (0.6)	16 (8)	90 (45)	1 (0.7)	10 (7)
0.5	4 (2)	1.6 (0.8)	16 (8)	52 (26)	256 (200)	>143 (>100)
10	16 (8)	3 (1.5)	16 (8)	30 (15)	>256 (>200)	>143 (>100)
40	ND[b]	12 (6)	ND	32 (16)	ND	>143 (>100)

[a] Results are micrograms per milliliter with micromolar concentrations in parentheses. IC_{50} are the geometric means determined in a transglycosylase assay with UDP-MurNAc-pentapeptide substrate from concentration-response curves that were analyzed by nonlinear regression with a four-parameter logistic equation (Prism; GraphPad, Inc.). Each point was determined from the results from at least three separate experiments.
[b] ND, not determinable.

Interaction with D-Ala-D-Ala residues. The affinity of telavancin and THRX-881620 for binding D-Ala-D-Ala residues of peptidoglycan intermediates was determined by measuring binding affinities to the cell wall-mimicking tripeptide dKAA by ACE and ESI-MS (Table 2). The binding affinity constants (K_A) determined for vancomycin are in good agreement and are consistent with values reported previously for both these (2, 17, 27, 52) and other methods (41, 44). The K_A values determined for telavancin by the two methods are also in good agreement and indicate that telavancin's affinity for this substrate surrogate is four- to sixfold less than that of vancomycin. The affinity of THRX-881620, as determined by ESI-MS, was reduced by about 25-fold compared to telavancin. Under the ACE assay conditions employed, we did not detect formation of a THRX-881620–dKAA complex. These data are consistent with reduced binding affinities reported previously with hexapeptide derivatives of vancomycin and related glycopeptides (4, 21).

Compelling evidence supporting a substrate-dependent mechanism for vancomycin derives from studies demonstrating the ability of excess D-Ala-D-Ala-containing ligands to suppress inhibitory activities (42). To further investigate a substrate-dependent mechanism for telavancin, we tested the ability of dKAA to antagonize activity in assays for inhibition of peptidoglycan synthesis in intact cells, transglycosylation, and bacterial growth. In peptidoglycan synthesis assays (Fig. 2), a significant molar excess of dKAA (0.5 mM) only partially antagonized telavancin activity (IC_{50} = 1.4 μM). Notably, the IC_{50} for inhibition of peptidoglycan synthesis by telavancin in the presence of 0.5 mM dKAA was similar to the IC_{50} for THRX-881620 (3.2 μM). The addition of dKAA to peptidoglycan synthesis assays containing THRX-881620 did not affect activity, whereas 0.5 mM dKAA completely abolished the inhibition of peptidoglycan synthesis by vancomycin (IC_{50} > 100 μM). In the cell-free assay for transglycosylase activity, increasing concentrations of dKAA also antagonized inhibition by both vancomycin and telavancin, while inhibition by THRX-881620 was not suppressed. There was, notably, a significant difference in the sensitivities of telavancin and vancomycin to antagonism by dKAA in this assay. The IC_{50} for vancomycin shifted greater than 10-fold in the presence of 0.5 mM dKAA, while the highest concentration of dKAA tested (40 mM) was required to increase the IC_{50} for telavancin 10-fold (Table 1). The antibacterial activity of telavancin, as measured by MIC determination, was more potent than that of vancomycin. MICs of the two compounds were 0.5 μg/ml (0.3 μM) and 1 μg/ml (0.6 μM), respectively, for MRSA ATCC 33591 (Table 1). However, as observed for antagonism of peptidoglycan synthesis activity, there was a significant difference in the ability of dKAA to antagonize antibacterial activity of the two compounds. The activity of vancomycin was completely suppressed (MIC > 256 μg/ml) by 10 mM dKAA, while telavancin retained significant activity (MIC = 16 μg/ml) in the presence of 10 mM dKAA (Table 1). The MIC for THRX-881620 (16 μg/ml) was not altered in the presence of 10 mM dKAA (Table 1), consistent with the low binding affinity of THRX-881620 for D-Ala-D-Ala-containing peptide substrates. The results presented in Fig. 2 and Table 1 indicate that peptidoglycan synthesis inhibition by telavancin occurs by a substrate-dependent mechanism that requires interaction with the terminal D-Ala-D-Ala residues of lipid II.

TABLE 2. Solution-phase binding affinities for dKAA

Glycopeptide	K_A (M^{-1})[a] determined by:	
	ACE	ESI-MS
Vancomycin	$(5.2 \pm 0.6) \times 10^5$	$(13.5 \pm 0.3) \times 10^5$
Telavancin	$(0.96 \pm .03) \times 10^5$	$(3.2 \pm 0.4) \times 10^5$
THRX-881620	ND[b]	$(0.13 \pm 0.006) \times 10^5$

[a] Values shown are means ± standard deviations of data from three separate experiments.
[b] ND, not determinable.

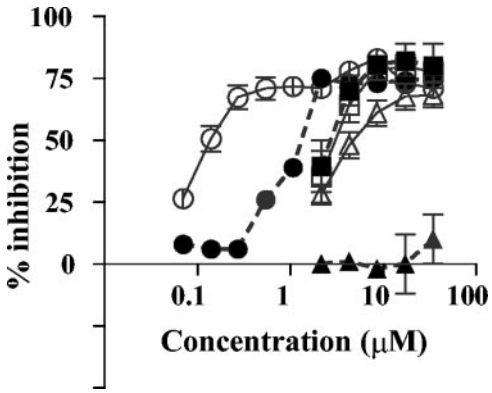

FIG. 2. Inhibition of peptidoglycan synthesis in MRSA 33591 cells. The percentages of peptidoglycan synthesis inhibited by various concentrations of telavancin (circles), THRX-881620 (squares), and vancomycin (triangles) in the absence (open symbols) and presence (closed symbols) of 0.5 mM dKAA are plotted. Each data point is the mean ± standard deviation of 3 estimates. The IC_{50}s determined from these concentration-inhibition curves in the absence and presence of dKAA are 0.14 and 1.4 μM for telavancin, 3.2 and 3.3 μM for THRX-881620, and 4.8 and >100 μM for vancomycin.

Effects of telavancin on cell membrane integrity and function. To investigate interactions with the bacterial membrane, we monitored the effect of telavancin on MRSA membrane potential by using the DiSC3(5) assay as described previously (55). In this assay, the partitioning of DiSC3(5) between the cell membrane and assay medium is proportional to the membrane potential such that the degree of fluorescence indicates the degree of reduction in the membrane potential. The results

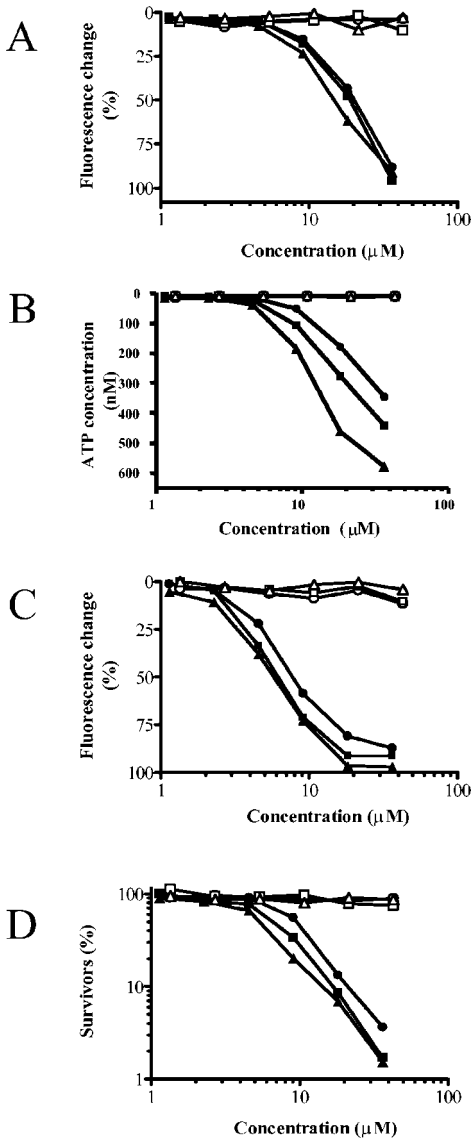

FIG. 3. Correlation between change in membrane potential (A), ATP leakage (B), permeability (C), and cell viability (D) at various times after antibiotic addition. ●, 15 min; ■, 30 min; ▲, 60 min; closed symbols, telavancin; open symbols, vancomycin. Representative curves are shown from single experiments that were reproduced in at least three independent determinations.

(Fig. 3A) demonstrate that telavancin dissipated the membrane potential in both a concentration- and time-dependent manner. A significant change in membrane potential was observed at a concentration as low as 9 μM, even in the presence of 10^8 CFU of bacteria/ml. Vancomycin had no effect on cell membrane potential even at the highest concentration (44 μM) tested (Fig. 3A). Assays performed under these high-inoculum (10^8 CFU/ml) conditions resulted in MICs of telavancin and vancomycin of 2 μg/ml (~1 μM) and 8 μg/ml (~5 μM), respectively.

The observed effect of telavancin on membrane potential prompted us to examine other aspects of membrane function. We employed the K^+-sensitive fluorescent probe PBFI to detect K^+, and a luciferase-based assay for ATP detection, to test whether exposure to telavancin resulted in release of cytoplasmic K^+ and ATP into the extracellular medium. Telavancin triggered the release of both K^+ (data not shown) and ATP (Fig. 3B) in a concentration-dependent manner that correlated with effects on cell membrane potential. To measure perturbation of plasma membrane integrity in real time, we developed a highly sensitive permeability assay based upon the nucleic acid-specific viability dyes SYTO9 and propidium iodide (PI) of the commercially available LIVE/DEAD *Bac*Light kit with fluorescence detection by FLIPR. Viable cells with an intact plasma membrane are stained by SYTO9, a membrane-permeant green fluorescent dye. Upon membrane permeabilization, SYTO9 fluorescence is quenched by entry of PI to the cytoplasm. This can only occur if the cytoplasmic membrane is compromised (14, 22). Cells exposed to telavancin, but not vancomycin, exhibited a rapid onset of membrane permeability (Fig. 3C). Notably, the observed effects of telavancin on membrane integrity correlated well with a reduction in cell viability (Fig. 3D). THRX-881620 also caused an increase in membrane permeability (data not shown), although the concentration required was higher (~4-fold) than that observed for telavancin.

The SYTO9-PI assay of membrane permeability was also used to assess the effect of dKAA on membrane permeabilization caused by telavancin. The addition of dKAA antagonized the permeability increase induced by telavancin in a concentration-dependent manner (Fig. 4), suggesting a role for D-Ala-D-Ala binding in either targeting telavancin to the membrane or in facilitating interactions that ultimately result in altered cell permeability. The concentration of dKAA required to fully antagonize activity in the membrane permeabilization assay was less than that required to partially antagonize activity in either the transglycosylase or the antibacterial assay (Table 1 and Fig. 4).

Binding and fractionation studies. The data presented above suggested that a direct physical interaction with the bacterial cell membrane may be responsible for the effects of

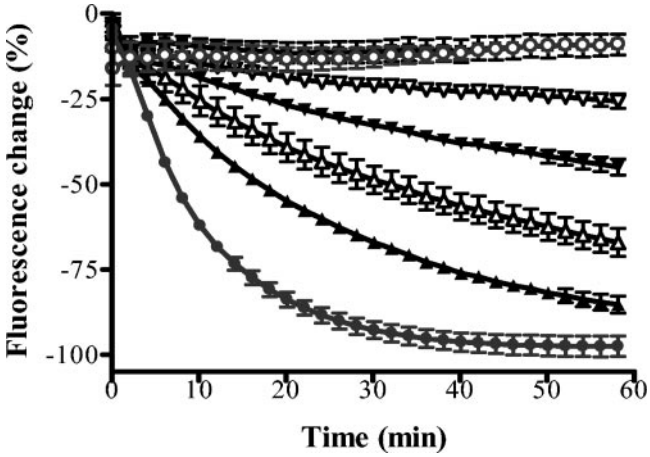

FIG. 4. Antagonism of telavancin-induced membrane permeability by dKAA. Membrane permeability was induced by 8 μM telavancin in the absence (●) or presence (added 10 min prior to telavancin) of the following concentrations of dKAA: 31.25 μM (▲), 62.5 mM (△), 125 μM (▼), 250 μM (▽), 500 μM (■). There was also a permeability control with no telavancin and no dKAA (○). Representative curves are shown from a single experiment that was repeated three times.

TABLE 3. Binding of [^{3}H]telavancin and [^{3}H]vancomycin to MRSA cells and cellular fractions

Drug	dKAA (mM)	Amt (ng) in total culture[a]	Amt (ng) (%)[b] in cellular fraction:	
			PG	Protoplast
Telavancin	0	30	6.8 ± 1.5 (23)	23 ± 2 (77)
	0.4	2.8	0.8 ± 0.07 (29)	2 ± 0.1 (71)
Vancomycin	0	13	12 ± 2 (95)	0.6 ± 0.1 (5)
	0.4	0.32	0.3 ± 0.06 (94)	0.02 ± 0.007 (6)

[a] Amount of compound recovered in the combined peptidoglycan and protoplast fractions derived from 75 ml of cell culture incubated with 375 ng of compound.

[b] Amount of compound recovered in the indicated fraction ± standard deviation of the mean from three separate experiments, with the percentage of total bound compound recovered in the indicated fraction noted in parentheses. PG, peptidoglycan.

telavancin on membrane function. To determine whether telavancin binds differentially to the cell membrane compared to vancomycin, we measured the compound recovered in cell wall and cell membrane fractions isolated from mid-exponential-phase cultures of MRSA exposed to [^{3}H]telavancin or [^{3}H]vancomycin (Table 3). Notably, more telavancin (8% of input) was found associated with unfractionated cells than was vancomycin (<3.5% of input). Of the cell-bound telavancin, <25% was recovered in the peptidoglycan fraction, whereas >75% remained associated with the resulting protoplast (cells devoid of cell wall). In contrast, >90% of the cell-bound vancomycin was recovered in the peptidoglycan fraction and <10% remained associated with protoplasts. The presence of 0.4 mM dKAA decreased the ability of both drugs to bind by more than 90%; however, the relative distribution of each antibiotic between the two fractions was unaltered. These data demonstrate the preferential interaction of telavancin with the cell membrane and the importance of the interaction with D-Ala-D-Ala to both cell wall and cell membrane binding.

DISCUSSION

Telavancin is a novel lipoglycopeptide antibiotic in late-stage clinical development for the treatment of serious infections caused by gram-positive bacteria. Despite its structural similarity to vancomycin, telavancin exhibits superior antibacterial potency and rapid in vitro bactericidal activity against a broad spectrum of gram-positive pathogens (25, 31, 43). The studies reported in this communication were undertaken to explore the mechanistic basis for the enhanced antibacterial properties of telavancin.

The antibacterial activity of vancomycin is determined by its ability to bind to D-Ala-D-Ala-containing peptidoglycan precursors (54). This interaction results in inhibition of peptidoglycan polymerization (transglycosylation) and subsequent cross-linking (transpeptidation) steps. We anticipated that the glycopeptide core of telavancin would, like vancomycin, inhibit peptidoglycan synthesis at the transglycosylation step through a substrate-dependent mechanism. To test this hypothesis, we measured the ability of telavancin to inhibit peptidoglycan synthesis in intact MRSA cells and polymerization of peptidoglycan catalyzed by a particulate membrane fraction. Telavancin was a potent inhibitor of peptidoglycan synthesis in intact cells (IC_{50} = 0.14 μM). This activity was antagonized 10-fold by the addition of a molar excess of the tripeptide ligand dKAA (Fig. 2). This observation, together with the finding that activity of the hexapeptide derivative of telavancin, THRX-881620, was reduced over 40-fold in this assay (Fig. 2), suggests a substrate-dependent mechanism of inhibition. We determined that telavancin inhibited peptidoglycan synthesis at the transglycosylase step (IC_{50} = 0.6 μM). THRX-881620 was 75-fold less potent at inhibiting this reaction (IC_{50} = 45 μM) (Table 1). No inhibition was observed by telavancin when the natural substrate UDP-MurNAc-pentapeptide was replaced with UDP-MurNAc-tetrapeptide. These observations, together with the ability of dKAA to antagonize telavancin activity in this assay, confirmed the importance of substrate binding to transglycosylase inhibition.

Telavancin was over 10 times more active than vancomycin at inhibiting both synthesis of peptidoglycan in intact MRSA cells and polymerization of peptidoglycan catalyzed by a membrane fraction isolated from *E. coli*. Despite this, telavancin exhibited an approximately fivefold-reduced affinity for binding to the D-Ala-D-Ala-containing ligand dKAA in solution (Table 2). Others have shown that N-alkyl substitution of glycopeptides confers membrane binding and have proposed this as a mechanism to explain enhanced potency (3, 9, 19). We speculate that such attachment of telavancin to a second site effectively increases the binding affinity for D-Ala-D-Ala-containing targets on the bacterial cell surface. Our studies examining antagonism of activity with the dKAA tripeptide are consistent with this model. Telavancin required, relative to vancomycin, a greater molar excess of dKAA to antagonize activity (for example, antibacterial activity of telavancin was suppressed only 32-fold by 10 mM dKAA, whereas vancomycin activity was suppressed 256-fold by only 0.5 mM dKAA) (Table 1). Simultaneous (intramolecular) interaction with the cell membrane and cell wall substrates would render telavancin recalcitrant to antagonism by dKAA in much the same manner as has been proposed for other N-alkylated glycopeptides (9, 34). We propose that telavancin is a multivalent antibiotic. The decylaminoethyl side chain promotes interaction with the cell membrane, and this interaction provides improved binding affinity of the glycopeptide core for D-Ala-D-Ala-containing peptidoglycan intermediates by localizing the molecule to the bacterial cell surface. This interpretation is supported by telavancin's 10-fold-greater potency toward inhibition of peptidoglycan synthesis despite a lower calculated affinity relative to vancomycin for binding a D-Ala-D-Ala-containing target in solution.

Inhibition of cell wall synthesis by the mechanism outlined above would be expected to result, as in the case for vancomycin, in a slow bactericidal effect (51). This differs markedly from results obtained from time-kill studies with telavancin that demonstrate rapid, concentration-dependent bactericidal activity (43). While the increased antibacterial activity of telavancin may be explained by increased potency for late-stage peptidoglycan synthesis inhibition, several lines of evidence suggest that telavancin possesses additional and possibly separate mechanism(s) of antibacterial action. First, it has been demonstrated by others that inhibition of the transglycosylase reaction in gram-positive bacteria fails to elicit a rapid lethal response (7). Second, THRX-881620 retains modest antibacterial activity that is not antagonized by a vast molar excess of

dKAA tripeptide. Third, telavancin possesses in vitro antibacterial activity against glycopeptide-intermediate susceptible *S. aureus*, vancomycin-resistant *S. aureus*, and vancomycin-resistant enterococci (28, 31, 33). And finally, we have been unable to select spontaneous mutants resistant to high levels of telavancin (K. Krause, D. Debabov, J. Pace, and K. Kaniga, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1810, p. 96, 2003).

Based upon our own observations (above) and those provided by others working with related lipoglycopeptides (3, 9, 19), we have proposed that telavancin interacts with the bacterial membrane. We investigated this interaction further by examining the ability of telavancin to perturb cell membrane function, since membrane depolarization and permeabilization have been implicated in the mode of action of antibacterial peptides (56, 57), including, most recently, daptomycin (49). Telavancin triggered rapid (within 15 min) concentration-dependent dissipation of cell membrane potential (Fig. 3A). This activity appeared to require interaction with peptidoglycan intermediates (Fig. 4), suggesting a molecular basis for bacterial membrane selectivity. Membrane depolarization was only detected at concentrations significantly (10-fold) higher than the MIC. However, the direct correlation (both concentration and time dependent) observed between membrane potential and viability suggests that this mechanism may contribute to, or be solely responsible for, the characteristic rapid bactericidal activity of telavancin. Membrane depolarization was also accompanied by leakage of cytoplasmic ATP (Fig. 3B) and K^+ ions, events that were also determined to occur in a concentration- and time-dependent manner correlating with reduced cell viability. Interestingly, cell membrane permeabilization occurred at concentrations closer to the MIC (two- to fourfold) and prior to the onset of detection of membrane depolarization and cell killing (Fig. 3C). These results suggest that membrane permeabilization preceded these other events.

Direct binding of telavancin to the cell membrane was demonstrated by using radiolabeled compounds and cell fractionation (Table 3). The ability of dKAA to inhibit the binding of both vancomycin and telavancin by >90% attests to the importance of the interaction with D-Ala-D-Ala-containing intermediates. However, telavancin differed from vancomycin in that the majority of the compound was associated with the membrane rather than the cell wall. These findings are consistent with the proposed multivalent mode of binding, including the contribution of both the carboxylate binding pocket in dictating interaction with D-Ala-D-Ala motifs and the decylaminoethyl side chain in promoting interaction with the cell membrane.

The close temporal correlation observed between membrane depolarization and cell killing suggests that we have identified a novel and important additional mechanism to explain telavancin's pharmacodynamic activity. This is in contrast to the apparent mechanism of cell killing achieved by many antimicrobial cationic peptides, where rapid cytoplasmic membrane depolarization is followed by a delayed (1 to 2 h) onset of cell killing (56, 57). Additionally, and also in contrast to the mechanism we describe, recent investigation of the antibacterial mechanism of the lipopeptide daptomycin suggests the occurrence of bactericidal activity prior to the onset of membrane depolarization (29). Interestingly, direct interaction with lipid II is a feature that telavancin shares with the lantibiotic nisin (12). Both agents exhibit concentration-dependent membrane permeabilization and are rapidly bactericidal (13, 43). Recent work with nisin demonstrates that it assembles into a complex comprised of 4 lipid II molecules and 8 nisin molecules that constitutes a transmembrane pore (E. Breukink, personal communication). Although its hydrophobic side chain is of insufficient length to span the membrane, telavancin may recruit lipid II by a conceptually similar mechanism to induce membrane permeability.

Thus, we have described a novel additional mechanism of action for the lipoglycopeptide antibiotic telavancin. Whether or not this mechanism is operative for related lipoglycopeptides remains to be determined. Although others have demonstrated interaction of lipoglycopeptides with protoplasts and isolated membranes (2, 3), to our knowledge, this is the first report of the physiological consequences of interaction of a lipoglycopeptide with the cytoplasmic membrane of intact cells. These findings provide a rational mechanistic basis for the improved pharmacodynamic activity and low potential for resistance development observed for telavancin both in vitro and in vivo (26, 43).

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