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Inhibition of D-Ala Incorporation into Wall Teichoic Acid in *Staphylococcus aureus* by Desleucyl-Oritavancin

J. Chang^a, L. Coffman^a, and S. J. Kim^a

Department of Chemistry, Baylor University, One Bear Place #97348, Waco, Texas 76798 United States. Tel: 1-254-710-2531

Abstract

Mode of action for desleucyl-oritavancin was investigated by adding the antibiotic to *Staphylococcus aureus* during growth in a defined media containing L,D-[1-¹⁵N]Ala and L-[1-¹³C]Lys, or D-[1-¹⁵N]Ala. ¹³C{¹⁵N} and ¹⁵N{¹³C} rotational-echo double resonance NMR determined that desleucyl-oritavancin inhibited the incorporation of D-[1-¹⁵N]Ala into wall teichoic acid.

Peptidoglycan (PG) and wall teichoic acid (WTA) are two major components of the cell wall in *Staphylococcus aureus*. A PG-repeat unit (Fig. 1a) consists of a disaccharide *N*-acetylglucosamine-*N*-acetyl-muramic acid (GlcNAc-MurNAc), a pentapeptide stem, and a pentaglycine-bridge structure. The cell wall is assembled through polymerization of PG-repeat units into a glycan chain by transglycosylase, and then these glycan chains are interconnected by cross-linking. Cross-link is a peptide bond formed between the N-terminus of pentaglycine bridge from one chain to the carboxyl carbon of D-Ala at the fourth position of a PG-stem in a neighbouring chain. As each successive PG-stem in the glycan chain is rotated 90° relative to the previous stem's orientation, cross-links form in all four directions in respect to the glycan propagation and result in a lattice structure.^{1, 2} The other major component of cell wall is WTA, which is an anionic polymer consisting of approximately 40-repeat units of ribitol-phosphate with ester-linked D-Ala (Fig. 1b). WTA is covalently attached to the MurNAc of PGs and they are interwoven to form the cell wall (Fig. 1c).

Vancomycin (Fig. 2a) is a potent glycopeptide antibiotic that targets the PG biosynthesis in *S. aureus* by binding to the D-Ala-D-Ala terminus of PG-stem in lipid II, a membrane bound PG-repeat unit utilized for delivering PG precursors from the cytoplasm. Vancomycin binding to lipid II inhibits the transglycosylation step of PG biosynthesis and prevents the regeneration of a critical lipid transporter bactoprenol phosphate, resulting in cytoplasmic accumulation of Park's nucleotide.³ Vancomycin has been indispensable in treatment of serious infections by multi-drug resistant Gram-positive pathogens, but its utility has been hampered by the emergence of vancomycin-resistant enterococci (VRE). In VRE, the D-Ala-D-Ala terminus of PG-stem is replaced by a depsipeptide D-Ala-D-Lac,⁴ which

Correspondence to: S. J. Kim.

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introduces an electrostatic repulsion and reduces the vancomycin binding affinity by a 1000-fold.⁵

Oritavancin (Ori) is a semi-synthetic lipoglycopeptide antibiotic (Fig. 2b) developed by Eli Lilly (Indianapolis, IN) to treat infections by VRE and methicillin-resistant *S. aureus*. The parent compound of Ori is chloroeremomycin, which shares the identical heptapeptide-core structure with vancomycin. The chemical structure of oritavancin differs from that of vancomycin by the addition of a chlorobenzylphenyl side chain to the 4-epi-vancomsamine on the fourth amino acid, and an additional vancomsamine at the sixth amino acid of the aglycon.⁶ Unlike the bacteriostatic activity of vancomycin, Ori exhibits rapid bactericidal activity against both vancomycin-susceptible and vancomycin-resistant pathogens including VRE, vancomycin-intermediate and vancomycin-resistant *S. aureus*.^{7–9} Unlike vancomycin which inhibits only the transglycosylation, Ori exhibit dual inhibitions of both transglycosylation and transpeptidation steps of PG biosynthesis.¹⁰

Ori's potent activity against vancomycin-resistant pathogen is attributed to a new mode of action that does not involve enhanced D-Ala-D-Lac binding as it shares an identical heptapeptide-core structure with vancomycin, and its hydrophobic sidechain does not improve binding to either dipeptide nor depsipeptide.¹¹ For vancomycin, removal of the first residue (*N*-methyl-leucine) through Edman degradation destroys the D-Ala-D-Ala binding site and abolishes all antimicrobial activity (Fig. 2a).¹⁰ In contrast, same degradation product of Ori (Fig. 2b), desleucyl-oritavancin (des-Ori), retains the potent antimicrobial activity.^{12–14} Solid-state NMR studies have determined that des-Ori can still bind to PG in the cell wall of *S. aureus* despite the loss of its primary D-Ala-D-Ala binding site. This is due to the hydrophobic sidechain of des-Ori, which forms a secondary-binding site that targets the cross-linked pentaglycine-bridge structure.^{15–19} In this study, we investigate the *in situ* mode of action of this secondary-binding site in des-Ori by measuring the changes in PG and WTA compositions of *S. aureus* grown in presence of des-Ori at sub-inhibitory concentration using ¹³C{¹⁵N} and ¹⁵N{¹³C} rotational-echo double resonance (REDOR) NMR.

For solid-state NMR analysis, des-Ori was added to *S. aureus* during the midexponential growth to final concentration of 20 µg/mL. To ensure that des-Ori at 20 µg/mL does not disrupt *S. aureus* membrane, ATP-leakage assays were performed (Fig. 2c). Various concentrations of vancomycin (gray), Ori (blue), and des-Ori (red) were added to *S. aureus* cultures at OD_{600nm} of 1.5. Following a 20 min incubation with the antibiotic, membrane disruption was determined by measuring the amount of ATP leaked using a reagent containing luciferin and luciferase. The breakdown of luciferin in presence of ATP releases photons at 560 nm, and integration of the luminescent intensity was directly proportional to the amount of leaked ATP. We found that glycopeptide antibiotics do not readily induce significant ATP leakage. In contrast, the positive control with the addition of daptomycin, a well-known membrane targeting cyclic depsipeptide, resulted in a significant ATP leakage (Fig. 2c, black).

To characterize the effects of des-Ori on PG biosynthesis, the PG-stem link was ¹³C-¹⁵N pair labelled (Fig. 3a) by growing *S. aureus* in a chemically defined media containing L,D-

[^{15}N]Ala and L-[1- ^{13}C]Lys. $^{13}\text{C}\{^{15}\text{N}\}$ REDOR full-echo spectra (S_0) of untreated and des-Ori treated *S. aureus* at 1.6 ms dipolar evolution are shown in Fig. 3b (bottom). The S_0 spectra are normalized to the 175-ppm peak intensity. In the difference spectra (ΔS) only the ^{13}C -lysyl carbonyl carbon of L-[1- ^{13}C]Lys in PG-stem link is visible at 175 ppm (Fig. 3b, top). To validate that the observed 175-ppm dephasing is from the PG-stem link, observed REDOR dephasings from des-Ori treated *S. aureus* are plotted as a function of dipolar evolution (Fig. 3c, filled circles). The calculated $^{13}\text{C}\{^{15}\text{N}\}$ REDOR dephasing curve (black line) is for the combined distances of 1.3 (red dotted line) and 3.3 Å (red solid line) with equal dephasing plateaus, and observed dephasings are consistent with this calculated dephasing curve as shown in Fig. 3a. Thus, the dephased 175-ppm intensity (Fig. 3b, dotted line) is directly proportional to the total number of PG stems. Since both the untreated and des-Ori treated *S. aureus* have the identical 175-ppm dephasing value of 25%, des-Ori did not inhibit the D-[^{15}N]Ala incorporation into the PG-stem structure.

Figure 3d shows the $^{15}\text{N}\{^{13}\text{C}\}$ REDOR spectra of *S. aureus* grown in a defined media containing L,D-[^{15}N]Ala and L-[1- ^{13}C]Lys in absence (left) and presence of des-Ori (right) at 1.6 ms dipolar evolution. In the ΔS spectra, only the covalently bonded ^{15}N -alanyl amide from the PG-stem link at 93 ppm is dephased. Observed dephasings (Fig. 3e, black circles) are fitted to a calculated $^{15}\text{N}\{^{13}\text{C}\}$ REDOR curve (black line) for combined distances of 1.3 (dotted red line) and 3.3 Å (solid red line). This confirmed that the ΔS 93-ppm intensity (Fig. 3b, dotted line) is directly proportional to the amount of L-[1- ^{13}C]Lys from pentapeptide PG-stem structures. As the ^{15}N -alanyl-amide dephasings at 93 ppm for treated and untreated *S. aureus* are equal at 20%, we conclude that des-Ori does not inhibit the incorporation of L-Lys into PG in *S. aureus*.

While the addition of des-Ori had no visible effects on D-[^{15}N]Ala and L-[1- ^{13}C]Lys incorporation into PG, we found that the D-[^{15}N]Ala incorporation into WTA was critically impaired. Enlarged overlapped S_0 spectra of des-Ori treated *S. aureus* show reduced ester-linked ^{15}N -alanyl amine peak intensity at 15 ppm (Fig. 4a, red line), which indicated that *S. aureus* in response to des-Ori treatment inhibited the D-Ala incorporation into WTA while maintaining the utilization of D-Ala for the PG biosynthesis.

To confirm that des-Ori inhibits the D-Ala incorporation into WTA, we grew *S. aureus* in a defined media containing D-[1- ^{13}C]Ala in presence of alanine racemase inhibitor alaphosphin (5 $\mu\text{g}/\text{mL}$). Alaphosphin prevented the conversion of D-[1- ^{13}C]Ala into L-[1- ^{13}C]Ala by the racemase, ensuring that only the exogenous D-[1- ^{13}C]Ala is incorporated to PG and WTA. ^{13}C -CPMAS spectra of intact whole cells of *S. aureus* labelled with D-[1- ^{13}C]Ala grown both in absence and presence of des-Ori are shown in Fig. 4b. The superimposed spectra centred at 175 pm reveals a difference in the lineshape. Subtraction of the des-Ori treated spectrum from the untreated results in the difference spectrum (Fig. 4c, right), where the increased 175-ppm intensity corresponds to the ^{13}C -alanyl carboxyl carbon of D-[1- ^{13}C]Ala in Park's nucleotide,^{20, 21} and the decreased 170-ppm intensity to the reduced ester-linked D-[1- ^{13}C]Ala in WTA.¹⁰

Edman degradation of type I glycopeptide antibiotics removes *N*-methyl-leucine from the aglycon structure, which damages the damaged D-Ala-D-Ala binding site and abolishes

antimicrobial activities. But the same degradation products of lipoglycopeptides^{10, 17} retain their antimicrobial activities against both vancomycin-susceptible and resistant pathogens.^{10, 12, 13} Solid-state NMR studies have determined that the hydrophobic sidechain of desleucyl-lipoglycopeptides forms a secondary-binding site targeting the pentaglycine-bridge structure of PG in *S. aureus*^{10, 17, 22, 23} despite the damaged aglycon structure.^{15, 16, 24, 25} This binding of lipid II by des-Ori inhibits the transglycosylation step of PG biosynthesis, which results in accumulation of Park's nucleotide²⁵ and prevents the regeneration of bactoprenol phosphate, the lipid transporter for both PG and WTA biosyntheses. Despite the shared lipid transporter, our results show that the D-Ala incorporation into WTA was impaired while the incorporation to PG biosynthesis was unaffected. This suggests that *S. aureus* responded to cell wall stress from des-Ori by preferentially routing all available D-Ala into PG biosynthesis, leaving very little D-Ala for WTA biosynthesis. Similar WTA biosynthesis inhibition by vancomycin and oritavancin at sub-MIC,²⁶ as well as by cyclic antimicrobial peptides plusbacin A₃²⁰ and amphomycin²¹ have been observed. This inhibition of WTA biosynthesis has far reaching consequences on *S. aureus* growth, cell division,²⁷ host-cell binding, biofilm formation, susceptibility to antibiotics,²⁸ autolytic activity,²⁹ and fitness. Thus, the ability of lipid II targeting antibiotics to inhibit both WTA and PG biosynthesis provides a new insight into their potent antibacterial activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Chemical structure of *S. aureus* peptidoglycan-repeat unit (a) and wall-teichoic acid (b). (c)
Schematic representation of covalently linked WTA and PG in cell wall.

Figure 2.

Chemical structures of vancomycin and desleucyl-vancomycin (a), and oritavancin and des-Ori (b). (c) Membrane depolarization assay. Glycopeptide antibiotics were added to *S. aureus* harvested at OD_{600nm} of 1.5. After 20 min of incubation with the antibiotic, the amount of leaked ATP was quantified using luciferase assay by measuring the luminescence. Des-Ori did not induce significant ATP leakage at any concentrations tested. In contrast, the positive control of *S. aureus* treated with daptomycin (100 μg/mL) showed large ATP leakage consistent with membrane disruption. The arrow indicates the des-Ori concentration (20 μg/mL) for which solid-state NMR samples were prepared. The error bars represent 95% confidence interval determined from 8 independent repeat measurements.

Figure 3.

(a) Isotope labelling scheme of *S. aureus* PG-stem structure using L,d-[¹⁵N]Ala and L-[1-¹³C]Lys. Only the d-[¹⁵N]Ala incorporated into PG results in ¹³C-¹⁵N pair labelling. (b) ¹³C{¹⁵N} REDOR at 1.6 ms dipolar evolution of d-[¹⁵N]Ala and L-[1-¹³C]Lys labelled *S. aureus* grown in absence (left) and presence of des-Ori (right). At 1.6 ms, only the covalently bonded lysyl-carbonyl carbons from ¹³C-¹⁵N pair from the PG stem-links is dephased and appears in the ¹³C S at 175 ppm. Des-Ori did not affect the ¹³C S 175-ppm peak intensity of *S. aureus* grown in presence of the drug. (c) ¹³C{¹⁵N} REDOR dephasings of 175 ppm from des-Ori treated *S. aureus* (filled circles) are plotted as a function of dipolar evolution. Calculated ¹³C{¹⁵N} REDOR dephasing curve for the combined distances of 1.3 and 3.3 Å is shown in black, and calculated dephasing curves for two separate distances of 1.3 and 3.3 Å are individually shown as red dotted and red solid lines. (d) ¹⁵N{¹³C} REDOR of L,d-[¹⁵N]Ala and L-[1-¹³C]Lys labelled *S. aureus* grown without (left) and with des-Ori (right) at 1.6 ms dipolar evolution. Peak intensities of the ¹⁵N-alanyl amide from d-[¹⁵N]Ala at 93 ppm in the ¹⁵N S spectra are constant, unaffected by the addition of des-Ori. (e) ¹⁵N{¹³C} REDOR dephasings of 93 ppm (filled circles) from des-Ori treated *S. aureus* plotted as a function of dipolar evolution. Calculated ¹⁵N{¹³C} REDOR dephasing curve for the combined distances of 1.3 and 3.3 Å is shown in black, and the dephasing curves for two separate distances of 1.3 and 3.3 Å are individually shown as red dotted and red solid lines.

Figure 4.

(a) d-[¹⁵N]Ala incorporation into WTA observed as ¹⁵N-alanyl amine at 15 ppm in the ¹⁵N{¹³C} REDOR S₀ spectra from Figure 3d. Reduction of 15-ppm intensity in des-Ori treated *S. aureus* is consistent with des-Ori inhibition of the d-[¹⁵N]Ala incorporation into WTA. (b) Incorporation of d-[¹³C]Ala into WTA of *S. aureus* in the presence of alanine racemase inhibitor alaphosphin. ¹³C-CPMAS spectra of d-[¹³C]Ala labelled *S. aureus* grown in absence (black) and presence of des-Ori (red). (c) The overlapped spectra of ¹³C-carbonyl carbon peaks show the changes in the line shape for des-Ori treated *S. aureus* (left). Spectral subtraction of des-Ori treated sample from the untreated results in the difference spectrum (right) with a positive peak at 175 ppm (accumulation of Park's nucleotide) and a negative peak at 170 ppm (reduction of ester-linked d-[¹³C]Ala from WTA).