Fluorescence Microscopy Demonstrates Enhanced Targeting of Telavancin to the Division Septum of \textit{Staphylococcus aureus}^{\dagger \ddagger}

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Received 12 November 2009/Returned for modification 14 December 2009/Accepted 8 February 2010

The cellular binding patterns of fluorescent conjugates of telavancin and vancomycin were evaluated in \textit{Staphylococcus aureus} by fluorescence microscopy and ratio imaging analysis. Telavancin showed enhanced binding at the division septum compared to vancomycin. This result is consistent with observations that telavancin binds with higher affinity to lipid II than to \(\text{d-Ala-d-Ala}\) residues in the cell wall, thus demonstrating the preferential binding of telavancin to the site of active cell wall biosynthesis.

Telavancin is a bactericidal, semisynthetic lipoglycopeptide with activity against Gram-positive bacteria, including methicillin-resistant \textit{Staphylococcus aureus} (MRSA), vancomycin-intermediate \textit{S. aureus} (VISA), and heterogeneous VISA (hVISA) (5, 8, 9). Telavancin is indicated in the United States and Canada for the treatment of complicated skin and skin structure infections (cSSSI) due to Gram-positive pathogens and is under investigation as a once-daily treatment for nosocomial pneumonia. The bactericidal activity of telavancin results from a mechanism of action that combines the inhibition of cell wall synthesis and the disruption of membrane barrier function (6, 10). Telavancin inhibits cell wall synthesis by binding to the terminal \(\text{d-alanyl-d-alanine (d-Ala-d-Ala)}\) residues of peptidoglycan precursors, including lipid II, in a manner similar to vancomycin. The lipophilic substituent on the vancomycin sugar confers additional properties to telavancin, specifically, preferential targeting to lipid II and concomitant association with the bacterial membrane (10). By binding lipid II and the membrane simultaneously, telavancin disrupts bacterial membrane function and potently inhibits cell wall synthesis. Here, we describe the results of a fluorescence microscopy study with live \textit{S. aureus} cells exposed to fluorescent conjugates of telavancin and vancomycin. Our findings expose one of the key differences between telavancin and vancomycin: the ability to access the division septum, the site of active cell wall biosynthesis.

(This work was presented in part at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 12 to 15 September 2009 [11].)

Fluorescent conjugates of telavancin (TLV-FL) and vancomycin (VAN-FL) were prepared by PyBOP chemistry, purified by reversed-phase high-performance liquid chromatography (HPLC) and characterized by mass spectrometry. BODIPY-FL was linked at the carboxylate of the glycopeptide core present in both vancomycin and telavancin. BODIPY-FL was selected as the fluorophore for its small size, neutral charge, and enhanced stability. MICs were determined by the CLSI broth microdilution method (2). The antibacterial activity of TLV-FL and VAN-FL differed no more than 2-fold from that of the parent compounds, consistent with previous reports of fluorescently tagged vancomycin with \textit{S. aureus} (7).

\textit{S. aureus} cultures (MRSA ATCC 33591) were grown at 37°C with agitation in cation-adjusted Mueller-Hinton II broth (MHB) (Difco, Detroit, MI) and maintained in MHB throughout staining and imaging to support metabolic activity. Cells were grown to an optical density at 625 nm (OD\textsubscript{625}) of 0.3 and stained with TLV-FL or VAN-FL at a concentration of 0.12 \(\mu\text{M}\) (approximately 0.25 \(\mu\text{g/ml}\)) for 6 min prior to imaging. This concentration is below the MICs of the fluorescently tagged antibiotics (TLV-FL, 0.5 \(\mu\text{g/ml}\); VAN-FL, 1 \(\mu\text{g/ml}\)), and was optimal for labeling the cell without inducing ultrastructural changes prior to image capture. Images of bacteria were captured on a Zeiss Axioskop microscope equipped with a Plan-Neofluar 100\(\times\)1.3 objective and with a Photometrics CoolsnapFx charge-coupled-device (CCD) camera. A field of suitable bacteria was selected by phase contrast and then imaged by fluorescence, using a fluorescein isothiocyanate (FITC) filter set.

Images of individual cells were used to calculate the ratio of fluorescence intensity at the division septum versus lateral cell wall (12). Intensity was quantified by acquiring the pixel-by-pixel values in a line perpendicular to the septum (Image-Pro Plus software), and three pixels at the septum and each wall were averaged prior to calculation of the septum/wall ratio. While the septum was often wider than three pixels, this method was selected as it best accommodated the cell wall width while capturing most of the septum, thus minimizing artifacts from differing widths of the septum and lateral wall. Based on the septum/wall ratio, cells were placed in one of three categories: septum > wall, septum = wall, or septum < wall. The entire population of cells in each field was analyzed, and at least 250 cells were evaluated for each experimental condition.

To visualize the spatial distribution of telavancin and vancomycin bound to the surface of \textit{S. aureus}, cells were exposed to...
fluorescent conjugates of each agent and imaged by fluorescence microscopy. For both TLV-FL and VAN-FL, fluorescence intensity was observed across the entire cell surface, representing the binding to D-Ala-D-Ala residues in peptidoglycan of the mature cell wall as well as peptidoglycan precursors at the division septum (Fig. 1). Notably, TLV-FL binding was localized predominantly at the division septum, the site of active cell wall biosynthesis in \textit{S. aureus}. In contrast, VAN-FL binding was distributed more evenly between the cell wall and the division septum (Fig. 1).

We used a ratio imaging approach, as described by Pereira and colleagues (12), to accurately quantify these observations. The cellular binding distribution of TLV-FL and VAN-FL was quantified by calculating the ratio of fluorescence intensity at the division septum versus the lateral cell wall (Fig. 2). This analysis revealed that 61\% of the bacterial population had TLV-FL bound predominantly at the septum, the site of active cell wall biosynthesis. In contrast, only 13\% of the VAN-FL-labeled population showed more septal staining than wall staining. The majority of VAN-FL was localized to the lateral cell wall or equally bound at septum and wall sites.

Cells were also stained with TLV-FL and VAN-FL at different concentrations. Visual observation of these cells showed that decreasing concentrations of VAN-FL resulted in similar patterns of binding, whereas a difference was noted with decreasing concentrations of TLV-FL. Specifically, TLV-FL localization at the lateral cell wall became fainter and then absent at reduced concentrations, whereas binding at the septum remained consistently visible.

\textit{S. aureus} is a coccoid bacterium that undergoes cell division by formation of a flat, circular division septum, which alternates to the perpendicular plane on the subsequent division cycle (15). Since the division septum is the only site of active cell wall biosynthesis in this organism, antibacterial agents such as glycopeptides that inhibit late-stage steps of cell wall synthesis must be able to access their specific molecular targets at the septum: the membrane-embedded cell wall precursor lipid II and nascent peptidoglycan (4, 13, 14, 17, 18). Vancomycin and telavancin bind to the terminal D-Ala-D-Ala residues of the pentapeptide, which exist in lipid II and newly synthesized peptidoglycan and within the mature cell wall across the surface of \textit{S. aureus}. A sufficient number of D-Ala-D-Ala residues remain in the mature cell wall of \textit{S. aureus} due to un-cross-linked stems and lack of carboxypeptidase activity (15). These free D-Ala-D-Ala residues act as a nonproductive binding reservoir for glycopeptides (3, 16). Fluorescence intensity observed across the cell surface demonstrates nonproductive binding in the mature cell wall, which is where the majority of vancomycin was bound. In contrast, fluorescence intensity at the septum indicates binding to the lethal targets (lipid II and nascent peptidoglycan). The majority of telavancin was bound at the septum.

Affinity capillary electrophoresis (ACE) and electrospray ionization mass spectrometry (ESI-MS) have shown that the affinity of telavancin for soluble D-Ala-D-Ala residues is weaker.
than that of vancomycin (6). Affinity for soluble D-Ala-D-Ala can be considered a surrogate measure for un-cross-linked cell wall residues. In contrast, as determined by isothermal titration calorimetry in a model membrane, the binding affinity of telavancin for lipid II is stronger than that of vancomycin. This results in telavancin exhibiting affinity for lipid II that is over 2 orders of magnitude higher than that for soluble D-Ala-D-Ala (1).

The unique affinity differential of telavancin—strong affinity for lipid II coupled with weaker cell wall affinity—translates to improved targeting of lipid II and the site of active cell wall biosynthesis. The binding distribution of telavancin across the surface of live S. aureus cells is consistent with the improved binding of telavancin to lipid II. Compared to vancomycin, telavancin showed substantially enhanced binding to the lipid II-rich division septum. The preferential binding of telavancin to the lethal target site likely contributes to the improved antibacterial potency of telavancin compared to that of vancomycin.

We thank M. Mammen and J. Jane for helpful discussions and critical review of the manuscript and Q. Xue and M. Leadbetter for chemistry assistance.

This work was supported jointly by Theravance, Inc., and Astellas Pharma Global Development, Inc.

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