Ultrastructural Effects of Oritavancin on Methicillin-Resistant *Staphylococcus aureus* and Vancomycin-Resistant *Enterococcus*[▽]

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The ultrastructural effects of the lipoglycopeptide oritavancin on methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE) were examined by transmission electron microscopy. Oritavancin but not vancomycin induced aberrant septum formation and loss of staining of nascent septal cross walls in MRSA. Septal distortions were also observed in VRE exposed to oritavancin.

Oritavancin is a semisynthetic lipoglycopeptide (1) with activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Its capacity to interact with the bacterial cell membrane, leading to loss of membrane potential and increased membrane permeability (14), confers rapid bactericidal activity (15) against exponentially growing MRSA within 15 min to 2 h (13). This distinguishes oritavancin from other glycopeptides which have been shown to only inhibit cell wall synthesis (4, 18) and which correspondingly exert bactericidal activity against susceptible strains typically only after 24 h (13).

Cryo-electron microscopy revealed in fine detail the growing septum in *S. aureus*: newly synthesized cell wall originates from the outer wall bridge and extends inwards to form the two nascent cross walls arranged in a parallel plane (12). Under lower resolution the newly synthesized cross walls compose the midline, an electron-dense staining area

within the septum (12, 20). Once the midline is fully formed, autolysins cleave the outer wall bridge, thereby releasing the daughter cells (10, 22). Antimicrobial agents such as β -lactams and glycopeptides that inhibit cell wall synthesis act mainly on newly synthesized cell wall of the division septum in replicating gram-positive cocci (16). In the current study, we explored the effects of oritavancin on the ultrastructure of *S. aureus* and VRE to gain a more complete understanding of oritavancin activity.

(Part of this work was presented at the 18th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 19 to 22 April 2008 [5].)

Oritavancin diphosphate powder (Targanta Therapeutics Corporation, Cambridge, MA) was dissolved in water containing 0.002% polysorbate 80 (7), and polysorbate 80 was maintained at 0.002% in all assays to minimize oritavancin loss to the surface of vessels during in vitro testing (2, 3). Vancomycin testing was also done in the presence of 0.002%

FIG. 1. Thin section of untreated control MRSA ATCC 4330 reveals typical features of exponential-phase *S. aureus*. A. *S. aureus* ATCC 43300 cell showing dense cytoplasm, condensed DNA, and highly apparent septal cross wall (midline [arrows]) within the nascent septum. B. The cell wall (arrow) of *S. aureus* ATCC 43300 exhibits a uniform thickness of 25 to 30 nm. Bars, 200 nm.

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FIG. 2. Oritavancin causes septal deformations, loss of the septal midline, and membrane inclusions in *S. aureus* ATCC 43300. A. Cell wall thickening (black arrow) and membrane inclusions (white arrows) are present around the periphery of this MRSA cell, which was exposed to oritavancin. B. The septum of the cell is deformed (arrow) and is lacking a distinct midline. C. The more advanced septum of this cell shows evidence of difficulty in joining so as to bisect the cell. Furthermore, no clearly defined midline is distinguishable within the septum. D. The boundaries of the half-formed septum in the oritavancin-treated cell are shown (arrows). Bars, 200 nm.

polysorbate 80, which has previously been shown not to affect assay results (2).

Exponential-phase ATCC 43300 cells were diluted to approximately 5×10^7 CFU/ml in CAMHB (cation-adjusted Mueller-Hinton broth) and exposed to 1 μ g/ml oritavancin for 10 min or to 16 μ g/ml vancomycin for 3 h (16 times their

respective broth microdilution MICs, following the guidelines of the Clinical and Laboratory Standards Institute [6, 7]). Under these conditions, oritavancin and vancomycin similarly reduced cell counts by 0.35 log and 0.26 log, respectively. Exponential-phase VRE (*Enterococcus faecalis* clinical isolate A5241515 [VanB phenotype], obtained from

FIG. 3. Exposure to vancomycin does not cause septal deformations or loss of the midline. A. The septum of this cell, which was exposed to vancomycin for 3 h, shows a distinct midline and no septal aberrations. B. A cross-cut of the septum of dividing daughter cells exposed to vancomycin for 10 min displays a uniform closing iris pattern of growth (arrows). Bars, 200 nm.

FIG. 4. Thin section of untreated control VRE clinical isolate A5241515 reveals typical features of exponential-phase *E. faecalis*. A. The dense cytoplasm, condensed DNA, distinct raised wall bands (arrows), and nascent division septum are highly apparent. Note the absence of membrane inclusions. B. The cell wall (arrow) exhibits a uniform thickness of 20 to 25 nm. Bars, 200 nm.

Joyce de Azavedo, Mount Sinai Hospital, Toronto, Canada) was likewise diluted to 5×10^7 CFU/ml and exposed to oritavancin at 0.12 $\mu\text{g/ml}$ or 1 $\mu\text{g/ml}$ (2 times and 16 times its broth microdilution MIC, respectively) for 10 min. These exposures inhibited growth compared to untreated control cells for up to 3 and 6 h, respectively. Bacteria were then

fixed and prepared for transmission electron microscopy as described previously (20).

Control cultures of MRSA grown in the presence of 0.002% polysorbate 80 exhibited typical characteristics of exponential-phase *S. aureus* (20); namely, a coccoid shape with dark cytoplasm filled with ribosomes, a highly con-

FIG. 5. Oritavancin causes membrane inclusions and deformed septa in the VRE clinical isolate A5241515. A. A large membrane inclusion (arrow) is apparent in this cell, which was exposed to oritavancin (1 $\mu\text{g/ml}$). B. The broadened septum (arrow) of this oritavancin-treated (0.12 $\mu\text{g/ml}$) cell is indicated. Note the fibrils extending from the cell wall around the right-side periphery of this cell. C. Extensive fibrils (arrows) extending from the raised wall bands of an oritavancin-treated (0.12 $\mu\text{g/ml}$) VRE cell are indicated. D. This cell ghost reveals that lysis occurred at septal sites (arrows). Bars, 500 nm (A and C) and 200 nm (B and D).

trasted septal midline (Fig. 1A), homogeneous cell wall (approximate thickness of 25 to 30 nm), and symmetrical bilayered cell membranes (Fig. 1B). All stages of septation were evident in the growing culture (data not shown), with the nascent midline (12, 20) clearly evident (Fig. 1A). Addition of 0.002% polysorbate 80 therefore did not elicit readily observable ultrastructural defects.

The majority of MRSA cells exposed to either oritavancin for 10 min or vancomycin for 3 h appeared normal at low magnification (data not shown). Cell wall thickenings and membrane inclusions were apparent in both oritavancin- and vancomycin-exposed cells (Fig. 2A shows these effects in an oritavancin-exposed cell). Cells that were affected by oritavancin exhibited deformed septa that were thickened and misshapen (Fig. 2B), and it appeared that more-advanced septa had difficulty completing (joining) the final stages of development (Fig. 2C). Similar effects on the septum have been described for the lipoglycopeptide telavancin (17) and the lipopeptide daptomycin (19). Exposure to oritavancin also caused loss of staining intensity of the septal midline (Fig. 2B and C), which has also been described following penicillin exposure (8). The high contrast of the midline results from autolysins that hydrolyze polymers of the nascent cross walls, exposing chemically reactive sites that interact with the heavy metal stain uranyl acetate (12, 20). Loss of midline staining could result from oritavancin inhibiting cell wall synthesis (4, 21) or altering autolysin activity via its ability to decrease membrane potential (14), believed to be important in the regulation of autolysis (11). Interestingly, a thin section that bisected the septum of oritavancin-exposed MRSA daughter cells revealed that only half of the septum had formed (Fig. 2D), suggesting that asymmetric initiation of septum formation that occurs in *S. aureus* (8) may represent a point of action of oritavancin. In contrast, the septal architecture in MRSA exposed to vancomycin for 3 h appeared normal with a highly visible midline (Fig. 3A), concordant with a previous report (19). Moreover, a cross-cut through the septum of a cell exposed to 16 µg/ml of vancomycin for 10 min (5) showed the typical "closing iris" septal growth pattern (12) (Fig. 3B). Further investigation of the perturbation of the coordination of septum growth by oritavancin is warranted.

Addition of 0.002% polysorbate 80 did not cause any obvious ultrastructural abnormalities in the VRE clinical isolate, as evidenced by the cell's typical "lancet" shape (9) (Fig. 4A) and uniform homogeneous cell wall (approximately 20 to 25 nm thick) (Fig. 4B). In contrast, oritavancin induced formation of large membrane inclusions (Fig. 5A) and septal distortions (Fig. 5B). Furthermore, fibrils could be seen extending from the cell wall (Fig. 5B) and clusters of these fibrils were often associated with the raised wall bands (Fig. 5C) which mark previous rounds of cross wall synthesis (9). These fibrils were not seen in the untreated control culture, indicating that they were unlikely pili and most probably derived from cell wall breakdown. Cell ghosts showed that these cells had lysed at septal sites (Fig. 5D).

In conclusion, transmission electron microscopy revealed the sensitivity of the septum in MRSA and VRE to oritavancin. That vancomycin did not cause septal defects may reflect the ability of oritavancin to disrupt membrane integrity (14) or its higher affinity for cell wall targets as a func-

tion of its capacity to dimerize and form cooperative interactions (1). Further investigation into the relationship between membrane depolarization by oritavancin and septation is warranted.

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