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Oritavancin Disrupts Transmembrane Potential and Membrane Integrity Concomitantly with Cell Killing in *Staphylococcus aureus* and Vancomycin-Resistant Enterococci

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Abstract

Background: Oritavancin (ORI), a semi-synthetic glycopeptide derived from chloroeremomycin (CEM), possesses potent bactericidal activity against Gram-positive cocci including vancomycin (VAN)-resistant *Staphylococcus aureus* and enterococci. Its mechanism of action is thought to involve inhibition of cell wall synthesis and interaction with cell membranes.

Methods: The mode of action of ORI was investigated in *S. aureus* and *Enterococcus* using fluorescent dyes that probe the membrane potential (ΔΨ) and the permeability of the cytoplasmic membrane. Mid log phase cells (10^6 CFU/ml) were exposed to ORI, CEM, or VAN at concentrations of 0.5 to 16x MIC in HEPES-glucose and fluorescence changes were followed using a TECAN Ultra microplate reader. Time-kill studies were performed by sampling the fluorescence assay from 1 min to 2 h post-challenge and plating bacteria on cation-adjusted Mueller-Hinton agar.

Results: ORI demonstrated time- and concentration-dependent membrane activity against *S. aureus* and enterococci. Both ΔΨ and permeability were affected by ORI at concentrations approximating its MIC (0.5 μg/ml) for *S. aureus*. Moreover, the effects of ORI on both ΔΨ and permeability were temporally correlated with changes in bacterial cell viability: challenge with 1x MIC ORI completely depolarized *S. aureus* cell membranes and decreased cell counts by 3.5 ± 0.2 log within 10 min. CEM, which lacks the chlorobiphenyl group of ORI, had no effect upon ΔΨ, membrane permeability, or cell viability at up to 16x MIC over a 2 h challenge.

Conclusions: ORI had a profound effect on the membranes of *S. aureus* and enterococci: ΔΨ and membrane permeability were rapidly and dramatically affected. These effects were temporally correlated with a loss of cell viability and required the chlorobiphenyl group of ORI. This new proposed mechanism of ORI action helps to explain its rapid, potent bactericidal activity.

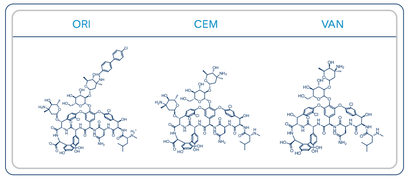

Figure 1.
Chemical structure of Oritavancin (ORI), Chloroeremomycin (CEM), and Vancomycin (VAN)

Introduction

Oritavancin (ORI) is a semi-synthetic glycopeptide antibiotic whose precursor chloroeremomycin is produced by *Amycolatopsis orientalis*. ORI demonstrates potent activity against a range of Gram-positive bacteria including vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant *Enterococcus* (VRE), and penicillin-resistant *Streptococcus pneumoniae* (PRSP). Typical glycopeptides including vancomycin demonstrate concentration independent killing of *Staphylococcus* and vancomycin-sensitive *Enterococcus* and are known to inhibit cell wall synthesis through blocking the enzymes necessary for transglycosylation and/or transpeptidation. ORI activity is maintained against vancomycin-intermediate and –resistant strains; furthermore, ORI possesses concentration dependent activity against both *Staphylococcus* and *Enterococcus*. This suggests that ORI likely possesses additional activities responsible for its rapid cidal activity. Here we investigate a possible supplementary mechanism by which ORI is able to rapidly kill bacteria, in addition to its previously identified cell wall inhibitory activity.

High level resistance to vancomycin and related "first generation" glycopeptides is now clinically prevalent and is typically achieved through alteration of the D-alaD-ala dipeptide to D-alaD-lac by the expression of either the VanA or VanB locus. In so doing, one of the hydrogen bonds can no longer form between the glycopeptide and its intended D-alaD-ala target and the effectiveness of the antibiotic is greatly reduced. While this provides high-level resistance to vancomycin, new lipoglycopeptides such as ORI continue to be effective against these organisms suggesting their binding to the dipeptides is maintained or alternatively their antimicrobial activity is not entirely dependent upon these hydrogen bonds. We sought to investigate the presence of an alternative mechanism of action for ORI responsible for the rapid killing of both vancomycin-sensitive and –resistant bacteria.

Methods

Membrane Potential(ΔΨ): ΔΨ was followed in bacteria using the fluorescent probe, 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$). Previous studies with the glycopeptide Telavancin (1) have successfully used this probe, which partitions into the plasma membrane in proportion to the ΔΨ, in order to demonstrate the membrane activities for this drug. Dissipation of the potential releases the probe leading to an increase in its fluorescence. Strains of *S. aureus* (ATCC 29213, ATCC 43300) and *Enterococcus* species (ATCC 29212, ATCC 51299, ATCC 51559) were chosen for testing in membrane studies. Strains were grown in subculture in CAMHB or BHI broth (from overnight cultures) to OD_{600}~0.3 whereupon the cells were washed twice in membrane buffer (10 mM HEPES-Cl pH 7.5, 5 mM Glucose, 50 μg/ml $CaCl_2$) and resuspended at OD_{600nm}0.25. $DiSC_3(5)$ was added to a final concentration of 1.5 μM and the solution was incubated in the dark at ambient temperature for 30 minutes to allow loading into cell membranes. After the loading period the cells were diluted 50-fold in membrane buffer. Membrane potential assays were initiated by the addition of antibiotics over a range of concentrations (0.5x MIC to 16x MIC) and were monitored in real time by fluorescence spectroscopy (λ_{ex}=612 nm, λ_{em}=665 nm) for a period of 20-30 minutes. Assays were repeated at least twice with similar results; results from one assay are presented.

Membrane Permeability: Membrane permeability was assayed using SYTO-9 (λ_{ex}=485 nm, λ_{em}=535 nm) and propidium iodide. SYTO-9 is a membrane permeable dye that during a loading phase enters the cells and fluoresces. As the membrane is damaged, the otherwise membrane impermeable dye propidium iodide enters the cell and displaces SYTO-9 leading to a loss in its fluorescence. Bacterial strains were prepared identically as described above with the exception that SYTO-9 was loaded at 5 μM. Assays were repeated at least twice with similar results; results from one assay are presented.

Cell Viability: Cell viability assays were performed on mid-log phase cells and monitored the loss of cell viability over a period of 1-2 h. Cells were grown overnight in CAMHB or BHI for *S. aureus* or *Enterococcus* strains, respectively, then subcultured at 1:100 in fresh media and grown to OD_{600}~0.3. Cells were washed twice in membrane buffer and resuspended in the same at OD_{600nm}0.25. Cells were incubated for 30 min (to mirror treatment of cells for membrane assay) and diluted 50-fold to a final OD_{600} of 0.005. Assays were initiated by the addition of antibiotics (0.5x MIC to 16x MIC). Aliquots were removed at time points (0, 1, 5, 10, 15, 30, 60 min), serially diluted in sterile saline and 5 μl was plated on CAMH agar or BHI agar where appropriate. Assays were repeated at least twice with similar results; results from one assay are presented.

Table 1.
Broth Microdilution MICs for Glycopeptide Antibiotics Against *S.aureus* and *Enterococcus* spp. (CLSI M7-A7 Guidelines Were Followed)

Strain	Resistance Status	Broth Microdilution MIC (μg/ml)		
		ORI	VAN	CEM
S.aureus ATCC 29213	MSSA	0.5	1	1
S.aureus ATCC 43300	MRSA	0.5	1	0.25
E.faecalis ATCC 29212	VSE	0.031	2	0.25
E.faecalis ATCC 51299	VanB VRE	0.25	256	4
E.faecium ATCC 51559	VanA VRE	1	>1024	N.D.

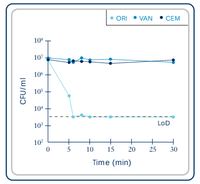

Figure 2a.
Oritavancin Exerts Rapid Cidal Activity Against MSSA (ORI at 1x MIC, VAN, and CEM at 8x MIC)

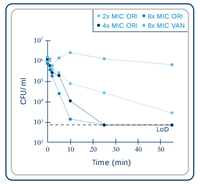

Figure 2b.
Oritavancin Exerts Rapid Cidal Activity Against VRE (ORI at 2x MIC, 4x MIC, 8x MIC, VAN at 8x MIC)

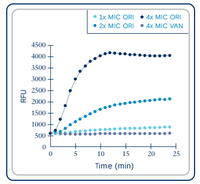

Figure 3a.
Oritavancin Rapidly Dissipates ΔΨ in MSSA ($DiSC_3$ (5) Assay)

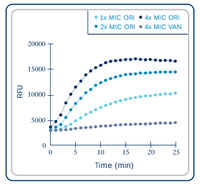

Figure 3b.
Oritavancin Rapidly Dissipates ΔΨ in VRE (VanA) ($DiSC_3$ (5) Assay)

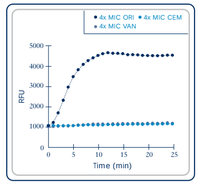

Figure 4.
Chloroeremomycin Lacks ΔΨ-Dissipating Activity Against MSSA ($DiSC_3$ (5) Assay)

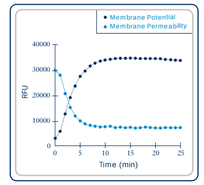

Figure 5.
Oritavancin Dissipation of ΔΨ and Permeabilization of MSSA Membranes are Temporally Correlated ($DiSC_3$ (5) assay (ΔΨ); SYTO-9 assay (Membrane Permeability))

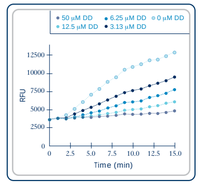

Figure 6.
Diacetyl-LysD-alaD-ala Peptide Inhibits Oritavancin Membrane Activity in MSSA (ORI at 1x MIC)

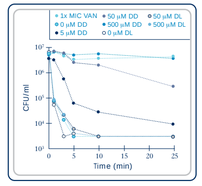

Figure 7.
Diacetyl-LysD-alaD-ala Peptide Inhibits Oritavancin Killing Activity of MSSA (ORI at 1x MIC)

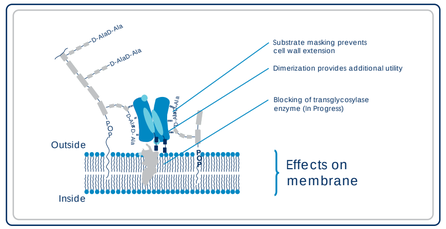

Figure 8.
Proposed Mechanisms of ORI Activity

Results

- At 1x MIC ORI causes a 3.5±0.2 log loss of cell viability for MSSA within 10 minutes (Figure 2a). Concentrations of 8x MIC for either VAN or CEM under similar conditions show no significant impact upon cell viability over the same period of time. At 8x MIC ORI causes a 3.0±0.5 log loss of cell viability for VRE (VanA) by 10 minutes (Figure 2b). Concentrations of 8x MIC for both VAN and CEM under identical conditions show no significant impact upon cell viability for greater than 3 h (data not shown).
- Depolarization of MSSA (Figure 3a) and VRE (Figure 3b) cytoplasmic membranes occurs upon exposure to 1x MIC concentrations of ORI. Under identical conditions neither VAN nor CEM (Figure 4), even when added in vast excess of their respective MICs, affect ΔΨ of either *S. aureus* or *Enterococcus*.
- At 1x MIC ORI disrupts cytoplasmic membrane integrity in MSSA and this is found to correlate temporally with the loss of membrane potential (Figure 5) and loss of cell viability. Identical effects are observed for *Enterococcus* spp. where upon exposure to low levels of ORI there is a rapid loss of cell membrane integrity (data not shown) and cell death. Neither VAN nor CEM at 16x MIC lead to loss of membrane integrity over the same time period. Rapid depolarization and increased permeability of cytoplasmic membranes and thus cell death is dependent upon the presence of the chlorobiphenyl substitution of ORI: CEM, the parent glycopeptide which lacks this chlorobiphenyl substitution has neither the membrane activity (Figure 4) nor the rapid cidal activity observed for ORI (Figure 2a).
- The loss of ΔΨ (Figure 6) and membrane integrity as well as cell viability (Figure 7) can be inhibited by the tripeptide diacetyl-Lys-D-alaD-ala but not D-alaD-lac residues and requires the des-N-methyl-leucyl amino acid of oritavancin (data not shown).

Conclusion

Here we demonstrate that ORI has a profound effect on membranes of *S. aureus* (MSSA and MRSA) and enterococci (VSE and VRE) where membrane potential and integrity are rapidly compromised leading to cell death. The current study suggests that ORI's chlorobiphenyl side chain, which is not present in the parent glycopeptide chloroeremomycin, is necessary for its rapid cidal activity.

It is unknown whether the membrane activity of ORI is the result of a direct perturbation of the bilayer by the hydrophobic chlorobiphenyl group or an indirect effect due to interaction with other cell surface targets. Others have demonstrated that a compound very similar to ORI (CEM containing a biphenyl sidechain) interacts directly with model membranes in the absence of D-alaD-ala substituents suggesting that the anchoring of the biphenyl group with the membrane is direct and presumably responsible for the loss of membrane function in susceptible bacteria (2,3). It has previously been demonstrated that glycopeptide antibiotics, including ORI interact with D-alaD-ala dipeptide on the growing peptidoglycan chains while replacement of the terminal D-ala with a D-lac residue renders the bacteria resistant to vancomycin antibiotics. We demonstrate here that the presence of D-alaD-ala residues (and not D-alaD-lac) in solution antagonizes the membrane activity of ORI and this correlates with the loss of cell killing activity. This suggests that ORI may be partially directed to the cell surface through an interaction with the D-alaD-ala portion of the Lipid II structure. In doing so this could position the hydrophobic tail of ORI close to the cell surface allowing disruption of the cytoplasmic membrane (Figure 8). While this interaction may assist in helping to direct ORI to its appropriate target on the cell surface its requirement is not absolute as evidenced by the potent activity of the antibiotic towards D-alaD-lac-containing strains and the inability of D-alaD-lac to inhibit ORI activity when added in solution.

We have demonstrated that the lipoglycopeptide Oritavancin in addition to its cell wall synthesis inhibitory activity possesses a rapid potent membrane disrupting function owing to its hydrophobic chlorobiphenyl substitution. This supplementary activity of Oritavancin makes this antibiotic a potent alternative to earlier glycopeptides and one might speculate that Oritavancin may be more refractory to the development of resistance due to its multiple modes of action.

References

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2. Sharman *et al*, The Roles of dimerization and membrane anchoring in activity of glycopeptide antibiotics against vancomycin-resistant bacteria. *J.Am.Chem.Soc*, 1997. 119: 12041-12047.
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