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Oritavancin Kills S. aureus by Increasing Membrane Permeability without Causing Extensive Cell Lysis: Comparative Studies with Vancomycin, Daptomycin, and Lysostaphin

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Abstract (revised)

Background. The lipoglycopeptide oritavancin (ORI; differing from VAN by addition of *p*-chlorophenylbenzyl and epivancosamine moieties), shows marked conc.-dependent bactericidal effects against stationary-phase *S. aureus* associated with alterations of membrane integrity (Belley et al, AAC 2009; 53:918). To further analyze the effects of ORI on *S. aureus* membranes and relate them to bacterial killing of non-growing bacteria, we compared ORI to VAN, DAP, and LYS for membrane permeabilization, cell lysis, and reduction of CFU using high density inocula.

Methods. 3x10^8 bact./mL (*S. aureus* ATCC25923) were incubated at 37°C (in 0.15 M NaCl buffered at pH 7.4) with ORI, VAN, DAP or LYS for 15 min at increasing multiples of their MIC (determ. accord. to CLSI guidelines), and examined for (i) membrane permeabilization to low molec. weight tracer (calcein [MW: 622]; Cotroneo et al, AAC 2008; 52:2223), gross cell lysis (turbidimetry), and change in CFU.

Results.

MICs against *S. aureus* ATCC25923, and activity of each agent at 64 x MIC measured at 15 min

agent	MIC (µg/mL) [a]	calcein remaining entrapped [b]	OD_{620nm} [c]	Δ log CFU [d]
ORI	0.06	36 ± 6 *	105 ± 1	-2.16 ± 0.00 *
VAN	1	69 ± 6	102 ± 1	+0.16 ± 0.01
DAP	0.25	77 ± 11	99 ± 2	-1.26 ± 0.03 *
LYS	0.06	0 [e,*]	3 ± 0 *	> -4.00 *

[a] determined in broth at 5x10^5 CFU/mL (with polysorbate-80 for ORI);
[b] intracell./extracell. calcein fluoresc. ratio (% of control value);
[c] % of initial value; [d] change from initial value;
[e] max. achievable (100 % release, already reached at 8 X MIC)
* significantly different from the control (p<0.05)

ORI caused conc.-dependent calcein release and killing, but no gross lysis; VAN was ineffective for all criteria; DAP caused conc.-dependent killing (consistent with its membrane depolarization mode of action) but neither calcein release nor gross lysis; LYS caused massive calcein release, gross lysis, and conc.-dependent killing.

Conclusions. ORI bactericidal activity on non-growing bacteria may be mediated by increase in membrane permeability to low molecular weight solutes but, in contrast to LYS, is not accompanied by extensive bacterial lysis; this will potentially avoid release of pyrogenic or pro-inflammatory cell wall constituents.

References

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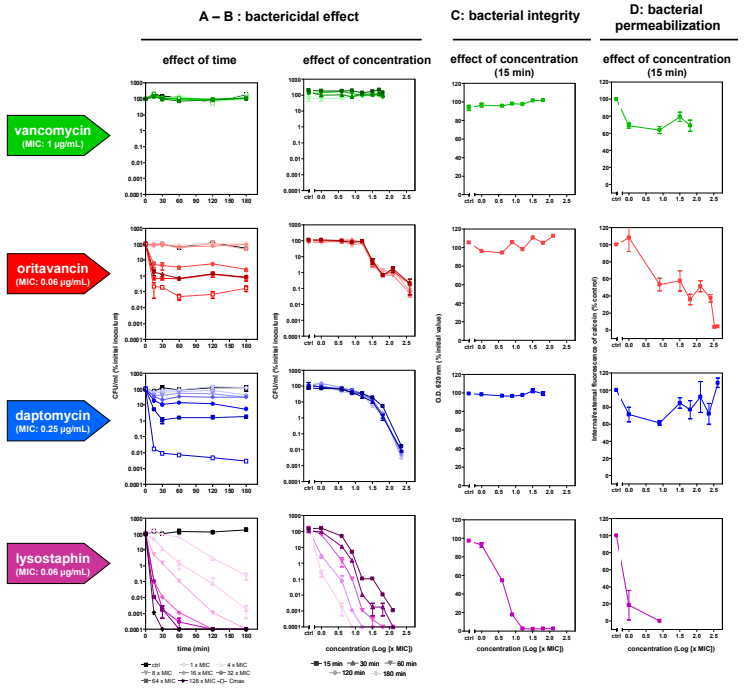
Introduction

- The lipoglycopeptide **oritavancin** is a semi-synthetic lipoglycopeptide, which differs from **vancomycin** by a marked concentration-dependent bactericidal effects against both growing and stationary-phase *S. aureus* (1,2). This may be related to its multiple modes of action (3). In particular, we have recently shown that oritavancin is capable of increasing membrane permeability in a model of liposomes (4).
- The lipopeptide **daptomycin** also exerts bactericidal effects against growing and non-growing bacteria (5), which most likely results from its ability to cause membrane depolarization (6).
- To further analyze the mechanism of bacterial killing exerted by oritavancin on non-growing bacteria, we compared the capacity of oritavancin, vancomycin, and daptomycin to induce membrane permeabilization and/or cell lysis, in high density inocula cultures, and correlated these effects to subsequent reduction in bacterial counts.
- **Lysostaphin** [EC 3.4.24.75], an endopeptidase that hydrolyses the -Gly-|-Gly- bonds in the pentaglycine inter-peptide link within staphylococcal peptidoglycan and causes thereby cell wall disruption and bacterial lysis (7), was used as a positive control.

Methods

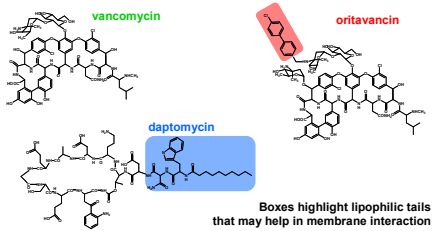
- **Bacterial strain**: the fully susceptible reference strain (ATCC25923; MSSA) was used throughout. All experiments were performed with an initial inoculum of 3x10^8 CFU/ml in PBS (Phosphate Buffered Saline; pH 7.4), supplemented by 0.002 % polysorbate-80 for oritavancin, 50 mg/L $CaCl_2$ for daptomycin, or 0.1 % BSA for lysostaphin.
- **Bactericidal effect** : bacteria were incubated at 37°C with one of the investigated drugs, aliquots were plated on TSA, and CFU were counted after overnight incubation at 37°C.
- **Bacterial integrity** : the absence of gross alteration in cell integrity was checked by following the Optical Density (620 nm) of the bacterial suspension during the experiments aimed at evaluating bactericidal effects.
- **Calcein release** : this technique was used to evaluate membrane permeabilization to a low molecular weight tracer (622 Da). Calcein can be loaded inside bacteria under an esterified form, which is cleaved by cytoplasmic esterases to regenerate the fluorescent, non diffusible probe (8).
  Bacteria were loaded with the acetoxymethyl ester of calcein (2 µM; 60 min), the non-internalized tracer was eliminated by centrifugation. Bacteria were incubated at 37°C with one of the investigated drugs; fluorescence was measured (λexc 472 nm ; λem 512 nm) for the whole sample (total fluorescence) and for the supernatant after eliminating bacteria by filtration. Data are expressed as the ratio between fluorescence associated to bacteria (total-supernatant) and supernatant fluorescence (8).

Results



A–**B**: change in bacterial counts (cfu); **A**, effect of time for increasing drug concentrations; **B**, effect of concentration at a fixed time (15 min);
C: change in optical density (620 nm) after 15 min exposure to increasing drug concentrations;
D: internal / external fluorescence ratio (see ref. 8) after 15 min exposure to increasing drug concentrations.
All data are expressed as % of the corresponding control (original inoculum [3 x 10^8 cfu/mL]; drug concentrations are multiples of their MICs.

Structure of antibiotics used



Boxes highlight lipophilic tails that may help in membrane interaction

Results

- **oritavancin:**
  - time- and concentration-dependent bactericidal effect
  - no gross bacterial lysis
  - release of calcein at high concentrations
- **vancomycin:**
  - no effect on bacterial viability
  - no bacterial lysis
  - no release of calcein
- **daptomycin:**
  - time- and concentration-dependent bactericidal effect
  - no gross bacterial lysis
  - no release of calcein (even at high concentrations)
- **lysostaphin:**
  - time- and concentration-dependent bactericidal effect
  - gross bacterial lysis (concentration-dependent)
  - massive release of calcein (concentration-dependent)

Conclusions

In sharp contrast to vancomycin, oritavancin exerts a time- and concentration-dependent killing effect on non-growing bacteria, as also observed with daptomycin. Significant release of medium-sized solutes (calcein) from bacteria is obtained at concentrations of 10 x the MIC or higher. While this concentration is larger than what is needed to permeabilize liposomes (4), it compares well with ATP-release data obtained with *S. aureus* exposed to telavancin (9), another bactericidal lipoglycopeptide with membrane destabilization effects (10).

The data therefore suggests that the bactericidal effect of oritavancin may involve alteration of membrane permeability. The lack of cell lysis could be an advantage by preventing the release of pyrogenic or pro-inflammatory cell wall constituents.

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