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Phagocytic activity and production of reactive oxygen species in J774 mouse macrophages exposed to increasing concentrations of oritavancin: comparison with vancomycin and azithromycin

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Abstract

Objectives. Oritavancin (ORI) is a lipoglycopeptide that accumulates to high levels in macrophages (up to 300-fold) and localizes in lysosomes. Although ORI shows extensive activity against intraphagocytic forms of *S. aureus* (Barcia-Macay et al, AAC, 2006), concerns have been raised about the possibility for the drug to also impair critical macrophage functions. Our aim was to assess the effect of ORI *in vitro* on phagocytosis of infectious organisms and production of reactive oxygen species (ROS) in macrophages at clinically relevant concentrations. Azithromycin (AZM, accumulating to high levels in lysosomes) and vancomycin (VAN, low cellular accumulation) were used as comparators.

Methods. J774 mouse macrophages were incubated for 3 h with increasing concentrations of ORI (0-50 mg/L; *Cmax* in patients: 25 mg/L) or VAN or AZM (0-100 mg/L) and then used to measure (i) the phagocytosis of serum-opsonized *P. aeruginosa* PA01 (CFU counting [MIC of ORI > 32 mg/L) and fluorescent red latex beads [2 µm diameter] (fluorimetry; subcellular localization assessed by confocal microscopy in comparison with the lysosomal tracer lysotracker green), and (ii) production of ROS by monitoring the cell permeant indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester in the presence of 0.5 % H_2O_2.

Results. As shown in the table, exposure of J774 macrophages to ORI (i) did not significantly inhibit their ability to engulf *P. aeruginosa* over the range of concentrations tested, or phagocytize latex beads (for conc. < 25 mg/L; these localized in lysosomes as in control cells [no change in latex beads engulfment seen for cells incubated with VAN or AZM up to 100 mg/L]); (ii) did not impair the production of ROS (for conc. < 40 mg/L).

Parameter studied	Values at increasing oritavancin extracellular concentrations					
	0 mg/L	1 mg/L	10 mg/L	25 mg/L	40 mg/L	50 mg/L
P. aeruginosa phagocytosis[a]	100 ± 0.1	99.9 ± 0.1	99.9 ± 0.1	99.9 ± 0.1	nd	99.9 ± 0.1
Latex beads phagocytosis[b]	100 ± 7.6	125.3 ± 8.4	106.5 ± 7.1	40.6 ± 17.9	nd	45.3 ± 19.4
ROS production[c]	117 ± 5.7	120.7 ± 8.5	118.2 ± 4.1	118.1 ± 1.5[d]	146.8 ± 1.8	nd

[a] percentage of control (10e6 CFU/mg prot.)
[b] percentage of control (10e6 beads/mg cell prot.)
[c] fluorescence signal at 25 min (in % of value measured at time 0 min)
[d] tested at 20 mg/L
nd, not determined

Conclusions. At extracellular conc. up to its human *Cmax*, ORI did not affect two critical functions of macrophages related to the handling and killing of infectious organism in vitro. Differences in conc. needed to affect phagocytosis of latex beads and *P. aeruginosa* cells may be related to opsonization of bacteria (receptor-mediated phagocytosis). Together with our previous observation that ORI at Cmax is bactericidal against *S. aureus* phagocytized by macrophages, the data presented here suggest that no untoward effect is to be expected from extensive accumulation of ORI in macrophages in a context of intracellular infection and handling of particulate matters, as long as its concentration in serum remains in this range or lower.

Introduction

Oritavancin is a lipoglycopeptide antibiotic accumulating to high levels in the lysosomes of eukaryotic cells (macrophages and fibroblasts).[1] It shows extensive activity towards intrapha-gocytic *forms of S. aureus* (including Methicillin- and Vancomycin-Resistant isolates),[2-3] yielding true bactericidal activity (≥ 3 $\log_{10}$ CFU decrease) towards intracellular forms against which vancomycin is only poorly effective.

However, macrophages exposed to oritavancin show conspicuous morphological alterations of the lysosomes and related vacuoles suggestive of a mixed-lipid storage disorder,[4] which could impair the host defenses mechanisms. The aim of our study was to evaluate the effect of oritavancin on phagocytosis and production of reactive oxygen species (ROS) by macrophages. Azithromycin (AZM, high cell. accumulation) and Vancomycin (VAN, low cell. accumulation) were used as comparators.

Methods

Cell line. J774 murine macrophages were used throughout and maintained in our laboratory as previously described.[5-6] These cells were incubated for 3h in RPMI 1640 medium (+ 10 % FCS) containing increasing concentrations of antibiotic (oritavancin, up to 50 mg/L; vancomycin and azithromycin, up to 100 mg/L). For experiments with oritavancin, the culture medium was supplemented with 0.002 % Tween-80 (to avoid absorption of the drug on plastic surfaces).

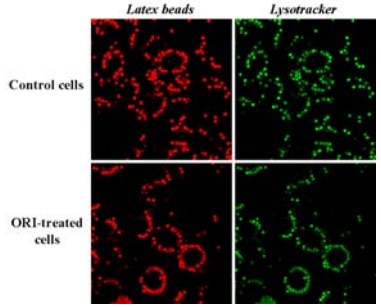
Phagocytosis of *P. aeruginosa* strain PA01. Phagocytosis of *P. aeruginosa* (oritavancin MIC > 32 mg/L) was determined using a previously described protocol.[6] Briefly, cells were washed free from antibiotic using phosphate buffered saline (PBS) and incubated for 1 h in the presence of the serum-opsonized bacteria (using a bacteria-per-macrophage ratio of 50) to allow phagocytosis. Elimination of adherent but non-phagocytized bacteria was achieved by incubation with 50 mg/L gentamicin for 45 min. Gentamicin was then eliminated by 3 successive washings with PBS. Cells were finally lysed, harvested by scraping, and enumeration of viable bacteria was performed after appropriate dilution by spreading on TSA plates and overnight incubation at 37°C.

Uptake of red fluorescent latex beads. Uptake of carboxylate-modified red fluorescent latex beads (2 µm in diameter; 270 particles/nL) was assessed by fluorimetry or confocal microscopy as previously described.[5] Labeling of acidic organelles was achieved by incubation with 50 nM Lysotracker green.

Production of intracellular Reactive Oxygen species (ROS). ROS were detected using the cell-permeant dye 5-(and-6)-chloromethyl-2',7'-dichlorohydrofluorescein diacetate acetyl ester (CM-H2DCFDA, Invitrogen). Briefly, cells were washed free from antibiotic, loaded with the fluorescent probe (5 µM, 37°C, 5 % CO_2) for 30 min, and then reincubated for 30 min in medium (to allow desterification of the probe). Intracellular ROS production was induced by addition of 0.5 % H_2O_2 and fluorescence intensities were measured using emission and extinction wavelength set at 485 and 530 nm, respectively (Packard fluorocount).

Results–1

Figure 1. Live cell imaging of control and oritavancin-treated J774 cells.

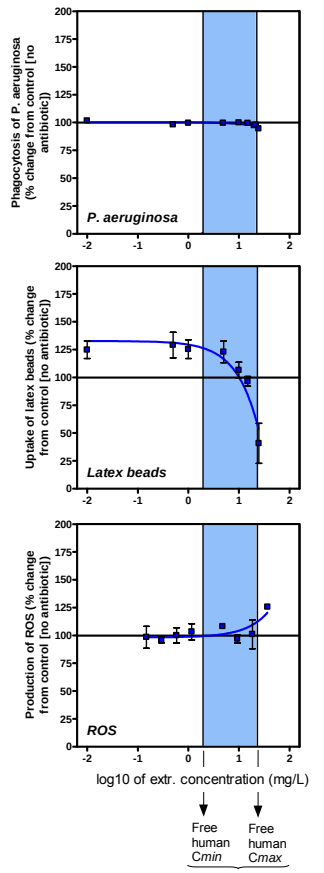


Control cells or cells exposed to 25 mg/L oritavancin for 3h (ORI-treated) were (i) washed with PBS; (ii) incubated with red-fluorescent latex beads for 1h, and (iii) incubated with Lysotracker green for 1 h.

Oritavancin-treated cells showed a clear co-localization of both tracers

Results–2

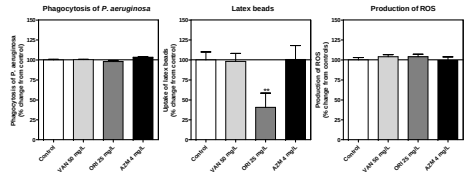
Figure 2. Comparative effect of oritavancin on phagocytosis and production of Reactive Oxygen Species (ROS)



Exposure of J774 cells for 3 h to oritavancin (i) did not affect phagocytosis of *P. aeruginosa*; (ii) did decrease phagocytosis of latex beads and increased ROS production but only at concentration equivalent (for latex beads) or above (for ROS production) the free human C_{max} ($p < 0.01$ at 40 mg/L).

Results–3

Figure 3. Influence of antibiotic on phagocytosis and production of ROS



Comparative effect of antibiotics on phagocytosis (*P. aeruginosa* strain PA01, red-fluorescent latex beads) and production of ROS in J774 macrophages exposed for 3 h to clinically-relevant conc. of antibiotics (Cmax; 50 mg/L [VAN], 25 mg/L [ORI, free Cmax for an IV administration of 1200 mg[7] and 4 mg/L [AZM]). Statistical analyses (Tukey test for multiple comparison, data with asterisks are significantly different from controls (P< 0.01)

Exposure of cells to ORI, VAN or AZM (using clinically-meaningful concentrations) has no effect on the capability of J774 cells to produce ROS or phagocytize *P. aeruginosa*, but ORI decreases the uptake of latex beads

Conclusions

- At clinically-meaningful concentrations, oritavancin does not affect two critical functions of macrophage - phagocytosis (as demonstrated with *P. aeruginosa* strain PA01), and the generation of Reactive Oxygen Species (ROS).
- Decreased uptake of latex beads was observed for higher concentrations of oritavancin, but without impairing the accessibility of latex beads to lysosomes.
- Differences in the concentration needed to affect phagocytosis of latex beads and *P. aeruginosa* may be related to bacterial opzonization (receptor-mediated phagocytosis).

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