Study of macrophage functions in murine J774 cells and human activated THP-1 cells exposed to oritavancin, a lipoglycopeptide with high cellular accumulation.

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Running title: Oritavancin and macrophage functions

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ABSTRACT

Oritavancin, a lipoglycopeptide antibiotic in development, accumulates to high levels in the lysosomes of eukaryotic cells. We have examined specific functions of macrophages (phagocytic capacity, lysosomal integrity, metabolic activity, production of reactive oxygen species [ROS]) in correlation with the cellular accumulation of the drug, using J774 mouse macrophages and THP-1 human monocytes differentiated into macrophages using Phorbol 12-Myristate 13-Acetate. Oritavancin did not affect *P. aeruginosa* phagocytosis, lysosomal integrity, or metabolic activity in cells incubated for 3 h with extracellular concentrations ranging from 5 to 50 µg/mL. At extracellular concentrations ≥ 25 µg/mL, oritavancin reduced latex beads phagocytosis by approximately 50% and doubled ROS production in J774 macrophages only. This may result from the fact that the cellular accumulation of oritavancin was 15 times higher in J774 cells than in activated THP-1 cells at 3h. Human pharmacokinetic studies estimate that the concentration of oritavancin in alveolar macrophages could reach approximately 560 µg/mL after administration of a cumulative dose of 4 g, which is below the cellular concentration needed in the present study to impair latex beads phagocytosis (1180 µg/mL) or to stimulate ROS production (15000 µg/mL) by J774 cells. The data therefore suggests that, in spite of its substantial cellular accumulation, oritavancin is unlikely to markedly affect macrophage functions in the conditions of use investigated in current phase III trials (single dose of 1200 mg).
INTRODUCTION

Phagocytes are part of the innate response to infection. Among their diverse functions, they engulf bacteria and kill them by producing a cocktail of microbicidal agents that include reactive oxygen species (ROS), nitric oxide, and hydrolytic enzymes (1).

Several antibiotic classes accumulate within phagocytes (see [2] for review). Studying their potential impact on phagocyte functions appears thus of prime importance to detect potential interferences with host cell defense. Previous studies have demonstrated that the macrolide azithromycin inhibits fluid-phase endocytosis (3), blocks autophagy (by preventing lysosomal acidification [4]), and modulates cytokine production (5). These effects occur at clinically-relevant concentrations and are claimed to explain increased susceptibility to mycobacterial infections and immunomodulatory properties of macrolides in cystic fibrosis patients receiving chronic treatment with this drug (4,5). Conversely, the fluoroquinolone moxifloxacin does not interfere with neutrophil functions (6) but stimulates the oxidative burst in monocytes (7) and modulates immune response (8), which may contribute to the beneficial effects of fluoroquinolones on infection control in the clinics (8).

Oritavancin, an investigational lipoglycopeptide antibiotic (see [9] for review), has completed Phase III clinical investigations for the treatment of acute bacterial skin and skin structure infection with a single dose of 1200 mg IV (www.clinicaltrials.gov; NCT01252719 and NCT01252732; [10]). In healthy volunteers having received a cumulative dose of 4 g (5 doses of 800 mg), the drug reaches concentrations in alveolar macrophages that are as high as 50-fold the serum level after 1 week (11). In vitro studies have also demonstrated that oritavancin accumulates to high levels in cultured cells and concentrates in their
lysosomes (12), where it exerts potent antibacterial activity against the intracellular forms of
Staphylococcus aureus [13,14]. Yet, this high accumulation is accompanied by
conspicuous morphological alterations of lysosomes and related vacuoles suggestive of a
mixed-lipid storage disorder, as demonstrated for murine J774 macrophages incubated
with extracellular concentrations ≥ 20-25 µg/mL (15). A recent study shows however that
oritavancin does not affect the capacity of macrophages to kill pathogens that are out of its
spectrum of activity, like Candida albicans or Acinetobacter baumannii (16).

The aim of the present study is to examine the effect of oritavancin on specific
macrophage functions (including endocytic and phagocytic capabilities, maintenance of
lysosomal pH gradient, oxido-reductive activity, or ROS production), using in parallel
murine J774 macrophages and activated THP-1 cells, which both display macrophage-like
activities (17-19). Vancomycin (reference glycopeptide with low cellular accumulation [12])
and azithromycin (lysosomotropic antibiotic with high cellular accumulation causing a
lysosomal storage disorder similar to that observed with oritavancin [20,21]) were used as
comparators.

The data show that oritavancin reduces phagocytosis of latex beads but not of
bacteria and increases ROS production. These effects were only observed in the mouse
macrophage cell line and at cellular concentrations that likely exceed those predicted to be
reached in treated patients.
MATERIALS AND METHODS

Antibiotics and main reagents

Oritavancin diphosphate (fully hydrated diphosphate salt; potency, 83.1%) and \[^{14}\text{C}\]labeled oritavancin (specific activity, 53 mCi/mmol; diluted 50-fold with unlabeled oritavancin to obtain a stock solution of 2 mg/mL) were obtained from The Medicines Company (Parsippany, NJ). In experiments investigating concentration-effect relationships, the extracellular concentration was limited to 50 µg/mL to avoid solubility issues. Vancomycin and gentamicin were obtained as the branded products complying with the provisions of the European Pharmacopoiea and commercialized in Belgium for human use as Vancocin® and Geomycine® (distributed in Belgium by GlaxoSmithKline s.a./n.v., Genval, during the period of the study). Azithromycin (dehydrated free base [microbiological standard]; potency, 94%) was supplied by Pfizer s.a. (Brussels, Belgium).

Cell-culture media and sera were from Invitrogen Corp. (Carlsbad, CA) and other reagents from Sigma-Aldrich Inc. (St-Louis, MO) or Merck KGaA (Darmstadt, Germany). For experiments with oritavancin, cell culture media were supplemented with 0.002 % Tween-80 to avoid adsorption of the drug on plastic surfaces (22) with the corresponding control media supplemented with the same concentration of Tween-80.

Bacteria and cell lines

Pseudomonas aeruginosa strain PAO1 (ATCC BAA-47) was used as a bacterium insensitive to oritavancin for studies evaluating cellular phagocytic capacities. Bacteria were grown in Mueller-Hinton broth and CFU counting was performed by plating on Tryptic
Soy Agar. Murine J774 (derived from a reticulosarcoma) and human THP-1 cells (ATCC TIB-202, a myelomonocytic cell line displaying macrophage-like activity) were both obtained from the American Tissue Culture Collection (Manassas, VA) and maintained in our laboratory as described previously (3,23) using RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS). THP-1 monocytes were differentiated into adherent macrophages (referred to hereunder as A-THP-1 cells) by incubation with Phorbol 12-Myristate 13-Acetate (PMA; 200 µg/L, Sigma-Aldrich) for 24 hours at 37°C. This treatment results in an increase in their phagocytic activity, production of superoxide, as well as in the expression of HLA-DR and interleukin 1β (17,18,24).

**Determination of the cellular content in oritavancin**

The cellular accumulation of oritavancin was determined in J774 and A-THP-1 macrophages following the procedures previously established (12,16). Briefly, cells incubated with [14C]-labeled oritavancin were washed 3-4 times in ice-cold PBS, collected by scraping in distilled water, and used for measuring cell-associated radioactivity (liquid scintillation counting) and assaying protein (25). The apparent cellular-to-extracellular concentration ratio was calculated by using a conversion factor of 5 µL cell volume per mg of cell protein, as in our previous studies dealing with cellular pharmacokinetic studies of drugs in these cell types (12,26,27).
Incubation of cells with antibiotics

Macrophages were incubated with oritavancin (or comparators) at 37°C for 3 h (unless stated otherwise) and then washed 3 times with Phosphate Buffered Saline (PBS) to eliminate the extracellular drug. This protocol was adopted to avoid any interference of the extracellular drug in the assays performed while at the same time maintaining a high cellular concentration of the drug. We previously showed that the efflux of oritavancin was very slow, with about 2/3 of the accumulated drug remained associated with the cells after transfer and re-incubation in a drug-free medium for 24 h (15).

Phagocytosis of latex beads

Latex beads have been widely used as a marker of non-specific phagocytosis in macrophages (17,28,29). Control or antibiotic-loaded cells were incubated for 1 h at 37°C with 270 x 10⁶ red-fluorescent, carboxylate-modified polystyrene latex beads (2 µm mean particle size, Sigma-Aldrich ref. number L3030) per mL of culture medium, washed 4 times with ice-cold PBS to eliminate non-internalized latex beads, and scraped in distilled water and lysed by sonication (3). Cell-associated fluorescence was determined using a SPECTRAmax GEMINI-XS fluorescence microplate reader (Molecular Devices LLC, Sunnyvale, CA) with excitation and emission wavelengths set at 584 and 612 nm (linearity of the assay between 0.13 x 10⁶ and 270 x 10⁶ beads per mL [R² = 0.997]) and data were normalized to the cell content in proteins [25]). Latex bead-loaded cells were observed in parallel by confocal laser scanning microscopy (Zeiss microscope [Oberkochen, Germany] coupled to an MRC 1024 confocal scanning device [Biorad, Hemel, UK]). Cells incubated for 1 h with latex beads were thereafter exposed to 50 nM lysotracker green (Molecular
Probes, Eugene, OR) for an additional hour to label acidic organelles and fixed with 3.7 % paraformaldehyde for 15 min. Data were expressed as the number of latex beads per mg of cell protein.

Phagocytosis of *P. aeruginosa* PAO1

In contrast to latex beads, bacteria are phagocytized by macrophages via a receptor-mediated process, which may involve several distinct proteins at the surface of host cells (29-31). Macrophage phagocytosis of bacteria was therefore examined in parallel. *P. aeruginosa* was selected as test organism because neither oritavancin nor comparators show antibacterial activity against Gram-negative bacteria (MICs > 100 µg/mL). Intracellular counts therefore reflect the number of bacteria taken up by cells that escape intracellular killing, with no or minimal interference of antibacterial effect of the accumulated drug. Control or antibiotic-loaded cells were incubated for 2 h in the presence of serum-opsonized bacteria (at a bacteria per macrophage ratio of 50) to allow phagocytosis. Adherent but non-internalized bacteria were then eliminated by 45 min incubation with 50 µg/mL gentamicin and washing four successive times with sterile PBS. This condition was demonstrated to fully eliminate extracellular bacteria in protocols of intracellular infection (13,32). Infected macrophages were collected in distilled water, harvested by scraping, and used for determination of viable bacteria (by plating and cfu counting) and cell protein content (25). Data were expressed as the log10 cfu per mg of cell protein.
Fluid-phase pinocytosis of FITC-dextran

Fluorescein-isothiocyanate (FITC)-dextran (average molecular weight of dextran: 59,000-77,000; Sigma-Aldrich reference number FD70), was used as a marker of fluid-phase pinocytosis (33). Control or antibiotic-loaded cells were incubated for 3 h with 250 µg/mL FITC-dextran, after which the extracellular dye was eliminated by 4 successive washings with ice-cold PBS. Macrophages were collected and lysed by scraping in distilled water, fully dispersed by sonication, and used for determination of cell-associated FITC-dextran by measuring its fluorescence signal (LS30 fluorimeter [Perkin Elmer, Waltham, MA]; excitation and emission wavelengths were set at 488 and 512 nm; linearity between 0.2 and 1.2 µg/mL [R² = 0.998]) and of protein content (25). Data were expressed as µg of FITC-dextran per mg of cell protein.

Assessment of maintenance of lysosomal pH gradient

Neutral red was used to assess lysosomal integrity (34-36). This dye is a weak base that accumulates by a mechanism of diffusion-segregation in the acidic compartments of cells (primarily the lysosomes [37,38]) as long as these maintain the activity of the vacuolar H⁺-ATPase (39) to a level allowing to generate the corresponding pH gradient. Control or antibiotic-loaded cells were incubated for 2 h in the presence of 100 µg/mL neutral red in RPMI 1640 cell culture medium (adjusted to a pH of 6.4 by addition of 100 mM KH₂PO₄). After elimination of the extracellular dye by 4 successive washings with ice-cold PBS, macrophages were resuspended in 50 % ethanol - 1 % acetic acid by gentle shaking (10 min at 130 rpm). Absorbance was recorded at 570 nm using a
Novapath Microplate Reader (Biorad, Hercules, CA) and data were expressed in
percentage of the value measured for control cells.

**Assessment of cell oxido-reductive activity**

The metabolic capacity of macrophages was evaluated by measuring the formation
of purple formazan crystals from the yellow soluble dye 3-[4,5-dimethylthiazol-2-yl]-2,5-
diphenyl tetrazolium bromide (MTT) by cellular dehydrogenases associated mainly with
mitochondria, but also with non-mitochondrial or cytosolic membranes
(endosome/lysosome compartment and plasma membrane; (40,41). J774 cells were
exposed to oritavancin for 3h, washed in ice-cold PBS and then incubated for 1 h at 37°C
with 0.5 mg/mL MTT, after which DMSO was added to dissolve the formazan crystals;
absorbance was measured at 570 nm and data expressed in percentage of the value
recorded for control cells.

**Generation of Reactive Oxygen Species (ROS)**

ROS production by macrophages was evaluated using the cell-permeant, oxidation-
sensitive fluorescent probe, 5-(and-6)chloromethyl-2'-7'-dichloroethylfluorescein diacetate
acetyl ester (CM-H₂DCFDA [Invitrogen]). Control or antibiotic-loaded cells were incubated
with 5 μM CM-H₂DCFDA for 30 min at 37°C, washed twice with PBS and then reincubated
for 30 min in dye-free cell culture medium (allowing de-esterification of the probe). Cells
were then washed once (using PBS) and incubated in Hank’s Balanced Salt Solution
(HBSS) supplemented with 0.5 % hydrogen peroxide. Fluorescence intensities were
recorded using a Packard fluorocount microplate reader (Perkin Elmer, Waltham, MA;
excitation and emission wavelength set at 485 and 530 nm, respectively) for 25 min. Results were expressed as the change in fluorescence signal as compared to time 0.

Curve fitting and statistical analyses

Curve fittings were made using GraphPad Prism version 4.03, GraphPad Software, San Diego, CA, USA. Statistical analyses were performed using GraphPad Instat version 3.06 (GraphPad Software). Recursive partitioning analysis was performed using JMP version 10.0.2 from the SAS Institute, Cary, NC, USA.
RESULTS

Cellular accumulation of oritavancin

In a first series of experiments, we compared the cellular accumulation of oritavancin in murine (J774) and human (A-THP-1) macrophages as a function of time and concentration (Figure 1). In accordance with our previous study (12), we confirmed that the uptake of oritavancin (a) proceeded slowly over time in J774 macrophages and (b) was increased upon incubation with higher extracellular concentrations (left panel). The right panel shows that the apparent accumulation of oritavancin (cellular-to-extracellular concentration ratio) was increased upon incubation with higher extracellular concentrations, which was previously interpreted as denoting a saturation in protein binding (12). We show here that the A-THP-1 cells exhibit similar uptake kinetics (right panel), although the cellular levels reached were systematically lower than in J774 cells, being approximately 15 times lower at 3 h and 2-3 times lower at 24 h.

Influence of oritavancin on macrophage phagocytic capacity

We first examined whether the high level of accumulation of oritavancin in lysosomes (12) could affect the phagocytic capabilities of the cells. We used in parallel latex beads (taken up by non-specific phagocytosis), and P. aeruginosa PAO1 (poorly susceptible to oritavancin and entering the cells via receptor-mediated phagocytosis). Results are shown in Figure 2. Inhibition of phagocytosis of latex beads was observed in J774 macrophages incubated with 25 µg/mL oritavancin, which reached approximately 50% in cells pre-incubated for more than 1 h with the drug (left panel). Confocal microscopy, however,
showed that the ability of latex beads to reach lysosomes was not impaired, as they still
colocalized with LysoTracker Green (Figure 3). When the extracellular concentration of
oritavancin was reduced to 5 µg/mL or less, there was a trend (not statistically significant)
of an increase in latex bead uptake. In A-THP-1 cells, modest inhibition of latex bead
phagocytosis was observed at the highest concentrations tested (25 and 50 µg/mL) but this
effect was not statistically significant. Furthermore, the ability of cells to phagocytize
P. aeruginosa was not impaired (Figure 2, middle and right panels) as we did not observe
any change in the number of cell-associated bacterial counts in oritavancin-treated
macrophages compared to control macrophages, for either mouse or human cell types.

Influence of oritavancin on macrophage pinocytic activity

We also examined the potential effect of oritavancin on the uptake of FITC-dextran, a
tracer of fluid-phase pinocytosis, focusing on J774 macrophages since these appeared
more sensitive to the effects of oritavancin than A-THP-1 macrophages. As illustrated in
Figure 4 (left panel), FITC-dextran endocytosis tended to decrease in cells incubated with
concentrations > 10 µg/mL, but this effect never reached statistical significance.

Influence of oritavancin on the maintenance of lysosomal pH gradient

Because oritavancin accumulates in the lysosomes (12), we then examined whether the
high accumulation in these organelles could affect their integrity. To this end, we
measured the uptake of neutral red. Cells exposed to 50 % DMSO were used as a positive
control of toxicity and showed a 53 ± 4 % reduction in neutral rate accumulation. As shown
in Figure 4 (middle panel), pre-incubation of cells with oritavancin did not affect their
capacity to accumulate neutral red in the range of concentrations investigated (0.8-
50 µg/mL).

Influence of oritavancin on cell oxido-reductive activity
Reduction of MTT is commonly used for evaluating drug cellular toxicity, as it reflects
mainly, but not exclusively, the oxido-reductive metabolism of mitochondria (40,41). We
therefore measured the capacity of cells pre-exposed for 3 h to oritavancin to catalyze the
reduction of MTT. No significant difference was seen over the whole range of
concentrations used (Figure 4, right panel).

Influence of oritavancin on ROS production
Oxidant species constitute a major defense mechanism of host cells against bacteria (42).
We therefore examined the potential influence of oritavancin on ROS production by
macrophages (Figure 5). The left panel shows the production of ROS in J774 cells pre-
exposed for 3 h to oritavancin at increasing concentrations and then exposed for 5 to 25
minutes to H2O2. Oritavancin caused a concentration-dependent increase in ROS
production in J774 cells pre-exposed to concentrations ≥ 20 µg/mL, which reached a
maximum after 10 minutes of incubation with H2O2. The middle panel shows ROS
production in J774 cells pre-incubated for 3, 6, or 24 h to oritavancin and then exposed for
25 minutes to H2O2. Similar effects were observed; however, the amount of ROS produced
was higher when cells were pre-incubated for 24 h with oritavancin. The right panel shows
the results of the same experiment performed with human A-THP-1 cells where the
increase in ROS production induced by oritavancin never reached statistical significance.
Comparison of the effects of oritavancin, vancomycin, and azithromycin on macrophage functions in J774 mouse cells

In this last series of experiments, we compared the effects of oritavancin on phagocytosis/pinocytosis, maintenance of a lysosomal pH gradient, cell oxido-reductive metabolism, and ROS production with those of vancomycin and azithromycin (Table 1). Experiments were performed with mouse J774 macrophages exposed for 3 h to fixed concentrations of antibiotics. Concentrations were selected to be in the order of magnitude or slightly higher than the free human serum Cmax, namely 25 µg/mL for oritavancin (projected value: 19.4 µg/mL based on population pharmacokinetic data for a unique dose of 1200 mg [43]), 50 µg/mL for vancomycin (based on Cmax value mentioned in the Summary of Product Characteristics [63 µg/mL for total drug] and on a free fraction varying between 10 and 90 % [44,45]), and 1 µg/mL for azithromycin (based on Cmax ranging from 0.4 µg/mL and 2 µg/mL for total drug after administration of 500 mg by oral or intravenous route respectively and on a low protein binding [12%; 46,47]). Whereas oritavancin significantly impaired latex-bead phagocytosis, both azithromycin and vancomycin induced a slight and similar increase in ROS production.
DISCUSSION

The present study documents that despite its substantial cellular accumulation, the lipoglycopeptide oritavancin only minimally affects the endocytic activity and the maintenance of an acid pH in lysosomes and cell oxido-reductive capabilities in mouse macrophages, while causing no significant changes in human macrophages.

In a phase I study, the cellular concentration of oritavancin in alveolar macrophages of healthy volunteers having received a cumulative dose of 4 g of oritavancin (800 mg/day for 5 days) reached levels of 560 µg/mL (11) (i.e. ~ 2.8 µg/mg protein assuming that 5 µL of cell volume corresponds to 1 mg of cell protein [27,48]) vs. 180 µg/mL (total drug) in the serum. In the macrophage cell lines A-THP-1 and J774, cellular concentrations of oritavancin following exposure to static extracellular concentrations of 20 µg/mL for 3 h reached approximately 78 and 1450 µg/mL, respectively (see Figure 1, right lower panel).

This suggests that the anticipated cellular concentrations observed in patients receiving a single 1200 mg dose of oritavancin would be markedly lower than those observed in the phase I study (11) as well as those measured here in the macrophage cell lines (despite exposure to oritavancin concentrations approximating the projected free peak concentration in serum [19.4 µg/mL; 43]). The fact that oritavancin accumulation is much higher in vitro could be explained by (i) the maintenance of a constant extracellular concentration over time in our in vitro model and (ii) the higher free concentration of oritavancin in a culture medium containing only 10 % serum (12).

We previously showed that oritavancin causes a mixed-lipid storage disorder in the lysosomes of both phagocytic (mouse J774 macrophages) and non-phagocytic (rat embryo...
fibroblasts) cells (15). We demonstrate here an impairment of latex-bead phagocytosis in mouse macrophages but no change in fluid-phase pinocytosis (in contrast with what has been observed with azithromycin [3]). On the contrary, phagocytosis of bacteria was not impaired in both mouse and human macrophages. While the reasons for these discrepant effects need to be further examined, we argue that phagocytosis of bacteria requires specific interactions with many proteins at the cell surface (49), which is not the case for latex-bead uptake. Moreover, a recent study highlighted major differences in the proteome of infected phagosomes and of latex bead-loaded phagosomes (50), supporting the hypothesis that both processes proceed by mechanisms that could be differentially affected by a drug.

Recursive-partitioning analysis of the concentration-effect responses pooled for both cell lines and presented in Figure 6 shows that inhibition of latex-bead phagocytosis becomes significant at cellular concentrations higher than 5.9 µg/mg protein (1180 µg/mL) while ROS production is significantly increased for cellular concentrations higher than 75 µg/mg protein (15000 µg/mL). These values are respectively approximately 2- and 27-fold higher than the cellular concentrations measured in alveolar macrophages of healthy volunteers having received a cumulative oritavancin dose of 4 g (11). Of interest, our previous analysis of oritavancin-induced lipidosis (15) shows that overload with phospholipids and cholesterol becomes significant only at cellular concentration exceeding 31 µg/mg protein [6200 µg/mL]), which is approximately 11-fold higher than the cellular concentrations observed in phase I studies (11).

Oritavancin accumulates in the lysosomes by a process of adsorptive endocytosis (12), with no demonstrated association with mitochondria. MTT reduction is considered as
a marker of mitochondrial metabolism, but also as a general cytotoxicity test denoting a loss of viability that is more sensitive than other assays like lactate dehydrogenase release, trypan blue exclusion assay, or neutral red relocalisation (35,51,52). The MTT assay did not reveal any effect of oritavancin on mouse macrophages exposed to extracellular concentrations up to 50 µg/mL in vitro, which suggests a low toxic potential for this drug, especially if considering its cellular accumulation. In addition, the absence of neutral-red relocalisation in cells incubated with oritavancin argues against a loss of lysosomal pH gradient (through impairment of the activity of the V-ATPase or mere permeabilisation of the lysosomal membrane). This contrasts with observations made for other drugs that accumulate in lysosomes like chloroquine (53) or aminoglycosides (54,55), which neutralize their pH, or increase the permeability of their membrane.

ROS production is another major function of macrophages related to their role in pathogen clearance. We observed here that oritavancin actually increases production of ROS, but only in J774 mouse macrophages and at extracellular concentrations ≥ 20 µg/mL. Whether this may contribute to its activity against intracellular Gram-positive pathogens remains to be established. We recently demonstrated that (i) exposure of bacteria to an oxidant species (H2O2) did not change oritavancin MICs; (ii) its intracellular potency was similar in naïve THP-1 and A-THP-1 cells vs cells in which oxidative defenses have been boosted by PMA, (iii) its activity was only slightly impaired by N-acetylcysteine, a general scavenger of oxidant species (56). Thus, in contrast to several other bactericidal antibiotics (57), the ability of oritavancin to kill bacteria may be independent of the stimulation of oxidative stress.
An interesting observation made in the context of this study is that J774 mouse macrophages seem more susceptible to the toxic effects of oritavancin than human THP-1 cells. This difference is most probably related to the fact that J774 cells accumulate the drug to a much larger extent than human cells. This is illustrated in Figure 6, in which data pertaining to parameters modified by incubation of cells with oritavancin have been replotted as a function of its cellular concentration.

In conclusion, our data support that changes in macrophage functions are unlikely to occur upon treatment with a single 1200 mg dose of oritavancin as the anticipated cellular concentrations are expected to be lower than both the levels measured in alveolar macrophages from patients receiving a cumulative dose of 4000 mg and those that affected macrophage functions (latex-bead phagocytosis and ROS production in mouse cells) in vitro.
Acknowledgments

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519 5 (TLR5), IL-1beta secretion, and asparagine endopeptidase are critical factors for
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Table 1. Comparative effects of oritavancin, vancomycin and azithromycin on macrophage functions in mouse J774 cells pre-incubated for 3h at the indicated concentrations. All values are expressed as percentage of the corresponding control (no drug added).

<table>
<thead>
<tr>
<th>Macrophage function</th>
<th>Tracer</th>
<th>Percentage of control value</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Oritavancin (25 µg/mL)</td>
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<tr>
<td>Phagocytosis</td>
<td>P. aeruginosa uptake</td>
<td>95.3 ± 1.4 ***</td>
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<td></td>
<td>Latex beads uptake</td>
<td>43.1 ± 17.2 *</td>
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<tr>
<td>Fluid-phase</td>
<td>FITC-dextran uptake</td>
<td>81.0 ± 2.5</td>
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<td>endocytosis</td>
<td></td>
<td>106.5 ± 9.6</td>
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<td>Lysosomal integrity</td>
<td>Neutral red uptake</td>
<td>106.5 ± 9.6</td>
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<tr>
<td>Mitochondrial</td>
<td>MTT metabolism</td>
<td>96.4 ± 31.1</td>
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<tr>
<td>functionality</td>
<td></td>
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<tr>
<td>ROS production</td>
<td>CM-H2DCFDA fluorescence</td>
<td>117.2 ± 4.4 *</td>
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a 2 h incubation with 100 µg/ml-L neutral red (n = 2-3)
b 2 h incubation with P. aeruginosa (50 bacteria/cell) or 270 x 10^6 latex beads/ml (n = 3)
c 3 h incubation with 250 µg/m FITC-dextran from control (n = 2-3)
d 1 h incubation with 0.5 mg/mL MTT (n = 2-3)
e 30 min incubation with CM-H2DCFDA followed by 25 min incubation in HBSS supplemented by 0.5 % H2O2 (n = 4)

Statistical analysis [ANOVA; Tukey multiple comparison test]: *, P < 0.05; **, P < 0.01; ***, P < 0.001 as compared to the control condition (no pre-exposure to antibiotic); $, P < 0.05; $$, P < 0.01; $$$, P < 0.001 as compared to oritavancin.
Figure 1
Influence of time of incubation and of oritavancin extracellular concentration on the cellular accumulation of oritavancin in murine (J774; both panels) and human (A-THP-1; right panel) macrophages. Data are means ± SD of 2 independent determinations. Left panel: Kinetics of uptake in J774 macrophages exposed for up to 24 h to increasing extracellular concentrations of oritavancin; data are expressed in apparent cellular concentrations. Right panels: Comparison of the accumulation level (cellular to extracellular concentration ratio) in J774 cells (closed symbols) and A-THP-1 cells (open symbols) incubated for 3 h (lower panel) or 24 h (upper panel) with increasing extracellular concentrations.
Figure 2

Influence of time of incubation and of oritavancin extracellular concentration on phagocytosis of latex beads (all panels) or of *P. aeruginosa* strain PAO1 (middle and right panel) in murine (J774; left and middle panels) and human (A-THP-1; right panel) macrophages. Results are expressed in percentage of the value recorded for the control (no drug added) at the same time point. Data are means ± SD of 2-3 independent determinations. Statistical analysis (ANOVA; Dunnett multiple comparison test): *: P < 0.05; **, P < 0.01; ***, P < 0.001 as compared to control conditions (no pre-exposure to oritavancin).
Figure 3
Cell imaging in laser scanning confocal microscopy of J774 macrophages incubated for 3 h in the absence (control; top) or in the presence (bottom) of 25 µg/mL oritavancin, and then exposed to red-fluorescent latex beads for 1 h before addition of the acidotropic marker LysoTracker green (1 h).
Influence of oritavancin on specific cell functions

- **Fluid-phase pinocytosis**
- **Maintenance of lysosomal pH gradient**
- **Oxido-reductive activity**

**Figure 4**

Influence of oritavancin extracellular concentration on fluid-phase endocytosis of FITC-dextran (left panel), on maintenance of a lysosomal pH gradient (neutral red uptake; middle panel), and on cell oxido-reductive metabolism (MTT reduction: right panel). J774 cells were incubated for 3 h with oritavancin and then exposed for 3 h to 250 µg/mL FITC-dextran (left), for 2 h with 100 µg/mL neutral red (middle) or for 1 h with 0.5 mg/mL MTT (right). Results are expressed in percentage of the value recorded for the control (no drug added) at the same time point. Data are means ± SD of 2-3 independent determinations.

Statistical analysis (ANOVA; Dunnett multiple comparison test): no statistical difference (p > 0.05) vs. control conditions (no pre-exposure to oritavancin) over the whole range of concentrations investigated.
Figure 5

Left panel: influence of time of incubation with hydrogen peroxide on ROS generation by J774 cells preincubated during 3 h with oritavancin at increasing concentrations. Other panels: influence of time of incubation with oritavancin at increasing extracellular concentrations on ROS production by J774 cells (middle panel) and by A-THP-1 cells (right panel). Cells were incubated for the indicated times with oritavancin at increasing concentrations and then exposed to 5 µM CM-H2DCFDA for 30 minutes, washed and re-incubated in HBSS supplemented with 0.5 % hydrogen peroxide for the indicated times (left panels) or for 25 minutes (other panels). Results are expressed in percentage of the H2DCFDA fluorescence value recorded at time 0. Data are means ± SD of 2-4 independent determinations. Statistical analysis (ANOVA; Dunnett multiple comparison test): **, P < 0.01 as compared to control conditions (no pre-exposure to oritavancin).
Figure 6
Correlation between the cellular concentration of oritavancin and its effect on latex-bead phagocytosis (left) or ROS production (right). Constructed based on data from Figures 1 and 5 for J774 cells and THP-1 cells. The grey zone highlights the range of cellular concentrations observed in alveolar macrophages of healthy volunteers having received a daily dose of 800 mg IV oritavancin for up to 5 days (cumulative dose= 4g) (11). The vertical dotted line corresponds to the optimized split value obtained by recursive partitioning analysis of each concentration-response curve, which is the lowest cellular concentration for which a significant effect of oritavancin is observed (5.9 µg/mg prot. for inhibition of latex beads phagocytosis [LogWorth value: 187.9; p < 0.001]; 75.2 µg/mg prot. for stimulation of ROS production [LogWorth value 149.2; p < 0.001]).
Influence of oritavancin on phagocytosis

J774 cells; 25 mg/L oritavancin

Tracer uptake (% control value)

- latex beads

J774 cells; 3 h incubation

A-THP-1 cells; 3 h incubation

log_{10} extracellular concentration oritavancin (mg/L)