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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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Oritavancin, a lipoglycopeptide antibiotic in development, accumulates to high levels in the lysosomes of eukaryotic cells. We examined specific functions of macrophages (phagocytic capacity, lysosomal integrity, metabolic activity, and 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 *Pseudomonas 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 of ≥25 μg/ml, oritavancin reduced latex bead 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 3 h. 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 bead phagocytosis (1,180 μg/ml) or to stimulate ROS production (15,000 μg/ml) by J774 cells. The data, therefore, suggest that, in spite of its substantial cellular accumulation, oritavancin is unlikely to markedly affect macrophage functions under the conditions of use investigated in current phase III trials (a single dose of 1,200 mg).

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 includes reactive oxygen species (ROS), nitric oxide, and hydrolytic enzymes (1).

Several antibiotic classes accumulate within phagocytes (see reference 2 for a review). Studying their potential impacts on phagocyte functions thus appears to be of prime importance to detect potential interference 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 the 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 clinics (8).

Oritavancin, an investigational lipoglycopeptide antibiotic (see reference 9 for a review), has completed phase III clinical investigations for the treatment of acute bacterial skin and skin structure infection with a single dose of 1,200 mg intravenously (i.v.) (www.clinicaltrials.gov; NCT01252719 and NCT01252732) (10). In healthy volunteers who received a cumulative dose of 4 g (5 doses of 800 mg each), the drug reaches concentrations in alveolar macrophages that are as high as 50 times 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). However, 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 of ≥20 to 25 μg/ml (15). A recent study showed, 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 was to examine the effect of oritavancin on specific macrophage functions (including endocytic and phagocytic capabilities, maintenance of the lysosomal pH gradient, oxidoreductive activity, or ROS production), using in parallel murine J774 macrophages and activated THP-1 cells, which both display macrophage-like activities (17–19). Vancomycin (a reference glycopeptide with low cellular accumulation [12]) and azithromycin (a 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 observed only in the mouse macrophage cell line and at cellular concentrations that likely exceed those predicted to be reached in treated patients.

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MATERIALS AND METHODS

Antibiotics and main reagents. Oritavancin diphosphate (fully hydrated diphosphate salt; potency, 83.1%) and ^{14}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 Pharmacopoeia and commercialized in Belgium for human use as Vancocin and Geomycine (distributed in Belgium by GlaxoSmithKline s.a./n.v., Genval, Belgium, 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. The 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 (ATCC TIB-202; a myelomonocytic cell line displaying macrophage-like activity) cells 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 here as A-THP-1 cells) by incubation with phorbol 12-myristate 13-acetate (PMA) (200 μg/liter; Sigma-Aldrich) for 24 h at 37°C. This treatment results in an increase in their phagocytic activity and production of superoxide, as well as in the expression of HLA-DR and interleukin 1β (17, 18, 24).

Determination of the cellular content of oritavancin. The cellular accumulation of oritavancin was determined in J774 and A-THP-1 macrophages following procedures established previously (12, 16). Briefly, cells incubated with ^{14}C-labeled oritavancin were washed 3 or 4 times in ice-cold phosphate-buffered saline (PBS), collected by scraping in distilled water, and used for measuring cell-associated radioactivity (liquid scintillation counting) and assaying protein (25). The apparent cellular/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 otherwise indicated) and then washed 3 times with 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 remaining associated with the cells after transfer and reincubation in a drug-free medium for 24 h (15).

Phagocytosis of latex beads. Latex beads have been widely used as a marker of nonspecific phagocytosis in macrophages (17, 28, 29). Control or antibiotic-loaded cells were incubated for 1 h at 37°C with 270×10^6 red-fluorescent, carboxylate-modified polystyrene latex beads (2-μm mean particle size; Sigma-Aldrich reference number L3030) per ml of culture medium, washed 4 times with ice-cold PBS to eliminate noninternalized latex beads, 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 was between 0.13×10^6 and 270×10^6 beads per ml [$R^2 = 0.997$]), and data were normalized to the cell content of proteins (25). The 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 [Bio-Rad, Hemel, United Kingdom]). The 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. The data were expressed as the number of latex beads per milligram 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 surfaces of host cells (29–31). Macrophage phagocytosis of bacteria was therefore examined in parallel. *P. aeruginosa* was selected as the test organism because neither oritavancin nor the 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 from antibacterial effect of the accumulated drug. Control or antibiotic-loaded cells were incubated for 2 h in the presence of serum-opsonized bacteria (at 50 bacteria per macrophage) to allow phagocytosis. Adherent but noninternalized bacteria were then eliminated by 45 min of incubation with 50 μg/ml gentamicin and washing four successive times with sterile PBS. These conditions were 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 $\log_{10}$ CFU per mg of cell protein.

Fluid-phase pinocytosis of FITC-dextran. Fluorescein isothiocyanate (FITC)-dextran (average molecular weight of dextran, 59,000 to 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 [PerkinElmer, Waltham, MA]; excitation and emission wavelengths were set at 488 and 512 nm; linearity, between 0.2 and 1.2 μg/ml [$R^2 = 0.998$]) and protein content (25). The data were expressed as micrograms of FITC-dextran per milligram of cell protein.

Assessment of maintenance of the lysosomal pH gradient. Neutral red was used to assess lysosomal integrity (34–36). The 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 they maintain the activity of the vacuolar H^+-ATPase (39) at a level allowing the generation of 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 pH 6.4 by addition of 100 mM KH_2PO_4). After elimination of the extracellular dye by 4 successive washings with ice-cold PBS, the 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 (Bio-Rad, Hercules, CA), and the data were expressed as a percentage of the value measured for control cells.

Assessment of cell oxidoreductive 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 nonmitochondrial or cytosolic membranes (endosome/lysosome compartment and plasma membrane) (40, 41). J774 cells were exposed to oritavancin for 3 h, washed in ice-cold PBS, and then incubated for 1 h at 37°C with 0.5 mg/ml MTT, after which dimethyl sulfoxide (DMSO) was added to dissolve the

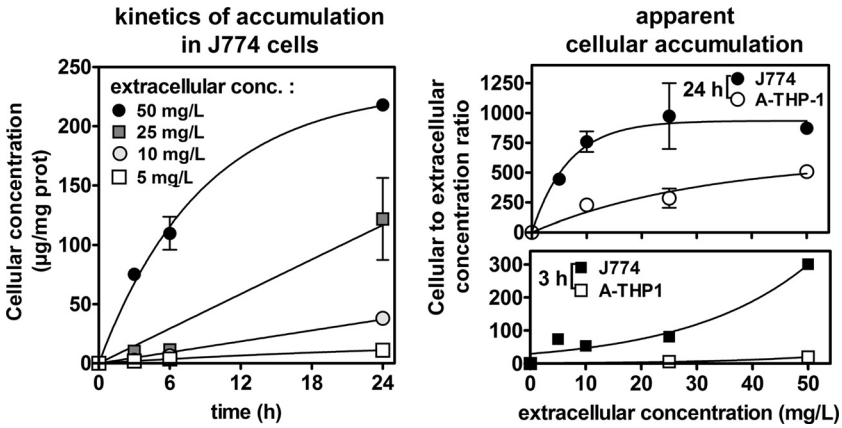

FIG 1 Influence of incubation time and of oritavancin extracellular concentration on the cellular accumulation of oritavancin in murine (J774) and human (A-THP-1) macrophages. The data are means ± standard deviations (SD) of 2 independent determinations. (Left) Kinetics of uptake in J774 macrophages exposed for up to 24 h to increasing extracellular concentrations of oritavancin; the data are expressed as apparent cellular concentrations. (Right) Comparison of the accumulation levels (cellular/extracellular concentration ratio) in J774 cells and A-THP-1 cells incubated for 3 h (bottom) or 24 h (top) with increasing extracellular concentrations.

formazan crystals; absorbance was measured at 570 nm, and data were expressed as a percentage of the value recorded for control cells.

Generation of ROS. ROS production by macrophages was evaluated using the cell-permeant, oxidation-sensitive fluorescent probe 5-(and-6)chloromethyl-2′-7′-dichlorohydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) (Invitrogen). Control or antibiotic-loaded cells were incubated with 5 μM CM-H_2DCFDA 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). The 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 (PerkinElmer, Waltham, MA) (excitation and emission wavelengths were set at 485 and 530 nm, respectively) for 25 min. The results were expressed as the change in fluorescence signal compared to time zero.

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, using a single-pass decision tree method with node splitting based on the LogWorth statistic (for details and justification, see http://www.jmp.com/support/notes/35/addl/fusion_35411_1_montecarlocal.pdf).

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 (Fig. 1). In accordance with our previous study (12), we confirmed that the uptake of oritavancin (i) proceeded slowly in J774 macrophages and (ii) was increased upon incubation with higher extracellular concentrations (Fig. 1, left). Figure 1, right, shows that the apparent accumulation of oritavancin (the ratio of cellular to extracellular concentrations) was increased upon incubation with higher extracellular concentrations, which was previously interpreted as denoting saturation in protein binding (12). We show here that the A-THP-1 cells exhibit similar uptake kinetics (Fig. 1, right), although the cellular levels reached were systematically lower than in J774 cells, being approximately 15 times lower at 3 h and 2 to 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 nonspecific phagocytosis) and *P. aeruginosa* PAO1 (poorly susceptible to oritavancin and entering the cells via receptor-mediated phagocytosis). The results are shown in Fig. 2. Inhibition of phagocytosis of latex beads was observed in J774 macrophages incubated with 25 μg/ml oritavancin, which reached approximately 50% in cells preincubated for more than 1 h with the drug (Fig. 2, left). Confocal microscopy, however, showed that the ability of latex beads to reach lysosomes was not impaired, as they still colocalized with LysoTracker Green (Fig. 3). When the extracellular concentration of oritavancin was reduced to 5 μg/ml or less, there was a trend (not statistically significant) of increasing 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 (Fig. 2, middle and right), as we did not observe any change in the 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 they appeared to be more sensitive to the effects of oritavancin than A-THP-1 macrophages. As illustrated in Fig. 4, left, FITC-dextran endocytosis tended to decrease in cells incubated with concentrations of >10 μg/ml, but this effect never reached statistical significance.

Influence of oritavancin on maintenance of the 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 for toxicity and showed a 53% ± 4% reduction in neutral rate accumulation. As shown in Fig. 4, middle,

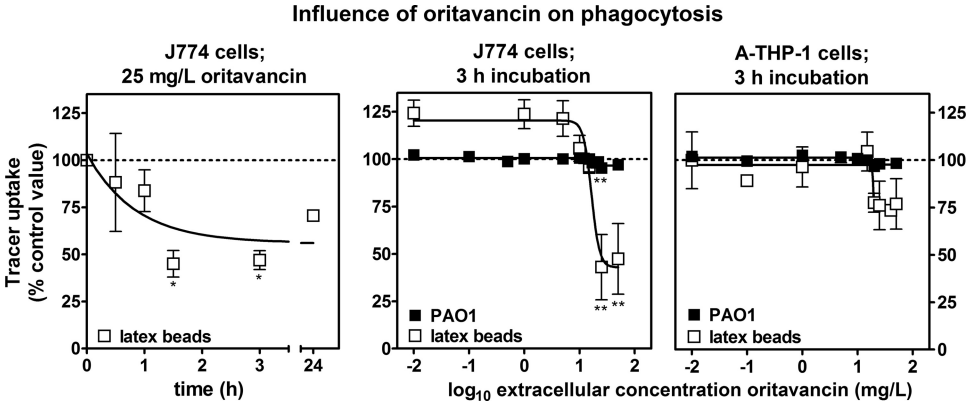

FIG 2 Influence of incubation time and of oritavancin extracellular concentration on phagocytosis of latex beads or of *P. aeruginosa* strain PAO1 in murine (J774) and human (A-THP-1) macrophages. The results are expressed as percentages of the value recorded for the control (no drug added) at the same time point. The data are means ± SD of 2 or 3 independent determinations. Statistical analysis (ANOVA; Dunnett multiple-comparison test): *, $P < 0.05$; **, $P < 0.01$ compared to control conditions (time 0 [left panel] or no preexposure to oritavancin).

preincubation of cells with oritavancin did not affect their capacity to accumulate neutral red in the range of concentrations investigated (0.8 to 50 μg/ml).

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

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 (Fig. 5). The left graph shows the production of ROS in J774 cells preexposed for 3 h to oritavancin at increasing concentrations and then exposed for 5 to 25 min to H_2O_2. Oritavancin caused a concentration-dependent increase in ROS production in J774 cells preexposed to concentrations of ≥20 μg/ml, which reached a maximum after 10 min of incubation with H_2O_2. The middle graph shows ROS production in J774 cells preincubated for 3, 6, or 24 h with oritavancin and then exposed for 25 min to H_2O_2. Similar effects were observed; however, the amount of ROS produced was greater when cells were preincubated for 24 h with oritavancin. The right graph 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 the last series of experiments, we compared the effects of oritavancin on phagocytosis/pinocytosis, maintenance of a lysosomal pH gradient, cell oxidoreductive metabolism, and ROS production with those of vancomycin and azithromycin. Experiments were performed with mouse J774 macrophages exposed for 3 h to fixed

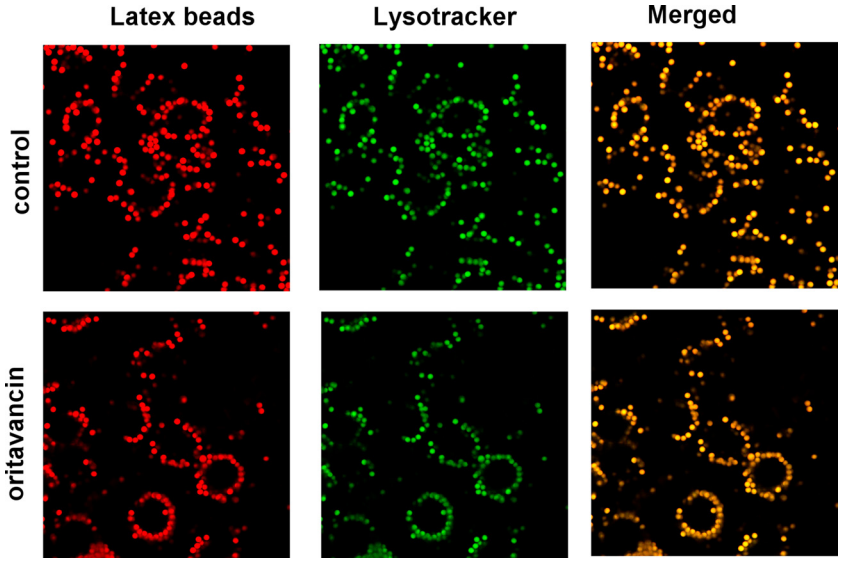

FIG 3 Cell imaging in laser scanning confocal microscopy of J774 macrophages incubated for 3 h in the absence (control) or presence (bottom) of 25 μg/ml oritavancin and then exposed to red-fluorescent latex beads for 1 h before addition of the acidotropic marker LysoTracker Green (1 h).

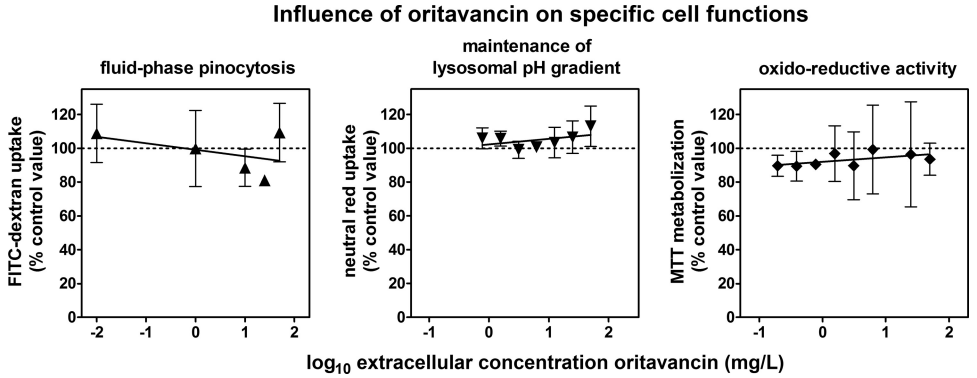

FIG 4 Influence of oritavancin extracellular concentration on fluid phase endocytosis of FITC dextran, on maintenance of a lysosomal pH gradient (neutral red uptake), and on cell oxidoreductive metabolism (MTT reduction). J774 cells were incubated for 3 h with oritavancin and then exposed for 3 h to 250 μg/ml FITC-dextran (left), for 2 h to 100 μg/ml neutral red (middle), or for 1 h to 0.5 mg/ml MTT (right). The results are expressed as percentages of the value recorded for the control (no drug added) at the same time point. The data are means ± SD of 2 or 3 independent determinations. Statistical analysis (ANOVA; Dunnett multiple-comparison test) showed no statistical difference ($P > 0.05$) versus control conditions (no preexposure to oritavancin) over the whole range of concentrations investigated.

concentrations of antibiotics. Concentrations were selected to be at the order of magnitude of or slightly higher than the maximum concentration of free drug in human serum (C_{max}), namely, 25 μg/ml for oritavancin (projected value, 19.4 μg/ml based on population pharmacokinetic data for a unique dose of 1,200 mg [43]), 50 μg/ml for vancomycin (based on the C_{max} 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 C_{max} values ranging from 0.4 μg/ml to 2 μg/ml for total drug after administration of 500 mg by the oral or intravenous route, respectively, and on low protein binding [12%] [46, 47]). Results are shown in Table 1. Whereas oritavancin significantly impaired latex bead phagocytosis, azithromycin and vancomycin induced slight and similar increases in ROS production.

DISCUSSION

The present study documents that, despite its substantial cellular accumulation, the lipoglycopeptide oritavancin only minimally affects endocytic activity and maintenance of an acid pH in lysosomes and cell oxidoreductive 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 who 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]) versus 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 1,450 μg/ml, respectively (Fig. 1, lower right). This suggests that the anticipated cellular concentrations observed in patients receiving a single 1,200-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 accumula-

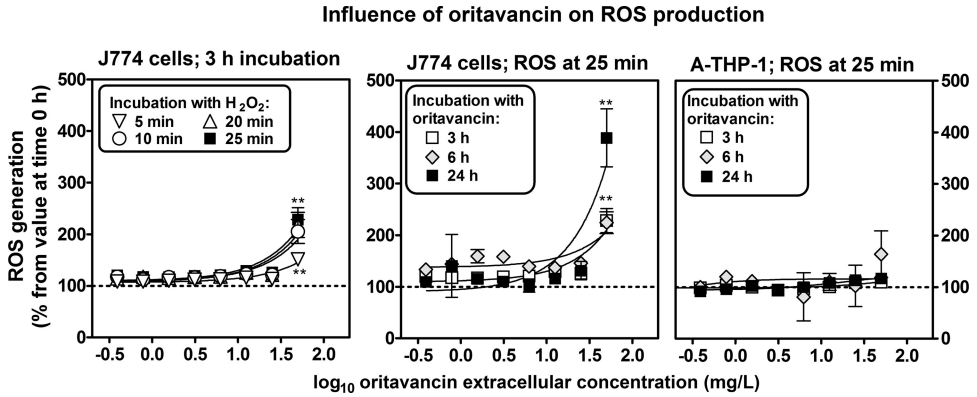

FIG 5 (Left) Influence of incubation time with hydrogen peroxide on ROS generation by J774 cells preincubated for 3 h with oritavancin at increasing concentrations. (Middle and right) Influence of incubation time with oritavancin at increasing extracellular concentrations on ROS production by J774 cells and by A-THP-1 cells. The cells were incubated for the indicated times with oritavancin at increasing concentrations and then exposed to 5 μM CM-H2DCFDA for 30 min, washed, and reincubated in HBSS supplemented with 0.5% hydrogen peroxide for the indicated times (left) or for 25 min (middle and right). The results are expressed as percentages of the H_2DCFDA fluorescence value recorded at time zero. The data are means ± SD of 2 to 4 independent determinations. Statistical analysis (ANOVA; Dunnett multiple-comparison test): **, $P < 0.01$ compared to control conditions (no preexposure to oritavancin).

TABLE 1 Comparison of effects of oritavancin, vancomycin, and azithromycin on macrophage functions in mouse J774 cells

Macrophage function	Tracer	% of control value[a]		
		Oritavancin (25 μg/ml)	Vancomycin (50 μg/ml)	Azithromycin (1 μg/ml)
Phagocytosis[b]	*P. aeruginosa* uptake	95.3 ± 1.4[g]	102.7 ± 2.8[j]	103.4 ± 0.5[h,j]
	Latex bead uptake	43.1 ± 17.2[i]	128.3 ± 10.1[k]	88.8 ± 19.7[l]
Fluid phase endocytosis[c]	FITC-dextran uptake	81.0 ± 2.5	95.2 ± 15.1	99.5 ± 4.9
Lysosomal integrity[d]	Neutral red uptake	106.5 ± 9.6	97.7 ± 2.2	98.0 ± 4.4
Mitochondrial functionality[e]	MTT metabolization	96.4 ± 31.1	81.4 ± 5.5	90.4 ± 28.5
ROS production[f]	CM-H_2DCFDA fluorescence	117.2 ± 4.4[i]	119.2 ± 2.6[i]	107.5 ± 10.8

[a] Cells were preincubated for 3h at the indicated concentrations. All values are expressed as percentages of the corresponding control (no drug added). Statistical analysis was by analysis of variance (ANOVA) with the Tukey multiple-comparison test.
[b] Two hours of incubation with *P. aeruginosa* (50 bacteria/cell) or 270 × 10^6 latex beads/ml ($n = 3$).
[c] Three hours of incubation with 250 μg/ml FITC-dextran from control ($n = 2$ or 3).
[d] Two hours of incubation with 100 μg/ml neutral red ($n = 2$ or 3).
[e] One hour of incubation with 0.5 mg/ml MTT ($n = 2$ or 3).
[f] Thirty-minute incubation with CM-H_2DCFDA, followed by 25-min incubation in HBSS supplemented with 0.5% H_2O_2 ($n = 4$).
[g] $P < 0.001$ compared to the control condition (no preexposure to antibiotic).
[h] $P < 0.01$ compared to the control condition (no preexposure to antibiotic).
[i] $P < 0.05$ compared to the control condition (no preexposure to antibiotic).
[j] $P < 0.001$ compared to oritavancin.
[k] $P < 0.01$ compared to oritavancin.
[l] $P < 0.05$ compared to oritavancin.

tion 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 nonphagocytic (rat embryo fibroblasts) cells (15). We demonstrated 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 other hand, 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 proteomes 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 Fig. 6 shows that inhibition of latex bead phagocytosis becomes significant at cellular concentrations higher than 5.9 μg/mg protein (1,180 μg/ml) while ROS production is significantly increased for cellular concentrations higher than 75 μg/mg protein (15,000 μg/ml). These values are, respectively, approximately 2- and 27-fold higher than the cellular concentrations measured in alveolar macrophages of healthy volunteers who received a cumulative oritavancin dose of 4 g (11). Of interest, our previous analysis of oritavancin-induced lipidosis (15) showed that overload with phospholipids and cholesterol becomes significant only at cellular concentrations exceeding 31 μg/mg protein (6,200 μg/ml), which is approximately 11-fold higher than the cellular concentrations observed in phase I studies (11).

Oritavancin accumulates in lysosomes by a process of adsorptive endocytosis (12), with no demonstrated association with mitochondria. MTT reduction is considered a marker of mitochondrial metabolism, but also a general cytotoxicity test indicating a loss of viability that is more sensitive than other assays, like lactate dehydrogenase release, the trypan blue exclusion assay, or neutral red relocalization (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 the drug, especially considering its cellular accumulation. In addition, the absence of neutral red relocalization in cells incubated with oritavancin argues against a loss of lysosomal pH gradient (through impairment of the activity of the V-ATPase or mere permeabilization of the lysosomal membrane).

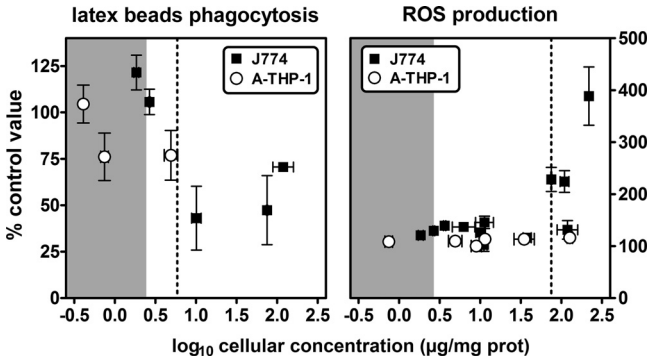

FIG 6 Correlation between the cellular concentration of oritavancin and its effect on latex bead phagocytosis or ROS production. The diagrams were constructed based on data from Fig. 1 and 5 for J774 cells and THP-1 cells. The gray zones highlight the range of cellular concentrations observed in alveolar macrophages of healthy volunteers who received a daily dose of 800 mg i.v. oritavancin for up to 5 days (cumulative dose, 4 g) (11). The vertical dashed lines correspond 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 was observed (5.9 μg/mg protein for inhibition of latex bead phagocytosis [LogWorth value, 187.9; $P < 0.001$]; 75.2 μg/mg protein for stimulation of ROS production [LogWorth value, 149.2; $P < 0.001$]).

This contrasts with observations made for other drugs that accumulate in lysosomes, like chloroquine (53) or aminoglycosides (54, 55), which neutralize the pH of lysosomes or increase the permeability of their membranes.

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 of ≥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 (H_2O_2) did not change oritavancin MICs; (ii) oritavancin intracellular potencies were similar in naive THP-1 and A-THP-1 cells in which oxidative defenses had been boosted by PMA; and (iii) the activity of oritavancin was only slightly impaired by *N*-acetylcysteine, a general scavenger of oxidant species (56). Thus, in contrast to what has been proposed for 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 greater extent than human cells. This is illustrated in Fig. 6, in which data pertaining to the parameters modified by incubation of cells with oritavancin have been replotted as a function of its cellular concentration.

In conclusion, our data support the notion that changes in macrophage functions are unlikely to occur upon treatment with a single 1,200-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 4,000 mg and those that affected macrophage functions (latex bead phagocytosis and ROS production in mouse cells) *in vitro*.

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