SUPPLEMENT ARTICLE

Macrophage Killing of Bacterial and Fungal Pathogens Is Not Inhibited by Intense Intracellular Accumulation of the Lipoglycopeptide Antibiotic Oritavancin

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Intact phagocytic effector function is fundamental to host defense against microbial pathogens. Concern has been raised regarding the potential that accumulation of certain agents, including cationic amphiphilic antibiotics, within macrophages could cause a mixed-lipid storage disorder, resulting in macrophage dysfunction in recipients. The ability of 2 macrophage cell lines (HL-60; RAW 264.7) to kill archetypal Gram-positive (*Staphylococcus aureus*), Gram-negative (*Acinetobacter baumannii*), and fungal (*Candida albicans*) pathogens was tested following exposure of the macrophages to the lipoglycopeptide antibiotic oritavancin. Oritavancin did not affect killing of *C. albicans* but markedly enhanced killing of *S. aureus* by both macrophages. Oritavancin modestly reduced killing of *A. baumannii* by HL-60 cells but not by RAW 264.7 cells. Thus, macrophage killing of microbes remains intact despite substantial intracellular accumulation with a lipoglycopeptide antibiotic.

Intact phagocytic effector function is fundamental to host defense against microbial pathogens, such as the Gram-positive coccus *Staphylococcus aureus*, the Gram-negative bacillus *Acinetobacter baumannii*, and the fungal pathogen *Candida albicans* [1–6]. Although many previous studies have focused on the role of neutrophils, the importance of macrophages in host defense against such pathogens has only been recently described [2, 7–9]. These studies are concordant with older literature confirming the early and marked clearance of extracellular pathogens, such as *C. albicans*, within minutes after bloodstream infection by phagocytes in the reticuloendothelial system of mammals, including rodents, lagomorphs, dogs, and humans [10].

One putative mechanism by which macrophage microbicidal function may be inhibited is the accumulation of complex lipid or carbohydrate-rich deposits within the phagocytes, as occurs in genetic metabolic storage diseases [11]. Oritavancin is an investigational lipoglycopeptide antibiotic with potent anti–*S. aureus* activity [12]. Oritavancin accumulates markedly within macrophages, where it causes deposition of concentric lamellar structures and finely granular material and other material, often in giant vesicles, consistent with a mixed-lipid storage disorder [13]. To determine whether marked intracellular accumulation of oritavancin alters killing of microbes, macrophage killing in the presence or absence of oritavancin was tested against *S. aureus*, as well as organisms against which the drug has no activity, including *C. albicans* and *A. baumannii*.

Human HL-60 cells and murine RAW 264.7 macrophage cells (both from American Type Culture Collection, Rockville, MD) were tested because they are known to be capable of killing microbes after differentiation [14–16]. The cells were cultured at 37°C in 5% CO_2 in

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Roswell Park Memorial Institute 1640 medium (Irvine Scientific, Santa Ana, California) with 10% fetal bovine serum, 1% penicillin, streptomycin, and glutamine (Gemini BioProducts), and 50 μmol/L β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). HL-60 cells were differentiated into macrophages by 5 days of growth in the presence of 50 μmol/L recombinant tissue plasminogen activator [14]. RAW 274.7 cells were activated by 3 days of exposure to 100 nmol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich). Activated HL-60 and RAW 264.7 macrophages were harvested after scraping with BD Falcon cell scrapers (Fischer Scientific).

Cellular accumulation of oritavancin was first confirmed in HL-60 cells at a fixed extracellular concentration (25 μg/mL) following procedures published elsewhere [17]. This concentration was chosen because it is also reflective of the predicted maximum free drug serum concentration achieved by administration of a 1200 mg clinical dose of the drug and approximates the limit of solubility of oritavancin at physiological pH in growth medium [18, 19]. Briefly, cells incubated with [^{14}C]-labeled oritavancin were washed 3 times in ice-cold phosphate-buffered saline, collected by scraping in distilled water, and used for radioactivity determination (liquid scintillation counting) and protein assay. The apparent cellular-to-extracellular concentration ratio was calculated by using a conversion factor of 5 μL cell volume per milligram of cell protein. Oritavancin accumulated substantially in HL-60 cells, reaching intracellular concentrations 200-fold above the extracellular concentrations after 24 hours incubation (Figure 1). This value is similar to what was observed earlier for murine J774 macrophages [20], which suggests that it corresponds to an intrinsic property of oritavancin in macrophages.

To test impact on cidal activity, HL-60 and RAW 264.7 cells were loaded with oritavancin (25 μg/mL) over 24 hours; as a negative control, other cells were loaded with azithromycin at 10 μg/mL, as described elsewhere [17]. After drug loading, the adherent cells were scraped and rinsed 3 times in Hanks Balanced Salt Solution (HBSS). As a positive control to suppress macrophage killing of microbes by inhibiting superoxide production, some cells were exposed to 1 nmol/L diphenylene iodonium (DPI) for 1 hour prior to cell harvesting [21–25].

To test macrophage killing, *S. aureus* strain LAC (methicillin-resistant clinical isolate) and *A. baumannii* HUMC1 (carbapenem-resistant, clinical bloodstream isolate) were cultured overnight in tryptic soy broth at 37°C, and *C. albicans* 15563 (clinical bloodstream isolate) was cultured overnight at 30°C in yeast peptone dextrose (YPD). The overnight cultures were passaged and organisms grown to mid log-phase prior to use in the killing assay. The killing assay was based on a modification of a method used elsewhere [26, 27]. In brief, macrophages were scraped and rinsed in HBSS as above. Microbes were cocultured in polystyrene snap cap tubes in a rotating drum at 37°C. On the basis of pilot studies,

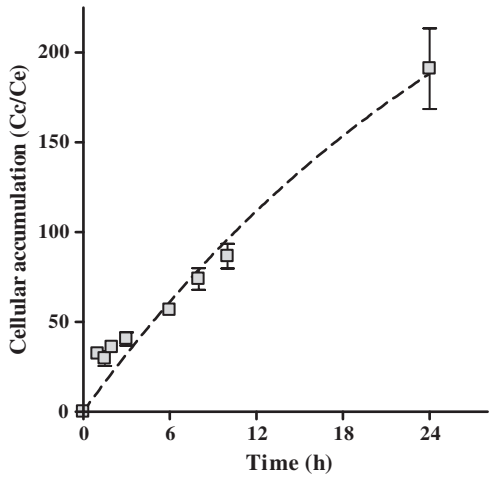

Figure 1. Influence of time on the cellular accumulation of oritavancin in HL-60 cells. Kinetics of the cellular accumulation of oritavancin (25 μg/mL) in HL-60 cells (complete culture medium, 10% fetal bovine serum). Results are shown as the apparent cellular to extracellular concentration ratio. Data are means ± standard deviations of 3 independent experiments. Abbreviation: Cc/Ce, cellular-to-extracellular concentration ratio.

HL-60 cells were cultured at a 200:1 ratio of macrophages to microbes, and RAW 264.7 cells were cultured at a 20:1 ratio. After a 1-hour incubation, the tubes were sonicated and quantitatively plated in tryptic soy agar for *S. aureus* and *A. baumannii* or YPD agar for *C. albicans*. Colony-forming units (CFUs) of the cocultured tubes were compared with CFUs of growth control tubes containing only microbes with no macrophages. Percent of killing was calculated as [1−(CFUs from coculture tubes/CFUs from growth control tubes)].

Both HL-60 and RAW 264.7 macrophages killed all 3 tested pathogens. Macrophage killing of *C. albicans* was not affected by oritavancin, whereas killing of *S. aureus* was substantially enhanced (Figure 2*A*). Oritavancin modestly reduced killing of *A. baumannii* by HL-60 cells (median [interquartile range (IQR)] killing = 24% [21%–28%] vs 16% [13%–18%], $P < .01$) but not by RAW 264.7 cells (median [IQR] killing = 37% [27%–52%] vs 35% [18%–50%], $P = .8$). DPI significantly reduced killing of all 3 organisms by both HL-60 and RAW 264.7 cells, including those compared with oritavancin-preloaded macrophages (Figure 2*B*).

Thus, in contrast to DPI, which suppressed production of reactive oxygen intermediates and which inhibited macrophage killing of all 3 extracellular pathogens, killing of *C. albicans* or *A. baumannii* remained intact after loading of macrophages with substantial levels of oritavancin or azithromycin. Although oritavancin mediated a modest reduction in killing of *A. baumannii* by HL-60 cells, the killing was similar to that following azithromycin; no similar reduction was seen with RAW cells, and there was no reduction against other pathogens. These

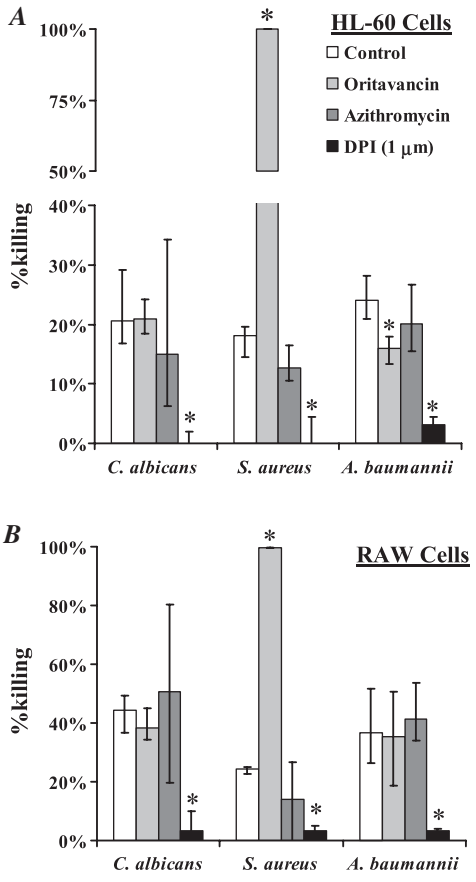

Figure 2. Macrophage killing of bacterial and fungal pathogens is not inhibited by oritavancin. Human HL-60 (*A*) or mouse RAW 264.7 (*B*) macrophages were cocultured with *Staphylococcus aureus, Acinetobacter baumannii*, or *Candida albicans* with or without preexposure to oritavancin, azithromycin, or diphenylene iodonium (DPI; a superoxide inhibitor). Median and interquartile ranges are shown from 6 to 12 samples each, performed in duplicate from 2 to 3 separate experiments. $^*P < .05$ versus killing by macrophages without preexposure to any substance.

data indicate that accumulation of lipoglycopeptides in macrophages does not necessarily correspond with dysfunction of macrophage killing of microbes, and provide reassurance that oritavancin accumulation does not prevent phagocytic killing of key pathogens.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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