Miltefosine Reduces the Cytolytic Activity and Virulence of *Acinetobacter baumannii*

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ABSTRACT Stagnation in antimicrobial development has led to a serious threat to public health because some *Acinetobacter baumannii* infections have become untreatable. New therapeutics with alternative mechanisms of action to combat *A. baumannii* are therefore necessary to treat these infections. To this end, the virulence of *A. baumannii* isolates with various antimicrobial susceptibilities was assessed when the isolates were treated with miltefosine, a phospholipase C inhibitor. Phospholipase C activity is a contributor to *A. baumannii* virulence associated with hemolysis, cytolysis of A549 human alveolar epithelial cells, and increased mortality in the *Gal-leria mellonella* experimental infection model. While the effects on bacterial growth were variable among strains, miltefosine treatment significantly reduced both the hemolytic and cytolytic activity of all treated *A. baumannii* strains. Additionally, scanning electron microscopy of polarized A549 cells infected with bacteria of the *A. baumannii* ATCC 19606^T strain or the AB5075 multidrug-resistant isolate showed a decrease in A549 cell damage with a concomitant increase in the presence of A549 surfactant upon administration of miltefosine. The therapeutic ability of miltefosine was further supported by the results of *G. mellonella* infections, wherein miltefosine treatment of animals infected with ATCC 19606^T significantly decreased mortality. These data demonstrate that inhibition of phospholipase C activity results in the overall reduction of *A. baumannii* virulence in both *in vitro* and *in vivo* models, making miltefosine a viable option for the treatment of *A. baumannii* infections, particularly those caused by multidrug-resistant isolates.

KEYWORDS *Acinetobacter baumannii* virulence, alkylphosphocholine drugs, antivirulence therapeutic, miltefosine, phospholipase C inhibition

The increasing occurrence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacteria has become a global problem in the health care setting that warrants immediate attention; however, antibiotic resistance in bacteria is not only a contemporary problem. *Staphylococcus* isolates resistant to penicillin were reported as early as 1940 (3 years before penicillin had become widely used throughout the population), likely because of the inherent presence of antibiotic resistance genes in natural microbial populations and/or the intermittent use of the drug (1–3). Following the discovery of penicillin, many other antibiotics were discovered, resulting in 20 new classes of antibiotics reaching the market by the 1970s (4, 5). Resistance to these antibiotics followed with increasing rapidity. Bacteria resistant to tetracycline, erythromycin, gentamicin, vancomycin, or imipenem were reported within 16 years following the introduction of each of these antibiotics (3). Resistances to methicillin, ceftazidime, linezolid, ceftaroline, and levofloxacin were all reported within

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FIG 1 Chemical structure of miltefosine.

2 years or less of their respective introductions, with resistance to levofloxacin being reported in the same year as its release (3). There are many reasons purported to have led to the current plight of antibiotic resistance; among these are inappropriate prescribing practices, the rampant use of antibiotics in agriculture, and the stagnation of novel antibiotic development primarily due to regulatory obstacles (3). The current situation should be considered especially dire when the occurrence of XDR and PDR organisms, such as XDR *Mycobacterium tuberculosis* (originally described in 2000) and PDR *Acinetobacter*, *Pseudomonas*, and *Enterobacteriaceae* (initially reported between 2004 and 2009), is taken into consideration (3).

When considering the increased prevalence of XDR and PDR isolates, the development of antibiotics to combat these organisms is of paramount importance. One explanation for the ever-increasing amount of antibiotic resistance reported is the mechanism by which bactericidal antibiotics function. Bactericidal antibiotics put tremendous selective pressure on the large number of bacterial cells involved in an infection to adapt or die, thus causing the selection of a small subset of bacteria that have undergone mutations or received a resistance gene from another bacterial species or even genus that allows them to survive and potentially thrive in medical settings (4). In order to circumvent this resistance problem, many researchers are turning to antivirulence strategies that are not bactericidal but instead impair the pathogen's ability to establish an infection, usually in a species-specific manner by targeting the biosynthesis of glycolipid surface structures, type III secretion systems, quorum sensing, or toxins (4). These antivirulence therapeutics reduce the virulence of a bacterium, allowing immune clearance or antibiotic clearance of the pathogen, if antibiotics are used in combination with the antivirulence agent.

Miltefosine, a phosphatidylcholine (PC) analog belonging to the class of alkylphosphocholine drugs, may be viewed as an antivirulence therapeutic (Fig. 1). Miltefosine was previously shown to have activity against both visceral and cutaneous leishmaniasis, trypanosomes, *Entamoeba histolytica*, *Acanthamoeba* species, *Trichomonas vaginalis*, *Schistosoma mansoni*, various fungi, HIV-1, *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and *Pseudomonas aeruginosa*, in addition to possessing both antineoplastic activity and immunomodulatory effects that induce cytokine release (6). Miltefosine competitively inhibits the phospholipase C (PLC) produced by *P. aeruginosa*, thus reducing the cleavage of choline-containing lipids and improving lung function during infection (7). Drug-resistant bacterial pathogens producing phospholipases that target choline-containing lipids in the host may therefore have the potential of being targeted using miltefosine.

Acinetobacter baumannii is a Gram-negative coccobacillus that produces two such phosphatidylcholine-specific phospholipase Cs (PC-PLCs) that play major roles in the hemolysis and cytolysis of host cells (8). This pathogen can cause severe nosocomial infections, such as bacteremia, meningitis, pneumonia, skin and soft tissue infections, and urinary tract infections, particularly in immunocompromised patients (9). Considered a severe human health threat by the CDC (<http://www.cdc.gov/drugresistance/threat-report-2013>) and a pathogen with a critical need for new antibiotic research and development by the World Health Organization (<http://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed>), *Acinetobacter*

strains cause 12,000 infections, 7,300 MDR infections, and 500 deaths per year in the United States. The resistance mechanisms of this pathogen have consequently been investigated; however, there is a paucity of information concerning this pathogen's molecular mechanisms of virulence, making the development of therapeutics difficult (10). The sparse data available pertaining to the virulence mechanisms do, however, offer potential research avenues that could result in the identification of bacterial targets for alternative antivirulence therapeutics. This report describes the use of miltefosine to decrease the hemolytic, cytolytic, and overall virulence properties of *A. baumannii* when tested using horse erythrocytes, A549 human alveolar epithelial cells, and the *Galleria mellonella* virulence model, respectively.

RESULTS

The effect of miltefosine on growth varies among *A. baumannii* strains. Supplementation of 12 μM miltefosine in Trypticase soy broth dialysate (TSBD) cultures of *A. baumannii* ATCC 19606^T, ATCC 17978, AB5075, or AYE resulted in variable growth changes based upon the isolate tested (Fig. 2). There were no significant changes in the growth of the 3494 strain when the culture was supplemented with 12 μM miltefosine (Fig. 2). At 20 h of incubation, there was, however, a significant reduction in the growth of the ATCC 19606^T strain ($P \leq 0.01$) and a significant increase in the growth of the AB5075 ($P \leq 0.05$) isolate. Strain-specific growth pattern variability was observed when cultures were supplemented with 300 nM miltefosine, similar to the results observed with supplementation of 12 μM miltefosine. Additionally, *A. baumannii* strains inoculated into M9 minimal medium containing only miltefosine as a potential carbon source were unable to grow (data not shown), indicating that miltefosine does not serve as a carbon source for strains shown to have increased growth in its presence. While miltefosine was shown to cause mathematically significant changes to *A. baumannii* growth, the biological significance of these growth changes and the mechanism(s) by which they occur remain unclear.

Miltefosine inhibits the hemolytic activity of *A. baumannii*. Horse erythrocytes were used in the hemolysis assays due to the phosphatidylcholine content of horse erythrocytes more closely mimicking that of human erythrocytes than that of sheep erythrocytes, which are typically used for diagnostics in clinical microbiology but which contain minimal phosphatidylcholine (8). Enumeration of the intact horse erythrocytes remaining in the TSBD culture medium after infection with ATCC 19606^T and treatment with increasing concentrations of miltefosine ranging from 0 μM to 12 μM demonstrated that miltefosine can competitively inhibit the hemolytic activity of this strain in a dose-dependent manner (Fig. 3A). In fact, the number of horse erythrocytes remaining after infection with ATCC 19606^T and administration of 12 μM miltefosine was not statistically different from that for the uninfected control (Fig. 3A). Enumeration of the horse erythrocytes remaining in the TSBD culture after infection with other *A. baumannii* isolates in the presence or absence of 12 μM miltefosine also demonstrated this therapeutic's ability to reduce hemolytic activity; however, the protective effect was the greatest with the ATCC 19606^T strain (Fig. 3B). Only the hemolytic activity of the 3494 strain, an ATCC 19606^T isogenic derivative which contains interruptions in both *plc-1* and *plc-2* (Table 1), was unaffected by miltefosine treatment (Fig. 3B). Since the hemolytic activity of *A. baumannii* has only recently been described (8, 11, 12), A549 cells were utilized as an established model to quantify the activity of miltefosine on *A. baumannii* virulence.

Miltefosine decreases the cytolytic activity of *A. baumannii* strains against A549 cells. Due to the prevalence of *A. baumannii*-associated pneumonia, the ability of miltefosine to mitigate the cytolysis of A549 human alveolar epithelial cells by *A. baumannii* isolates was also investigated.

The most efficacious dosage of miltefosine (12 μM), as determined by hemolysis assays, was used in the cytolysis assays. All tested *A. baumannii* strains were significantly reduced in their ability to lyse A549 cells, as assessed using the CellTiter-Glo assay (Fig. 4; $P \leq 0.05$).

FIG 2 *A. baumannii* growth curves with or without supplementation of 12 μ M miltefosine. *A. baumannii* ATCC 19606^T (A), ATCC 17978 (B), AYE (C), AB5075 (D), or 3494 (E) was propagated with or without 12 μ M miltefosine in TSBD. The OD₆₀₀ values of sextuplet cultures incubated at 37°C for 20 h with shaking at 200 rpm were recorded hourly. Error bars represent the standard error (SE) of the mean.

FIG 3 Hemolytic activity of *A. baumannii* with miltefosine supplementation. Horse erythrocytes were quantified following incubation with the ATCC 19606^T isolate (A) or one strain of a collection of *A. baumannii* clinical isolates (B) at 37°C for 20 h with shaking at 200 rpm in TSBD supplemented with miltefosine. The hemolytic activity of the ACICU strain was not determined (ND). Error bars represent the standard error (SE) of the mean.

Interestingly, incubation of A549 cells with miltefosine alone at concentrations ranging from 0 μM to 25 μM demonstrated that miltefosine can cause a statistically significant decrease in the number of viable A549 cells at concentrations at and above 390 nM (Fig. 5A; $P \leq 0.0001$). This is not surprising, however, considering the use of miltefosine as an anticancer drug (13) and the fact that A549 cells are adenocarcinoma cells isolated from the lung tissue of a 58-year-old Caucasian man (14). Due to this inherent limitation of the A549 cell model and the lack of a significant impact on the viability of A549 cells at miltefosine concentrations below 390 nM, a subset of *A. baumannii* strains was also treated using 300 nM miltefosine as the experimental dosage to determine the feasibility of using miltefosine in *A. baumannii*-associated lung

TABLE 1 Strains used in this study^a

<i>A. baumannii</i> strains	Relevant characteristic(s)	Source or reference
ATCC 17978	Clinical isolate	ATCC
ATCC 19606 ^T	Clinical isolate, type strain	ATCC
ATCC 19606 ^T 3494	<i>plc-1::ermAM plc-2::aph</i> derivative of ATCC 19606 ^T	8
AB5075	Clinical isolate	16
ACICU	Clinical isolate	36
AYE	Clinical isolate	ATCC
LUH 07672	Clinical isolate, EU clone III	37
LUH 08809	Clinical isolate, EU clone I	38
LUH 05875	Clinical isolate, reference strain, EU clone III	39
LUH 13000	Clinical isolate, EU clone II	L. Dijkshoorn
RUH 00134	Clinical isolate, reference strain, EU clone II	40
RUH 00875	Clinical isolate, reference strain, EU clone I	40

^aATCC, American Type Culture Collection; EU, European clone.

infections. As demonstrated at a dosage of 12 μ M, miltefosine at a dosage of 300 nM was also able to significantly decrease the cytolysis of A549 cells as a result of infections caused by *A. baumannii* ATCC 19606^T, ATCC 17978, AB5075, and AYE ($P \leq 0.05$ for all strains), albeit to a lesser degree, further highlighting the potential for the use of this compound as an effective therapeutic agent (Fig. 5B). Miltefosine's inability to decrease the cytolytic activity of the 3494 strain, which contains interruptions in both *plc-1* and *plc-2*, strongly implicates the protein products of these genes as targets for this therapeutic (Fig. 5B).

A. baumannii ATCC 19606^T and AB5075 were inoculated onto polarized A549 cells and imaged using scanning electron microscopy (SEM) to further examine the effect of miltefosine in an environment that closely mimics that of an *A. baumannii* infection of the lungs. Micrographs of uninfected polarized A549 cells left untreated or treated with 300 nM miltefosine demonstrated that the presence of miltefosine did not negatively affect either the formation of surfactant produced from A549 cells or the number of A549 cells present in the analyzed samples (Fig. 6A and B, respectively). SEM of polarized A549 cells infected with either ATCC 19606^T or AB5075 demonstrated that both strains not only are capable of causing damage to the A549 cells but also are able to cause an apparent reduction in the amount of surfactant covering these cells (Fig. 6C and E, respectively). In the presence of 300 nM miltefosine, however, the damage to

FIG 4 Cytolytic activity of *A. baumannii* at a miltefosine dosage of 12 μ M. A549 cell monolayers were infected with *A. baumannii* isolates in the presence of 0 μ M or 12 μ M miltefosine for 20 h at 37°C in 5% CO₂. CellTiter-Glo luminescent cell viability assays were used to determine the cytolytic activity of the *A. baumannii* isolates in the presence of miltefosine. Error bars represent the standard error (SE) of the mean for three different biological samples measured in duplicate ($n = 6$). RLU, relative luminescence units.

FIG 5 A549 cell culture assays. (A) The effect of the miltefosine dosage, which ranged from 0 to 25 μ M, on A549 cell viability was tested using the CellTiter-Glo luminescent cell viability assay. (B) A549 cell monolayers were infected with a subset of *A. baumannii* isolates, and CellTiter-Glo assays were performed to determine the cytolytic activity of these isolates in the absence or the presence of 300 nM miltefosine. A549 cells were maintained for 20 h at 37°C in 5% CO₂. Error bars represent the standard error (SE) of the mean for three different biological samples measured in duplicate ($n = 6$). RLU, relative luminescence units.

A549 cells and the breakdown of surfactant by either ATCC 19606^T or AB5075 were both visibly reduced to levels comparable to those detected in the noninfected samples (Fig. 6D and F, respectively). Taken together, these data indicate that miltefosine is efficacious in mitigating lung surfactant loss and damage to lung epithelial cells.

Miltefosine increases survival in the *in vivo* *G. mellonella* model. The *G. mellonella* virulence model shows that infection of larvae with ATCC 19606^T resulted in a 43% mortality rate, which is significantly higher than the 3% mortality rate ($P \leq 0.05$) of noninjected larvae or larvae injected with sterile PBS (17%), both of which were used as negative controls (Fig. 7). Additionally, larvae injected with ATCC 19606^T alone had a significantly higher mortality rate (43%) than larvae injected with ATCC 19606^T and miltefosine (27% mortality) ($P \leq 0.05$). In fact, the mortality rate of larvae injected with ATCC 19606^T together with 12 μ M miltefosine was not statistically significantly different from that of larvae injected with phosphate-buffered saline (PBS) alone. When taken together with the findings of the aforementioned cell-based assays, miltefosine demonstrates a capacity to decrease the cytolytic activity and overall virulence of *A. baumannii*, likely by reducing the ability of *A. baumannii* to lyse cells and degrade lung surfactant by competitively inhibiting phosphatidylcholine-specific phospholipase C activity.

DISCUSSION

Previous research utilizing a collection of *A. baumannii* isolates grown under iron-limiting conditions demonstrated that cell-free supernatants obtained from these bacteria exhibited PC-PLC activity, which is a critical component of *A. baumannii* virulence (8). This activity was attributed to the protein products of the *plc-1* and *plc-2* phospholipase genes that are present in the genomes of all sequenced *A. baumannii* isolates but absent in the genome of nonpathogenic *Acinetobacter baylyi* APD1. The PC-PLC activity of *A. baumannii* can specifically contribute to hemolysis, the cytolysis of alveolar epithelial cells, and overall virulence, determined using the *G. mellonella* virulence model, when this bacterium is propagated in an iron-limiting environment, such as that encountered during an infection of the human body (8). These previous findings supported investigation of the antivirulence properties of miltefosine, a phosphatidylcholine analog, against *A. baumannii* isolates differing in their source, time of collection, clonal lineage, and antibiotic susceptibilities. Our data show that miltefosine inhibited the hemolytic activity of ATCC 19606^T in a dose-dependent manner when

FIG 6 Scanning electron microscopy of polarized A549 cells infected with *A. baumannii* and treated with miltefosine. (A, B) SEM was performed to visualize uninfected polarized A549 cells cultured in the absence (A) or presence (B) of 300 nM miltefosine, both of which served as negative controls. The white arrow in panel A identifies surfactant. (C to F) Polarized A549 cells were infected with ATCC 19606^T and left untreated (C) or treated with 300 nM miltefosine (D) or infected with AB5075 and left untreated (E) or treated with 300 nM miltefosine (F). The white arrows in panels D and E identify an individual A549 cell and the Transwell membrane of the support plate, respectively. Polarized A549 cells treated with miltefosine were supplemented with this drug at 24-h intervals over the course of 72-h infections. Micrographs were captured at a magnification of $\times 5,000$. Bars, 2 μm .

tested using horse erythrocytes in iron-limiting TSBD. The hemolytic activities of all other tested *A. baumannii* isolates, with the exception of the 3494 isogenic insertion derivative, were also reduced when the isolates were grown in the presence of miltefosine. The lack of response of the 3494 strain to miltefosine is not surprising, as this ATCC 19606^T strain contains interruptions in both *plc-1* and *plc-2*, whose protein products most likely serve as targets for miltefosine. While the effect of miltefosine on the growth of *A. baumannii* isolates was variable between isolates, the effect of this drug on the cytolytic ability of *A. baumannii*, as assessed using A549 cells and the CellTiter-Glo assay, yielded consistent and comparably positive therapeutic results for all tested isolates. Scanning electron microscopy of polarized epithelial cells infected with either ATCC 19606^T or AB5075 and treated with 300 nM miltefosine over the course of 3 days visibly demonstrated that miltefosine treatment results in protection of A549 cell surfactant as well as decreased lysis of A549 cells proper during infection. Most notably, the AB5075 isolate caused A549 cell lysis and surfactant loss to the point of exposing the underlying Transwell membranes on which the polarized A549 cells were propagated, a phenomenon that was abrogated in the presence of miltefosine (Fig. 6E and F). This finding is not surprising, considering that the data presented in Fig. 4, 5B, and 6 demonstrate an increase in the number of viable A549 cells remaining after *A. baumannii* infections when the cells were treated with miltefosine, thus allowing more viable A549 cells present to produce surfactant. In this environment, miltefosine could be protective of lung surfactant, since our previous work has shown the ability

FIG 7 Effect of miltefosine on *A. baumannii* ATCC 19606^T virulence. *G. mellonella* larvae ($n = 60$) were injected with the ATCC 19606^T strain and incubated in the darkness at 37°C for 5 days. Uninjected larvae or larvae injected with sterile PBS alone served as negative controls. The number of dead larvae was recorded at daily intervals for 5 days, with the removal of dead larvae at each time point. This model shows that miltefosine significantly reduces the virulence of ATCC 19606^T ($P \leq 0.05$).

of *A. baumannii* to degrade phosphatidylcholine, a major component of lung surfactant (8, 15). These data parallel the results obtained with the *in vivo* *G. mellonella* virulence model, wherein the treatment of larvae infected with ATCC 19606^T with miltefosine resulted in a mortality rate that was significantly lower than that observed in larvae injected with ATCC 19606^T and left untreated. In fact, treatment with miltefosine decreased the mortality rate of infected larvae to a degree not significantly different from the mortality rate of larvae injected with PBS alone. We expect that similar results would be obtained if strains such as AB5075 were tested using the *G. mellonella* model, due to the similarity in PC-PLC activity (8) and hemolytic activity between these two *A. baumannii* strains. These findings are relevant when considering the lack of viable therapeutics to treat *A. baumannii* isolates with MDR phenotypes, such as the highly virulent multidrug-resistant AB5075 strain (16), and the occurrence of isolates causing severe infections, such as those associated with two lethal cases of necrotizing fasciitis (17).

Our results are particularly encouraging considering the dosages used in this study. At a concentration of 12 μM miltefosine, there were significant decreases in hemolysis, cytolysis, surfactant loss, and *G. mellonella* mortality rates resulting from *A. baumannii* experimental infections. This dosage of miltefosine is within the range tested to treat other pathogenic bacteria, such as *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and *P. aeruginosa*, and below those shown to be effective against trypanosomes and protozoans (6). The half-maximal effective concentration (EC_{50}) of miltefosine *in vitro* for *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense*, *Entamoeba histolytica*, and *Trichomonas vaginalis* was established to be 35.5 μM, 47 μM, 53 μM, and up to 40 μM, respectively (18–20). Miltefosine's mechanism of action against these trypanosomes and protozoans has not been definitively confirmed; however, it has been postulated that the activity is related to inhibition of phosphatidylcholine biosynthesis, membrane alteration, and/or inhibition of phospholipid signaling pathways (20–23). The MIC of miltefosine for *Streptococcus pneumoniae* strains ranged from 5 to 6.25 μM, while the MIC for *Streptococcus mitis* and *Streptococcus pyogenes* was 10 μM and the MIC for *Streptococcus agalactiae* and *Streptococcus mutans* was 20 μM (24). The MICs for methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), and vancomycin-resistant *Enterococcus* (VRE) were higher yet at 22 μM for MRSA and 44 μM for both MSSA and VRE (25). The mechanism by which miltefosine acts against *S. pneumoniae* involves the *in vitro* promotion of LysA autolysin activity

(24), whereas the mechanism against MRSA and VRE is not completely understood but has been theorized to involve disruption of lipid membranes via sphingomyelin synthesis inhibition, phosphatidylcholine synthesis/transport inhibition, or a reduction of choline uptake (25). It has also been demonstrated that miltefosine itself exhibits hemolytic characteristics at concentrations greater than 17.5 μM (25); however, the dose at which this property of miltefosine is achieved is limited to doses higher than those used in this study. While the doses of miltefosine tested in our study are not bactericidal to *A. baumannii*, they do have significant efficacy against this bacterium at dosages far below those required to treat other pathogens. Additionally, the dosage suggested for miltefosine by the United States Food and Drug Administration (FDA) is one 50-mg capsule twice daily for 28 consecutive days (<https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=bc387ac-2e90-4f5e-94b2-d3635190678e#boxedwarning>). While the plasma protein binding of miltefosine limits the availability of free miltefosine to 2% to 4% (26), studies in which individuals received the FDA-approved dosing regimen have demonstrated concentrations of miltefosine reaching 70 $\mu\text{g/ml}$ in the last week of treatment (27), a concentration that is far above the 300 nM (0.12- $\mu\text{g/ml}$) and 12 μM (4.8- $\mu\text{g/ml}$) concentrations used in this study.

The manner in which miltefosine has been shown to decrease *P. aeruginosa* virulence is likely most akin to the manner in which it acts against *A. baumannii*. Specifically, miltefosine has been shown to provide therapeutic benefits against pulmonary infections caused by *P. aeruginosa* without being bactericidal or bacteriostatic to this bacterium (7, 24). Miltefosine was protective of pulmonary function in *P. aeruginosa*-infected mice with pneumonia via inhibition of a phospholipase C/sphingomyelinase (PlcH), a known virulence factor of this pathogen that disrupts the physiological role of lung surfactant (7). Miltefosine was specifically shown to (i) inhibit PlcH from acting on sphingomyelin, thus preventing *P. aeruginosa* from causing hemolysis of sheep erythrocytes, (ii) protect against the loss of surfactant function by PlcH, and (iii) protect lung function in a murine model without changing the bacterial burden (7). These data coincide with our findings, in that miltefosine has an inhibitory effect on the PC-PLCs produced by *A. baumannii*, resulting in a dose-dependent decrease in hemolysis, decreases in A549 cell death and surfactant loss, as well as decreased mortality in the *G. mellonella* experimental infection model. Even when miltefosine does not reduce the growth of *A. baumannii* in infected alveolar epithelial cells, miltefosine is still able to significantly decrease the cytolysis of human cells and prevent the breakdown of surfactant. It is noteworthy that the dosages showing efficacy mitigating hemolysis in this study were lower than those shown to be efficacious in *P. aeruginosa* studies. This is likely due to the increased activity of the hemolytic phospholipase C produced by *P. aeruginosa* compared to that of the phosphatidylcholine-specific phospholipase Cs produced by *A. baumannii* strains (unpublished data). These data further underscore the potential role of miltefosine as an antivirulence therapy for *A. baumannii*-associated infections.

In conclusion, our work purports that miltefosine is a valuable antivirulence therapeutic that should not impart high selective pressure on *A. baumannii* and that is capable of decreasing the cytolysis of A549 cells, the loss of A549 cell-produced surfactant, and overall virulence, regardless of the isolate's source, time of collection, clonal lineage, or antibiotic susceptibility or miltefosine's effect on bacterial growth. With the ever-increasing incidence of MDR *A. baumannii* isolates, the severity of infections reported in the literature, and the scant availability of effective therapeutics to treat infections caused by this opportunistic pathogen, miltefosine offers an interesting therapeutic alternative that should not drive the acquisition of antibiotic resistance through selective pressure, an unfortunately common ability of this pathogen that could lead to the occurrence of isolates against which feasible antimicrobial treatment is lacking. While the therapeutic benefit of treating an *A. baumannii* infection with miltefosine alone remains to be seen, future research will study the ability of miltefosine to eradicate MDR isolates of *A. baumannii* when used in combination with currently utilized antibiotics.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The *A. baumannii* strains used in this work were routinely stored as Luria-Bertani (LB) broth glycerol stocks at -80°C (28) and are listed in Table 1. The *A. baumannii* ATCC 19606^T strain was used as the model organism throughout this study due to it being the *A. baumannii* type strain as well as its multidrug-resistant phenotype, as determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards, which demonstrated this isolate's resistance to three antibiotic classes clinically used to treat *A. baumannii*: (i) penicillins, (ii) cephalosporins, and (iii) aminoglycosides (29). *A. baumannii* strains were subcultured from LB agar into Chelex 100-treated Trypticase soy broth dialysate (TSBD) and grown for 24 h at 37°C with shaking at 200 rpm (30). Fresh TSBD or TSBD containing 10% defibrinated horse erythrocytes (Cleveland Scientific, Ltd.), both with the appropriate concentration of miltefosine (Sigma-Aldrich), was then inoculated with *A. baumannii* at a 1/100 ratio, and the bacteria were grown for 20 h at 37°C with shaking at 200 rpm, unless otherwise indicated. Culture medium supplemented with erythrocytes was prepared as previously described (31). A 5 mM stock solution of miltefosine was prepared in water and sterilized by filtration through a 0.22- μm -pore-size filter for use whenever miltefosine was required. *A. baumannii* growth curves were determined in sextuplet using 96-well microtiter plates containing TSBD under the aforementioned culturing conditions over a 20-h time period. The values of the optical density at 600 nm (OD_{600}) of these cultures were recorded hourly. Growth curve analyses of the *A. baumannii* isolates were also performed under the aforementioned culturing conditions over a 20-h time period in quadruplicate using 96-well microtiter plates containing phosphate M9 salts (3 g/liter KH_2PO_4 , 6 g/liter Na_2HPO_4 , 5 g/liter NaCl, and 1 g/liter NH_4Cl supplemented with 1.5 mg/liter CaCl_2 and 12 mg/liter MgSO_4 and adjusted to pH 7.4) or M9 salts supplemented with 0.5% (wt/vol) glucose alone or supplemented with both 0.5% (wt/vol) glucose and 0.2% (wt/vol) Casamino Acids in the presence or absence of miltefosine.

Hemolysis assays. Erythrocytes were diluted 1:1,000 in 0.22- μm -pore-size filter-sterilized FACSFlow sheath fluid (BD Biosciences) for enumeration using flow cytometry on a FACScan flow cytometer (BD Biosciences) following incubation with miltefosine alone or *A. baumannii* ATCC 19606^T and various concentrations of miltefosine together. Volumes were standardized among 5-s samplings through the addition of Flow Cytometry Absolute Count Standard beads (Bangs Laboratories, Inc.) at a 1:40 dilution. Forward and side scatter channels were used to gate erythrocyte populations. For analysis of miltefosine's influence on the hemolytic activity of *A. baumannii* isolates, in addition to that on the hemolytic activity of the ATCC 19606^T strain, erythrocytes were diluted 1:1,000 into 0.22- μm -pore-size filter-sterilized erythrocyte wash buffer (20 mM KH_2PO_4 , 60 mM Na_2HPO_4 , 120 mM NaCl, pH 8.0 [31]) and enumerated on an Attune NXT flow cytometer (Invitrogen) following incubation for 20 h with individual bacterial strains in the presence or absence of 12 μM miltefosine. Individual erythrocytes were quantified using forward and side scatter channels to distinguish intact erythrocytes from debris. Standardized volumes were analyzed and used to calculate the number of erythrocytes per milliliter.

Cytolysis assays. A549 human alveolar epithelial cell cytolysis was quantified as previously described (8). Briefly, A549 cells were passaged three times in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% ampicillin-streptomycin in the presence of 5% CO_2 at 37°C . A white, opaque, 96-well plate was seeded with approximately 1×10^5 A549 cells for a fourth passage and incubated under the aforementioned conditions without antibiotics. Miltefosine was applied to A549 cell monolayers at concentrations ranging from 0 to 25 μM to test the interaction between this therapeutic and A549 cells. Additionally, each *A. baumannii* strain, at a concentration of 1×10^6 bacterial cells/ml, was used to infect A549 cell monolayers in the presence or absence of miltefosine. The monolayers were incubated for 20 h at 37°C in the presence of 5% CO_2 . DMEM was used to wash the cell monolayers three times prior to cytolysis assays using the CellTiter-Glo luminescent cell viability assay (Promega) following the manufacturer's instructions. The relative number of viable A549 cells remaining following infection was reported as the ratio of the number of relative luminescence units (RLUs) produced following lysis of infected A549 cells versus the number produced following lysis of uninfected A549 cells. Cytolysis trials were repeated using three different biological samples in duplicate ($n = 6$).

Polarization, infection, and SEM imaging of A549 human alveolar epithelial cells. Scanning electron microscopic (SEM) imaging of polarized A549 cells infected with *A. baumannii* was performed to visualize the response of A549 cells to *A. baumannii* infection after treatment with miltefosine, while the aforementioned CellTiter-Glo assays served for quantification and statistical analysis of this response. Polarization and infection of A549 cells were performed as previously described (32). Briefly, A549 cells were passaged as described above prior to seeding of approximately 1×10^5 A549 cells/ml onto each Transwell membrane of a 24-well Transwell membrane support plate (Corning). Fresh DMEM without antibiotics was exchanged above and below the membrane daily for 7 days, at which time DMEM was aspirated from above and below the membrane and replaced with fresh DMEM below the membrane only to begin the polarization process. Fresh DMEM was exchanged from below the membrane for 14 days or until the cell layer was confluent, watertight, and secreting surfactant. Once polarized, the A549 cells were infected with 10^6 *A. baumannii* bacteria/ml for 72 h, and 0 nM or 300 nM miltefosine was added to the apical surface of infected polarized cells at 24-h intervals. The *A. baumannii*-infected polarized cells were fixed after 72 h and prepared for scanning electron microscopy as previously described (33).

***G. mellonella* virulence assays.** Assays with the *G. mellonella* virulence model were conducted to assess the efficacy of miltefosine in treating *A. baumannii* infections *in vivo*, as previously described (34). Briefly, six replicates each consisting of 10 randomly selected healthy final-instar *G. mellonella* larvae ($n = 60$) were injected with ATCC 19606^T bacteria or ATCC 19606^T bacteria and 12 μM miltefosine, both

of which were suspended in sterile phosphate-buffered saline (PBS). Larvae injected with 5 μ l of sterile PBS and noninjected larvae were included as negative controls. Larvae were incubated in the darkness at 37°C following injection, and the numbers of dead larvae were assessed at 24-h intervals over 5 days, with the removal of dead larvae at the times of inspection. If more than two deaths were observed in any of the control groups, the trials were repeated.

Statistical analyses. The Mann-Whitney, Wilcoxon matched-pairs, Student's *t* test, or analysis of variance (ANOVA) with the Tukey *post hoc* test, all provided as part of the GraphPad InStat software package (GraphPad Software, Inc.), was used to analyze the statistical significance of the data, as appropriate for the data set. Survival curves were plotted using the Kaplan-Meier method (35) and analyzed for statistical significance using the log-rank test of survival curves (GraphPad Software, Inc.). Significances for all data analyses were set *a priori* at a *P* value of ≤ 0.05 .

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