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Rapid Killing of *Acinetobacter baumannii* by Polymyxins Is Mediated by a Hydroxyl Radical Death Pathway

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*Acinetobacter baumannii* is an opportunistic pathogen that is a cause of clinically significant nosocomial infections. Increasingly, clinical isolates of *A. baumannii* are extensively resistant to numerous antibiotics, and the use of polymyxin antibiotics against these infections is often the final treatment option. Historically, the polymyxins have been thought to kill bacteria through membrane lysis. Here, we present an alternative mechanism based on data demonstrating that polymyxins induce rapid cell death through hydroxyl radical production. Supporting this notion, we found that inhibition of radical production delays the ability of polymyxins to kill *A. baumannii*. Notably, we demonstrate that this mechanism of killing occurs in multidrug-resistant clinical isolates of *A. baumannii* and that this response is not induced in a polymyxin-resistant isolate. This study is the first to demonstrate that polymyxins induce rapid killing of *A. baumannii* and other Gram-negatives through hydroxyl radical production. This significantly augments our understanding of the mechanism of polymyxin action, which is critical knowledge toward the development of adjunctive therapies, particularly given the increasing necessity for treatment with these antibiotics in the clinical setting.

*Acinetobacter baumannii* is an increasingly prevalent opportunistic pathogen that causes nosocomial infections (5, 6, 11, 34, 43, 47). This Gram-negative, aerobic, coccobacillus is responsible for a significant number of hospital-acquired infections, including those of the skin and bloodstream, as well as pneumonia and meningitis (5, 6, 18, 34, 47). Importantly, *A. baumannii* is able to persist on hospital surfaces for weeks to months, providing an environmental reservoir for its transmission (44–46). Compounding this problem, multidrug-resistant (MDR) strains of *A. baumannii* have been isolated with increasing frequency, and strains with pan-drug resistance (PDR) have been described as well, particularly among vulnerable patients within intensive care units or military hospitals (3, 11, 15, 33, 36, 39, 41). The polymyxin class of antibiotics is generally considered a final option of antibiotic therapy against MDR strains of *A. baumannii*, in large part due to the high potential for nephrotoxicity. Nonetheless, clinical use of the polymyxins, including polymyxin B and polymyxin E (colistin), to treat *A. baumannii* infection is increasing out of necessity due to antibiotic resistance (25, 26, 50).

Polymyxins are non-ribosomally synthesized, cationic antimicrobial peptides that bind to lipid A in the outer leaflet of the Gram-negative outer membrane (10, 32). Positively charged amino acid residues in the polymyxins form a ring that associates with negatively charged residues within lipid A through electrostatic interactions, causing membrane perturbations (10). In addition, polymyxins contain a string of hydrophobic amino acids which insert into the outer membrane, increasing bacterial membrane permeability (10). It has often been assumed that these membrane disruptions cause bacterial cell death directly through membrane lysis. However, reports from as far back as the late 1970s indicate that under certain conditions, polymyxins are capable of killing bacteria without lysis, suggesting that another mechanism of bacterial cell death may also be induced by treatment with these antibiotics (8, 21).

Recently, it has been demonstrated that a number of classes of antibiotics induce the production of lethal hydroxyl radicals within bacteria through the Fenton reaction (12, 22). Briefly, this reaction occurs when superoxides are converted to peroxides by superoxide dismutases present in the cell. Peroxides are capable of interacting with ferric iron associated with a number of biological molecules within the bacterial cell, oxidizing the iron and forming hydroxyl radicals in the process (16, 17, 22, 49). Ultimately, the concentration of hydroxyl radicals reaches levels that cannot be controlled, and the subsequent oxidative damage to DNA, lipids, and proteins eventually causes cell death (12, 22). Although hydroxyl radical-mediated cell death has been demonstrated with antibiotics that target intracellular proteins (12, 22), it is not known whether the classes of antibiotics that directly target the outer membrane (such as the polymyxins) cause cell death through a similar mechanism.

Here, we demonstrate that polymyxin B and colistin initiate rapid killing of both sensitive and MDR isolates of *A. baumannii*, as well as other Gram-negative species, through hydroxyl radical production. Treatment of *A. baumannii* with these antibiotics caused an increase in hydroxyl radicals and, furthermore, killing of *A. baumannii* by the polymyxins was delayed in the presence of inhibitors that both directly and indirectly block the production of oxygen radicals through the Fenton reaction. To our knowledge, this is the first demonstration of how the polymyxin family in-
duces rapid killing of *A. baumannii* and provides a rationale for previous observations of polymyxin-induced death without lysis observed in other species. With an increasing number of *A. baumannii* isolates demonstrating multidrug resistance, this study may provide clues as to how to exploit hydroxyl radical-mediated cell death to combat drug resistance in this and other drug-resistant bacterial pathogens.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All *A. baumannii* strains (ATCC 17978, CI-2, CI-3, and CI-4), as well as *Escherichia coli* DH5α (Invitrogen, Grand Island, NY), were routinely grown from frozen stock in Mueller-Hinton (MH) broth (BD Biosciences, Sparks, MD) at 37°C with aeration. For *Francisella novicida* U112 (a gift from Denise Monack, Stanford University), 0.2% l-cysteine was added to the growth medium. CI-2, CI-3, and CI-4 were kindly provided by Brandi Limbago, Division of Health Care Quality Promotion, Centers for Disease Control and Prevention, Atlanta, GA. CI-2 was isolated in the District of Columbia in 2005, CI-3 was isolated in Ohio in 2006 by endotracheal aspirate, and CI-4 was isolated in Mississippi in 2010 from sputum.

**Determination of MICs.** Identification and antimicrobial susceptibility testing were performed using the MicroScan WalkAway Plus automated system (Siemens Medical Solutions Diagnostics, Deerfield, IL). Organism suspensions were prepared using the Prompt system (Siemens Medical Solutions Diagnostics) to inoculate Neg Combo 41 conventional identification susceptibility panels (Siemens Medical Solutions Diagnostics). Manual susceptibility testing using Etest strips (bioMérieux, Durham, NC) on MH agar was performed for colistin since it is not included on the MicroScan panel. Multidrug resistance (MDR) was defined as resistance to at least one member of three or more classes of antibiotic: aminoglycosides, beta-lactams, cephalosporins, or fluoroquinolones (29). Pan-drug resistance (PDR) was defined as resistance to all antibiotic therapies tested, including colistin (29).

**Detection of hydroxyl radicals.** Overnight cultures were subcultured 1:50 into MH broth for 2 h to an optical density at 600 nm of ~2. Cultures were centrifuged at 5,000 × *g* for 10 min, washed twice in phosphate-buffered saline (PBS; Lonza, Walkersville, MD), and diluted to 10^7 CFU/ml in PBS. The cells were subsequently treated with polymyxin B (USB, Cleveland, OH) or polymyxin E (colistin; Sigma-Aldrich, St. Louis, MO) at a final concentration of 2 μg/ml or with hydrogen peroxide at a final concentration of 0.15%, followed by incubation at 37°C with gentle shaking for 30 min. After treatment, the hydroxyl radical–specific fluorescent dye 3′-(p-hydroxyphenyl) fluorescein (HFP; Life Technologies, Grand Island, NY) was added to treated or untreated cultures at a final concentration of 5 μM. Fluorescence was immediately measured in a BioTek Synergy MX plate reader (BioTek, Winooski, VT) with an excitation setting of 490 nm and an emission setting of 515 nm, both with a 9-nm bandwidth.

**Time-kill assays.** To determine the levels of killing by antimicrobial compounds, time-kill experiments were performed as previously described (28). Overnight cultures were subcultured as described above and then diluted to a final concentration of 10^6 CFU/ml in MH broth. Samples were treated with 2 μg of either polymyxin B or colistin/ml (or 400 μg of colistin/ml for the *F. novicida* experiments) and incubated with aeration at 37°C. At the indicated time points, aliquots of treated cells were harvested, suitable dilutions were performed, and then the cells were plated onto MH agar plates. After overnight incubation of the plates at 37°C, CFU were enumerated. Thiourea (Sigma-Aldrich) was added to cultures concurrently at the indicated doses. When 2,2′-dipyridyl (MP Biomedical, Solon, OH) was utilized, the cells were pretreated for 20 min at 37°C with the indicated doses, before treatment with polymyxins. The addition of thiourea or 2,2′-dipyridyl did not alter growth kinetics in broth (see Fig. S1A and B in the supplemental material).

**Statistics.** All experiments were analyzed using a two-tailed, unpaired Student *t* test.

**RESULTS**

**Polymyxins induce hydroxyl radical production.** Polymyxins are thought to kill Gram-negative bacteria by binding to lipid A in the outer membrane and subsequently disrupting the stability of both the outer and inner membranes, ultimately leading to cell lysis (10, 32). Due to the observations that bacterial antibiotic treatment induces the production of hydroxyl radicals within bacteria and that these radicals play a significant role in causing bacterial cell death through oxidative damage to DNA, lipids, and proteins (22), we sought to determine whether the last line polymyxin antibiotics induced hydroxyl radical-mediated cell death in *A. baumannii*.

In order to first establish a baseline of *A. baumannii* sensitivities to polymyxins, MICs were determined for the type strain, ATCC 17978 ([Table 1](#tab1){refig}). We next determined the kinetics of polymyxin B-mediated killing of *A. baumannii*. Time-kill assays revealed that polymyxin B-mediated killing of *A. baumannii* occurred quickly, with a >3-log reduction of viable cells within 30 min ([Fig. 1](#fig1)). Furthermore, similar rapid killing kinetics occurred upon treatment with colistin (polymyxin E) at an identical dose ([Fig. 1](#fig1)).

![FIG 1 Polymyxins induce rapid killing of *A. baumannii.* *A. baumannii* cultures were treated with 2 μg of polymyxin B/ml (■) or colistin/ml (●) or left untreated (△). At 0, 15, and 30 min, cultures were plated, and the CFU were enumerated. The data are representative of three independent experiments. Points represent the means and bars represent the standard deviation of triplicate samples. ***, P < 0.0001.](#fig1)
In order to determine whether hydroxyl radicals were induced during the observed rapid polymyxin killing, we utilized the cell permeable, hydroxyl radical-specific fluorescent dye, 3′-(p-hydroxyphenyl) fluorescein (HPF) (12). As positive controls, we treated cultures with hydrogen peroxide or kanamycin (previously demonstrated to induce hydroxyl radicals [22]) for 30 min and measured HPF fluorescence compared to untreated cultures (Fig. 2A and B). We observed a 2-fold increase in fluorescence, indicating an increase in hydroxyl radical production. We next assayed the A. baumannii cultures treated with either polymyxin B or colistin and observed that both treatments induced a 2-fold increase in fluorescence compared to the untreated control (Fig. 2C and D). To demonstrate that the increased fluorescence was not simply due to bacterial lysis, we sonicated A. baumannii and observed that the resulting lysates did not produce increased HPF fluorescence above the untreated control (see Fig. S2 in the supplemental material). These data indicate that lysis is not sufficient to cause an induction of HPF fluorescence and, furthermore, that hydroxyl radicals are induced by treatment with the polymyxins.

To determine whether polymyxin-mediated radical production was limited to its action on A. baumannii, we utilized the Gram-negative species Escherichia coli and Francisella novicida, a model intracellular pathogen (19). We first determined the kinetics of colistin-mediated killing in these species (see Fig. S3A and B in the supplemental material) and observed that both species were killed with similar kinetics as A. baumannii at the doses utilized. We subsequently measured hydroxyl radical production and observed a significant increase in HPF fluorescence following both kanamycin and colistin treatment of these strains (see Fig. S4A to D). Together, these data indicate that treatment with polymyxins induces the production of hydroxyl radicals, and that radical production is concurrent with rapid killing of these Gram-negative species by polymyxins.

**Rapid polymyxin killing of A. baumannii is mediated by hydroxyl radicals.** Due to our observations that polymyxin B and colistin treatment induced hydroxyl radical production in A. baumannii, we sought to determine whether hydroxyl radicals were required for the rapid killing of A. baumannii. We therefore utilized a hydroxyl radical scavenging compound, thiourea (35), and assessed its ability to prevent polymyxin killing of A. baumannii. If hydroxyl radicals do indeed mediate the rapid killing of A. baumannii, then concurrent treatment with thiourea would be hypothesized to prevent cell death. In fact, when we performed time-kill assays in the presence of thiourea, we found a striking decrease in the ability of both polymyxin B and colistin to kill A. baumannii compared to treatment with either antibiotic alone (Fig. 3A and B). Notably, treatment with thiourea rescued survival to nearly the level observed in bacteria not treated with either antibiotic and markedly inhibited the rapid decrease in viability. We further examined the ability of thiourea to rescue colistin-mediated killing in E. coli and F. novicida and observed similar magnitudes of rescue in these organisms as well (see Fig. S3A and B in the supplemental material). As an additional control, we directly assayed the ability of thiourea to prevent colistin-mediated radical formation. We observed that thiourea significantly dampened the

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**FIG 2** Polymyxins induce hydroxyl radical production. A. baumannii cultures were left untreated or were treated with 0.15% hydrogen peroxide (H₂O₂) (A), 5 μg of kanamycin/ml (B), 2 μg of polymyxin B/ml (C), or 2 μg of colistin/ml (D) for 30 min. After treatment, the hydroxyl radical specific fluorescent dye 3′-(p-hydroxyphenyl) fluorescein was added, and the fluorescence was measured (490 nm/515 nm). The data are representative of three independent experiments. Bars represent the means and standard deviations of triplicate samples. ***, P < 0.0001.
Polymyxins Kill through Hydroxyl Radical Induction

FIG 3 Polymyxin killing is delayed by hydroxyl radical quenching. A. baumannii cultures were treated with either 2 μg of polymyxin B/ml (A) or 2 μg of colistin/ml (B) alone (●) or in combination with either 300 mM thiourea (○) or 600 mM thiourea (■), or they were left untreated in culture medium (△). At 0, 15, and 30 min, the cultures were plated, and the CFU were enumerated. The data are representative of three independent experiments. Points represent the means and bars represent the standard deviations of triplicate samples. ***, P < 0.0001; *, P < 0.05.

amount of colistin-mediated radical formation in A. baumannii, as well as in E. coli and F. novicida (see Fig. S5A to C in the supplemental material). Since quenching hydroxyl radicals delays killing, these data indicate that the radicals induced by the polymyxins play a significant role in mediating rapid cell death in A. baumannii and other Gram-negative species.

Polymyxin killing of A. baumannii is delayed by inhibition of the Fenton reaction. Previous studies have indicated that reactive oxygen species initiate an exponential increase in hydroxyl radical production through an intracellular Fenton reaction (12, 16, 22). The iron chelator, 2,2'-dipyridyl (dipyridyl), has previously been shown to be a potent inhibitor of the Fenton reaction by sequestering available iron, thereby preventing its interaction with peroxides (16). In order to determine whether Fenton chemistry was indeed playing a role in the killing of A. baumannii during polymyxin action, we assayed the ability of dipyridyl to prevent polymyxin-mediated killing. Treatment of A. baumannii with dipyridyl significantly inhibited killing by both polymyxin B and colistin (Fig. 4A and B). Furthermore, we determined that dipyridyl was also capable of rescuing colistin-mediated killing of E. coli and F. novicida (see Fig. S3A and B in the supplemental material). We further examined whether dipyridyl prevented colistin-mediated radical production, in A. baumannii, E. coli, and F. novicida. Similar to our results with thiourea, we observed a significant decrease in colistin-mediated hydroxyl radical production following treatment with dipyridyl (see Fig. S5A to C in the supplemental material). Since dipyridyl is capable of inhibiting the rapid loss of viability observed during polymyxin-mediated death, as well as the induction of hydroxyl radicals, these data implicate the involvement of the Fenton reaction in the rapid killing of A. baumannii, as well as other Gram-negative organisms, by the polymyxins. Together with the ability of the hydroxyl radical scavenger thiourea to inhibit polymyxin-mediated death, these iron depletion data strongly suggest that polymyxins induce rapid killing of Gram-negative bacteria through Fenton chemistry-mediated hydroxyl radical production.

Drug-resistant clinical isolates of A. baumannii are killed through hydroxyl radicals after polymyxin treatment. MDR and PDR clinical isolates of A. baumannii have been identified with increasing frequency and present a significant problem in healthcare settings (3, 33, 36, 39, 41). We therefore sought to determine whether the hydroxyl radical-mediated cell death pathway was induced in recent clinical isolates recalcitrant to the majority of current therapies. These isolates would therefore represent potential candidates for treatment with the polymyxins, including colistin. We first examined the ability of colistin to induce hydroxyl radical production in two MDR clinical isolates of A. baumannii, CI-2 and CI-3, both of which are colistin sensitive (Table 1). Consistent with our previous results, following 30 min of treatment with colistin, we observed a significant increase in hydroxyl radical production (Fig. 5A and B). Furthermore, we examined whether treatment of a PDR isolate, CI-4, which has significant resistance to colistin (Table 1), would induce the production of hydroxyl radicals. Interestingly, colistin treatment did not cause an induction of hydroxyl radicals in this strain (Fig. 5C), indicating that sublethal levels of colistin are not able to induce hydroxyl radicals, which is consistent with data on other antibiotic treatments (22). Together, these data suggest that the induction of hydroxyl radicals following colistin treatment is conserved in recent clinical isolates, and that colistin resistance prevents their induction.

To determine whether these hydroxyl radicals were participating in rapid killing by colistin, we utilized time-kill assays and assessed the ability of thiourea and dipyridyl to prevent colistin-mediated death in these clinical isolates of A. baumannii. We observed that by quenching radical oxygen species or depleting iron to suppress the Fenton reaction, the rapid killing of CI-2 and CI-3...
by colistin was significantly inhibited (Fig. 6A and B). Furthermore, the PDR/colistin-resistant isolate CI-4 did not demonstrate a significant change in viability following treatment with colistin, alone or in combination with thiourea or dipyridyl (Fig. 6C). In total, these data demonstrate that the induction of hydroxyl radicals by colistin is responsible for the rapid killing of sensitive *A. baumannii* isolates, including clinically important MDR strains, and that resistance to the polymyxins prevents hydroxyl radical production.

**DISCUSSION**

Classically, the polymyxins have been thought to kill bacteria through membrane disruptions and, ultimately, cell lysis (10). However, there exist historical reports of polymyxins killing bacteria without actively lysing those cells (8, 21). Here, we demonstrated that treatment with polymyxins induces hydroxyl radical production through the Fenton reaction and that this radical production mediates the rapid killing of *A. baumannii*, as well as *E. coli* and *F. novicida*.

Other bactericidal antibiotics, including the quinolones, β-lactams, and aminoglycosides, have previously been shown to induce the production of hydroxyl radicals, which ultimately mediate killing of *Escherichia coli* and *Staphylococcus aureus* (12, 22). Notably, each of these antibiotic classes interact directly with enzymes involved in different aspects of bacterial physiology: DNA replication, cell wall synthesis, and translation, respectively. Conversely, the polymyxins are not known to interact with bacterial enzymes and instead target lipid A in the outer membrane of Gram-negative bacteria (10, 32). Thus, their ability to both induce hydroxyl radicals and kill *A. baumannii* through hydroxyl radical production is somewhat surprising. To our knowledge, this is the first demonstration that the polymyxins induce an oxidative cell death pathway.

The mechanism by which polymyxin treatment induces the
production of hydroxyl radicals in A. baumannii is not completely clear. Other bactericidal antibiotics were shown to induce a stress response in E. coli, disrupting the production of NADH by inhibiting the tricarboxylic acid cycle and thus ultimately causing aberrant respiration in the electron transport chain (12, 22). Disruptions in the electron transport chain promote the production of superoxide that can participate in the Fenton reaction and induce production of hydroxyl radicals (12, 16, 22). In addition, it has been demonstrated that the mistranslation of membrane proteins following treatment with aminoglycosides or the mammalian peptidoglycan recognition proteins activates the CpxAR two-component system in E. coli, which is involved in the bacterial envelope stress response, and subsequently triggers the depletion of NADH and production of hydroxyl radicals through the process described above (20, 23). It is therefore tempting to posit that the polymyxins may induce hydroxyl radicals in a similar fashion, by activating an envelope stress response in the bacterial cell, which shifts the metabolic state and causes aberrant electron transport. In fact, Vibrio cholerae treated with polymyxin B exhibits an increase in the transcriptional levels of rpoE, the sigma factor involved in envelope and oxidative stress responses (37). Interestingly, previous studies have indicated that polymyxin B treatment can induce aberrant oxidative respiration (31, 40, 42). In organisms with no experimentally identified CpxAR envelope stress-sensing two-component system, such as A. baumannii and F. novicida, this envelope stress response may be triggered by other, as-yet-identified, sensory systems. In total, these past observations are consistent with the data presented here and together suggest that killing of Gram-negative bacteria, including A. baumannii, by the polymyxins may follow the proposed conserved pathway of hydroxyl radical-mediated cell death. It is also interesting to consider the possibility that other membrane-targeting antibiotics, such as daptomycin, which depolarizes the bacterial membrane and also kills bacteria without causing lysis, induce this cell death pathway as well (7, 13).

Since polymyxin treatment is increasingly the last line therapeutic option for patients infected with MDR strains of A. baumannii, we further elucidated the conserved nature of hydroxyl radical-mediated killing in MDR clinical isolates. Not only did colistin treatment induce hydroxyl radical production in colistin-sensitive strains, but both thiourea and dipyridyl were able to inhibit the ability of colistin to kill these strains, indicating that hydroxyl radical-mediated cell death can occur in MDR nosocomial isolates of A. baumannii (Fig. 5 and 6). We also note that colistin resistance prevented the induction of hydroxyl radicals in A. baumannii.

Colistin resistance in A. baumannii has primarily been linked to changes in its lipid A, dampening or preventing the initial interaction between colistin and the bacterial envelope. These changes include complete loss of lipid A or additions of phospho-ethanolamine to mask negatively charged phosphate moieties (2, 4, 14, 30, 38). A. baumannii can become resistant through mutations in genes necessary to produce lipid A or through mutations in the PmrAB two-component system, which signals for lipid A alterations (1, 2, 4). The PmrAB system is activated by ferric iron, and growth in iron replete conditions has been shown to provide a slight increase in the A. baumannii MIC for colistin, likely due to PmrAB activation of lipid A alterations (1, 2, 4). This is not contrary to our data, which suggests that when colistin interacts with the bacterial envelope, intracellular iron potentiates killing through the Fenton reaction and the induction of hydroxyl radicals (16). Thus, as we demonstrate, iron depletion prevents Fenton chemistry from potentiating the production of hydroxyl radicals following polymyxin treatment. Notably, it has been demonstrated that some pathogenic bacterial species limit intracellular iron, and those with lower levels have increased resistance to oxidative killing (27). It is interesting to consider the possibility that this intracellular iron limitation may provide resistance to a broad range of host defenses, as well as the polymyxins.

With the increasing frequency of A. baumannii as a nosocomial pathogen, a rising percentage of these infections requiring treatment with polymyxins, as well as the growing cost of treatment of infections with this pathogen, understanding the precise mechanism of action of these last line therapeutics is imperative (3, 5, 6, 24, 34, 36, 43, 47). Our findings not only...
augment our knowledge of the mechanism of polymyxin action but also provide support to current concepts of utilizing hydroxyl radical-inducing agents as therapies against extensively drug-resistant pathogens (9, 48).

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