Pharmacodynamics of Polymyxin B against *Pseudomonas aeruginosa*

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The prevalence of antimicrobial resistance in gram-negative bacteria in hospitals is an increasing problem. *Pseudomonas aeruginosa* and *Acinetobacter* spp. are often implicated, and they are associated with significant morbidity and mortality. Multidrug resistance among these pathogens is especially worrisome, as the number of possible therapeutic options is severely limited. In a recent national surveillance of antimicrobial resistance in *P. aeruginosa* isolates obtained from intensive care units, the prevalence rate of multidrug resistance (defined as resistance to three or more of the following drugs: ceftazidime, ciprofloxacin, tobramycin, and imipenem) increased from 4% in 1993 to 14% in 2002 (20). Recently, there is much rekindled interest in using the polymyxins (polymyxin B and colistin) for the treatment of infections caused by multidrug-resistant (MDR) gram-negative organisms (6, 9, 11, 13, 15, 16, 23).

The polymyxins are polypeptide antibiotics isolated from *Bacillus polymyxa*, first made available for clinical use in the late 1950s and early 1960s. The polymyxins exert their bactericidal activity by binding to the bacterial cell membrane and disrupting its permeability, resulting in leakage of intracellular components. They also have antiendotoxin activity (2, 17). These agents are rapidly bactericidal against many gram-negative bacteria. Soon after their introduction into clinical use, concerns arose about adverse effects (e.g., nephrotoxicity, ototoxicity, and neuromuscular blockade) associated with their use. As antimicrobial agents with better safety profiles became available, the clinical use of the polymyxins was quickly abandoned due to perceived toxic side effects (5, 10).

As a consequence of the increasing rates of multidrug resistance in gram-negative bacteria, the polymyxins have increasingly become the last viable therapeutic option for MDR pseudomonal infections, despite very limited pharmacokinetic and pharmacodynamic data (5, 10). As we are faced with the possibility of returning to the preantibiotic era, the polymyxins are the agents of our last line of defense. It is therefore critical that they be used judiciously and optimally. If the pharmacodynamics of these agents are thoroughly understood, dosing regimens may be designed rationally to optimize patient outcomes and to minimize the emergence of resistance to these agents (4). The objectives of this study were to evaluate the in vitro pharmacodynamics of polymyxin B against *P. aeruginosa* with respect to its bactericidal activity and propensity to suppress spontaneous (non-plasmid-mediated) emergence of resistance.

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MATERIALS AND METHODS

Antimicrobial agent. Polymyxin B sulfate powder (USP) was purchased from Sigma (St. Louis, MO). A stock solution at 1.024 mg/liter in sterile water was prepared, aliquoted, and stored at –70°C. Prior to each susceptibility testing, an
aliquot of the drug was thawed and diluted to the desired concentrations with cation-adjusted Mueller-Hinton II broth (Ca-MHB) (BBL, Sparks, MD).

**Microorganism.** Four strains of *P. aeruginosa* were used in the study. A standard wild-type strain, ATCC 27853 (American Type Culture Collection, Rockville, MD), and three carbapenem-resistant clinical bloodstream isolates were examined. The bacteria were stored at −70°C in Protect (Key Scientific Products, Round Rock, TX) storage vials. Fresh isolates were subcultured twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at 35°C prior to each experiment. The clonal relatedness of the bacterial strains was assessed using rapid amplification of polymorphic DNA, using primer 208 (5′-ACGGCC GACC-3′) as described previously (14).

**Susceptibility studies.** Polymyxin B MICs were determined for different strains of *P. aeruginosa* in Ca-MHB using a broth macrodilution method as previously described (18). The final concentration of bacteria in each broth macrodilution tube was approximately 5 × 10^5 CFU/ml of Ca-MHB. Serial twofold dilutions of drug were used. The MIC was defined as the lowest concentration of drug that resulted in no visible growth after 24 h of incubation at 35°C in ambient air. Susceptibility to a screening panel of antimiobacterial agents (consisting of piperacillin, ceftazidime, aztreonam, imipenem, meropenem, levofloxacin, and tobramycin) was determined by E-test (AB Biodisk, Piscataway, NJ) according to the manufacturer’s instructions. The studies were conducted in duplicate and repeated at least once on a separate day.

**Time-kill studies.** Two to three bacterial strains were examined. Time-kill studies were conducted with different and escalating concentrations of polymyxin B. Seven clinically achievable concentrations of polymyxin B were used: 0 (control), 0.25, 0.5, 1, 2, 4, 8, and 16 mg/liter (5). An overnight culture of the isolate was diluted 30-fold with prewarmed Ca-MHB and incubated further at 35°C until reaching late-log-phase growth. The bacterial suspension was diluted with Ca-MHB accordingly based on absorbance at 630 nm. 15 ml of the suspension was transferred to 50-ml sterile conical flasks, each containing 1 ml of a drug solution at 16 times the target concentration. The final concentrations of the bacterial suspension in each flask at baseline were approximately 1 × 10^6 CFU/ml and 1 × 10^7 CFU/ml. In addition to the standard inoculum (1 × 10^6 CFU/ml), a high inoculum (1 × 10^7 CFU/ml) was also used to simulate the bacterial load in a severe infection. Furthermore, the high inoculum used would allow resistant subpopulations to likely be present at baseline. The experiment was conducted for 24 h in a shaker incubator at 35°C. At each sampling point (0, 2, 6, 12, and 24 h) were obtained from each flask over 24 h to characterize the effect of various drug exposures on the total bacterial population. Prior to culturing the bacteria quantitatively, the bacterial samples (0.5 ml) were centrifuged at 10,000 × g for 15 min and reconstituted with sterile normal saline to their original volumes in order to minimize drug carryover effect. Total bacterial populations were quantified by spiral plating of 10^5 serial dilutions of the samples (50 μl) onto cation-adjusted Mueller-Hinton agar (MHA) plates (Hardy Diagnostics, Santa Maria, CA). The medium plates were incubated in a humidified incubator at 35°C for 18 to 24 h, and the bacterial density from each sample was determined with a CASBA-4 colony scanner and software (Spiral Biotech, Bethesda, MD). The theoretical lower limit of detection was 400 CFU/ml. Each experiment was repeated at least once on a separate day.

**Hollow-fiber infection model.** The schematic of the hollow-fiber infection model system has been described previously (1). Drug was directly injected into the central reservoir to achieve the peak concentration desired. Fresh (drug-free) growth medium was infused continuously from the diluent reservoir into the central reservoir to dilute the drug, in order to simulate drug elimination in human. An equal volume of drug-containing medium was removed from the central reservoir concurrently to maintain an isovolumetric system. Bacteria were inoculated into the extracapillary compartment of the hollow-fiber cartridge (Fibercell Systems, Inc., Frederick, MD); the bacteria were confined in the extracapillary compartment but were exposed to the fluctuating drug concentration in the central reservoir by means of an internal circulatory pump in the bioreactor loop. The optional absorption compartment of the system was not used.

**Experimental setup.** Two strains (a wild-type strain, PA 27853, and an MDR clinical strain, PA 37) of *P. aeruginosa* were used. The inocula were prepared as described above. The bacteria (15 ml) were inoculated into the hollow-fiber infection models at a concentration of approximately 1 × 10^5 CFU/ml. Dose fractionation studies were conducted for 96 h in a humidified incubator set at 35°C. The bacteria were exposed to placebo and 3 dosing regimens (every 8 h [q8h], q12h, and q24h), simulating the steady-state pharmacokinetic profiles of unbound polymyxin B (terminal half-life = 6 h) resulting from a daily dose of 2.5 mg/kg of body weight (standard clinical dose) and 20 mg/kg (8 times the standard clinical dose), respectively (Fig. 1A) (5).
Microbiologic response. With the simulated standard dose (2.5 mg/kg/day), all active regimens showed a significant killing of both strains of bacteria at 4 and 8 h. However, regrowth was apparent with repeated dosing beyond 24 h (Fig. 4), similar to that observed in time-kill studies. Regrowth observed over time was likely due to amplification of resistant populations, as demonstrated in Fig. 5. Susceptible bacterial populations were selectively eradicated, resulting in unopposed growth of resistant subpopulations and consequently the emergence of resistance over time. As long as the total daily dose (exposure) remained the same, dosing schedules appeared to have little impact on the bactericidal activity of polymyxin B.

A higher dose (20 mg/kg/day, 8 times the clinical dose) was simulated to examine if resistance in P. aeruginosa could be counterselected. A sustained reduction in total bacterial burden and suppression of the resistant subpopulation were achieved over 96 h for the wild-type isolate (Fig. 6) but not for the MDR isolate (data not shown [similar to those in Fig. 4B]). The observed difference may be due to the difference in baseline mutation frequency between the two isolates. Despite the low inoculum used in the hollow-fiber infection model, we found an unexpectedly high mutation frequency (approximately 1 in 10^3 CFU) of the MDR strain at MIC (approximately 100 times that in the wild-type strain). As noted previously, dosing schedules did not have an impact on the propensity of polymyxin B in suppressing resistance emergence.

Studies on polymyxin B-resistant isolates. The resistant isolates were found to have an 8- to 16-fold increase in MIC of

<table>
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<tr>
<th>Strain</th>
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<tbody>
<tr>
<td>PA 27853</td>
<td>PB 1</td>
<td>PIP 1.5</td>
<td>CAZ 1</td>
</tr>
<tr>
<td>PA 3</td>
<td>0.5</td>
<td>6</td>
<td>3</td>
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<tr>
<td>PA 5</td>
<td>1</td>
<td>6</td>
<td>2</td>
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<tr>
<td>PA 37</td>
<td>1</td>
<td>&gt;256</td>
<td>3</td>
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a PB, polymyxin B; PIP, piperacillin; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; LVX, levofloxacin; TOB, tobramycin; COL, colistin (polymyxin E); ND, not determined. Bold type indicates resistant phenotypes based on NCCLS guidelines.
polymyxin B, compared to their parent strains. Cross-resistance to other antimicrobial agents in the screening panel was not observed, except for colistin (a 4- to 16-fold increase compared to wild-type parent strain). Upon serial passage on drug-free medium plates over 20 days, susceptibility reversal (to both polymyxin B and colistin) was observed in one of the three isolates investigated, suggesting that resistance to the polymyxins might be adaptive (nonmutational).

DISCUSSION

With the alarming increase in multidrug resistance in gram-negative bacteria, many antimicrobial agents are being rendered ineffective. The polymyxins (polymyxin B and colistin) are increasingly used clinically as our last viable therapeutic option. To date, clinical experience with polymyxin B is still very limited (21, 23; A. L. H. Kwa, P. L. Choo, A. Tan, J. Low, and B. H. Tan, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. K-701, p. 360, 2003), and relatively little is known about its efficacy in treating severe infections.

Most investigations on the pharmacodynamics of the polymyxins have focused on colistin (polymyxin E) so far (8, 12), and less is known about the pharmacodynamics of polymyxin B. Improved understanding of the pharmacodynamics of polymyxin B may help to design dosing regimens rationally in order to optimize patient outcomes and retard the emergence of resistance (4). To the best of our knowledge, this is the first study examining the pharmacodynamics of polymyxin B in a hollow-fiber infection model. We used a dose fractionation study design (same daily dose but various doses and different dosing intervals used) to delineate which pharmacodynamic parameter (e.g., ratio of area under the concentration-time curve to MIC [AUC/MIC], ratio of maximum concentration of drug in serum to MIC, percentage of the dosing interval that the drug concentration was above the MIC, etc.) was most closely linked to the bactericidal effect of polymyxin B. Our experimental data consistently revealed that altering the dosing schedule (with identical daily dose) did not appear to have influenced the killing or resistance suppression against two strains of *P. aeruginosa*. Furthermore, the feasibility of optimizing the pharmacodynamics of polymyxin B in suppressing the emergence of resistance was also examined.

Consistent with previous findings on colistin, we found that the killing of *P. aeruginosa* by polymyxin B in time-kill studies was concentration dependent (8, 12). As with colistin, initial killing was rapid, but regrowth was readily seen in all time-kill studies. In addition, the killing of *P. aeruginosa* was reduced if a higher inoculum was used at baseline, suggesting that polymyxin B might be susceptible to the inoculum effect. The explanation of regrowth was not specifically investigated, but it appeared that the killing activity of polymyxin B was not sustained.

We further explored the potential clinical utility of various concentration-time profiles of polymyxin B in a hollow-fiber infection model. We used a dose fractionation study design (same daily dose but various doses and different dosing intervals used) to delineate which pharmacodynamic parameter (e.g., ratio of area under the concentration-time curve to MIC [AUC/MIC], ratio of maximum concentration of drug in serum to MIC, percentage of the dosing interval that the drug concentration was above the MIC, etc.) was most closely linked to the bactericidal effect of polymyxin B. Our experimental data consistently revealed that altering the dosing schedule (with identical daily dose) did not appear to have influenced the killing or resistance suppression against two strains of *P. aeruginosa*, suggesting that the pharmacodynamics of polymyxin B was most closely linked to the AUC/MIC ratio.

Despite the fact that rapid and substantial initial killing was observed, the standard clinical dosing resulted in regrowth and resistance emergence over 4 days in both the wild-type and

![FIG. 2. Time-kill studies of polymyxin B with the standard inoculum (10^5 CFU/ml). Data are presented as means and standard deviations. WT, wild type.](http://aac.asm.org/DownloadedFrom/vol49/05/3627/3627.png)
MDR bacterial strains. By validating our simulated pharmaco-
kinetic exposures and using polymyxin B-supplemented me-
dium plates for quantitative cultures, we demonstrated that
regrowth was likely due to adaptation and/or selective ampli-
fication of resistant subpopulations (as opposed to degradation
of polymyxin B over time). This unexpected result cautions
against the standard polymyxin B daily dose of 2.5 mg/kg as
monotherapy in an immunocompromised host, as it may not be
adequate for the treatment of infections caused by *P. aerugi-
nosa*. Clinical experience with this cohort is limited. However,
data obtained with immunocompetent patients suggested that
polymyxin B therapy was reasonably efficacious against infec-
tions cause by MDR gram-negative organisms, if used in com-
bination with other agents (21, 23).

The mechanism of resistance to the polymyxins remains
poorly understood. It is believed to be due to loss of lipopoly-
saccharide (7) or replacement of magnesium by protein H1 in
the outer membrane (19, 22). In this study, we did not inves-
tigate the specific mechanism of polymyxin B resistance. How-
ever, cross-resistance between the polymyxins (polymyxin B
and colistin) was demonstrated, but not cross-resistance with
any of the β-lactams, quinolone, and aminoglycoside investig-
gated. More interestingly, there might be more than one type
of non-plasmid-mediated resistance mechanism, as suggested
previously (5). One of the polymyxin B resistance mechanisms
appeared to be stable (mutational), while the other might be
reversible upon removal of selective pressure (adaptive). While
mutational resistance occurs infrequently (more commonly
seen with an inoculum size greater than the mutation fre-
quency of resistance), adaptive resistance may occur more
readily (even with a standard inoculum size). This finding is
consistent with our observations in time-kill studies, in which
regrowth occurred readily (within 12 h) after the initial decline
in bacterial burden. In addition, in the hollow-fiber infection

![FIG. 3. Time-kill studies of polymyxin B with a higher inoculum (10^7 CFU/ml). Data are presented as means and standard deviations. WT, wild type.](image)

![FIG. 4. Biologic response observed in hollow-fiber infection models with standard dose (2.5 mg/kg/day).](image)
models, repeated (standard daily) dosing did not result in a sustained suppression of the bacterial burden.

We further explored the feasibility of using elevated drug exposure to counterselect resistance. We noted that polymyxin B-resistant isolates had an 8- to 16-fold increase in MIC, compared to their parent strain. Therefore, we investigated the effect of 8 times the standard clinical dose on the time courses of the bacteria. Using 8 times the standard clinical dose, all simulated dosing regimens would have a concentration of ≥8× MIC of the parent strain throughout the entire dosing interval (time above 8× MIC, 100%). We found that the emergence of resistance could be suppressed using this simulated daily dose for the wild-type strain, somewhat consistent with our postulation of regrowth due to adaptive resistance. However, we were still unable to prevent the emergence of resistance in the MDR strain, despite using 8 times the standard clinical dose.

This observation is consistent with those observed in time-kill studies, in which higher polymyxin B concentrations were necessary to achieve the same bacterial burden reduction in the MDR strain. However, given that the time-kill studies were conducted for only 24 h, there might not have been enough time for the resistant subpopulation to proliferate and dominate the entire bacterial population. On the other hand, the hollow-fiber infection model studies were conducted over 96 h. The resistant subpopulations were selectively amplified, resulting in regrowth over time despite using a higher daily dose. We noted that the baseline mutation frequency of the MDR strain to polymyxin B (at 3× MIC) was approximately 100 times higher than that observed in the wild type. In spite of an apparently low MIC, this strain was likely to be hypermutable and much more difficult to suppress, as reflected in our time-kill studies (Fig. 2D and 3D). Consequently, regrowth was likely due to a combination of both adaptive resistance and selective amplification of mutational resistance. Combination therapy may be considered for treatment of infections caused by this strain.

**Conclusion.** Our results suggested that polymyxin B exhibits rapid and concentration-dependent bactericidal activity against *P. aeruginosa*, which was attenuated by a higher inoculum. The pharmacodynamics of polymyxin B was most closely linked to the AUC/MIC ratio. In conjunction with toxicity data, a dose higher than the standard dose and/or combination therapy may be necessary to suppress *P. aeruginosa* resistance in immunocompromised hosts.

**REFERENCES**


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**FIG. 5.** Selective amplification of the resistant subpopulation observed with standard dose (2.5 mg/kg/day) in PA 27853.

**FIG. 6.** Biologic response observed in hollow-fiber infection models with 8 times the standard dose (20 mg/kg/day).