Polymyxin B Pharmacodynamics in the Hollow Fiber Infection Model: What You See May Not Be What You Get

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Abstract

Dose-range studies for polymyxin B (PMB) regimens of 0.75 to 12 mg/kg given every 12h (Q12h) were evaluated for bacterial killing and resistance prevention against an AmpC-overexpressing *Pseudomonas aeruginosa* (PA) and a *bla*$_{KPC-3}$-harboring *Klebsiella pneumoniae* (KP) in 10-day *in-vitro* hollow fiber models. An exposure-response was observed. But all regimens failed due to regrowth. Lower-dose regimens amplified isolates that expressed transient, lower-level adaptive resistance to PMB (MICs: $\leq$ 4 mg/L). Higher PMB dosages amplified isolates that expressed this resistance mechanism, a higher-MIC "moderately stable" adaptive resistance, and a higher-MIC stable resistance to PMB. Failure of the highest dose regimens was solely due to subpopulations that expressed the two higher-level resistances. Total and bioactive PMB concentrations in broth declined below targeted PK profiles within hours of treatment initiation and prior to bacterial regrowth. With treatment failure, the total PMB measured in bacteria were substantially higher than in broth. But the bioactive PMB in broth and bacteria were low to non-detectable. Together these findings suggest a sequence of events for treatment failure of the clinical regimen. First, PMB concentrations in broth are diluted as PMB binds to bacteria, resulting in total and bioactive PMB in broth that are lower than targeted. Bacterial regrowth and treatment failure follow, with emergence of subpopulations that express transient lower-level adaptive resistance to PMB and possibly higher-level adaptive and stable resistances. Higher-dose PMB regimens can prevent the emergence of transient lower-level adaptive resistance but they do not prevent treatment failure due to isolates that express higher-level resistance mechanisms.

Introduction

Over the last decade polymyxin B (PMB), along with colistin, has played a central role as an antibiotic class of last resort for the therapy of patients infected with multidrug-resistant Gram-negative bacteria, especially those that are carbapenem-resistant.

Polymyxin antibiotics, including PMB, are cationic lipopeptides that bind to the anionic charged lipid A moiety of the outer membrane localized lipopolysaccharides (LPSs) of many Gram-negative bacteria, to displace membrane-associated Mg^{+2} and Ca^{+2}. This destabilizes and disrupts the outer and inner membranes, leading to microbial death (1, 2).

Resistance to polymyxin antibiotics by Gram-negative bacteria is primarily due to a change in the ionic charge of the outer membrane, preventing or reducing PMB binding to the outer membrane and subsequent bacterial death. Multiple resistance mechanisms have been shown to protect *P. aeruginosa* and *K. pneumoniae* from the antimicrobial effects of PMB. These include adaptive resistance, in which the bacteria transiently and reversibly alter the structure of the LPS on their outer membranes in response to certain stimuli, including low concentrations of PMB and colistin, to decrease polypeptide drug binding (3). For stable PMB resistance, the bacterium can alter the structure of the lipid A moiety of LPS by adding 4-amino-4-deoxy-L-arabinose (L-ARA4N) to a phosphate group, decreasing the net charge of lipid A to 0 or by replacing the phosphates with phosphoethanolamine (PEtN), changing the net charge of lipid A from -1.5 to -1.0 (3, 4). These changes reduce PMB binding to LPS that, in turn, decreases the microbiological activity of this antibiotic. *K. pneumoniae* may also

increase the production and shed capsular polysaccharides to which PMB can bind, thus reducing the interaction of PMB with Lipid A (5). Further, *P. aeruginosa* may increase the production of the outer membrane protein H1, which replaces Mg^{+2} and Ca^{+2} at the binding sites of LPS (6).

The clinical response of Gram-negative infections to PMB treatment has varied substantially (7-9) and most clinicians feel that, when employed, it should be used as part of a combination regimen (10). This antibiotic has attracted substantial study as multiple laboratories have tried to delineate its pharmacodynamic drivers for treatment optimization and resistance prevention (11-13). Despite these efforts, the efficacies of PMB-alone regimens have been suboptimal.

We decided to explore the use of PMB against a multidrug-resistant *Pseudomonas aeruginosa* strain that is a hyperproducer of AmpC β-lactamase and a *bla*$_{KPC-3}$ harboring *Klebsiella pneumoniae* strain in a hollow fiber infection model to elucidate the reasons why the clinical outcomes with this polymyxin antibiotic may be suboptimal. These isolates were selected because they express resistance mechanisms that are important causes of treatment failure in the clinic.

Results

MICs and mutational frequencies to resistance of PMB. Agar dilution and broth microdilution MICs using 2-fold geometric dilutions of PMB were determined for a *P. aeruginosa* POA1 strain that is a hyperproducer of AmpC and a *K. pneumoniae* strain that harbors a *bla*$_{KPC-3}$ beta-lactamase. For the *P. aeruginosa* strain, the agar and broth geometric dilution MICs were both 1 – 2 mg/L for 5 trials (median MIC: 2 mg/L). For the

K. pneumoniae strain, the agar and broth geometric dilution MICs were both 1 mg/L for 5 trials. Mutation frequencies to 3x the median geometric dilution MIC of the *P. aeruginosa* and *K. pneumoniae* strains were 1 in 1.82×10^8 CFU and 1 in 7.76×10^7 CFU, respectively.

Dose-range studies for PMB against *P. aeruginosa* in the HFIM. In HFIM studies, bacteria inoculated into the peripheral compartment of hollow fiber cartridges are exposed to simulated concentration-time profiles of drugs reported in humans or other mammals. Quantitative cultures of bacterial samples collected from the peripheral compartment are performed on drug-free and antibiotic-infused agars to characterize the effect of these PK profiles and drug exposures on the killing of the bacterium and the killing or amplification of less-susceptible bacterial subpopulations (14 - 16). Broth samples are typically collected from the central reservoir for drug measurements to determine how closely the actual PK profile simulated in a HFIM arm simulates the desired profile.

The US-Food and Drug Administration (US-FDA) has licensed clinical doses of PMB as 0.75 to 1.25 mg/kg given intravenously every 12h (Q12h) while an international consensus guideline recommends dosages of PMB 1.25 to 1.5 mg/kg Q12h (17-18). For the 10-day dose-range HFIM study with *P. aeruginosa*, the experimental arms consisted of a no-treatment control and simulated free (non-protein bound) concentration-time profiles for five PMB regimens. They included the clinically prescribed regimen of PMB 0.75 mg/kg Q12h and PMB as 1.5 mg/kg Q12h, a regimen that was slightly greater than the highest dose regimen licensed by the US-FDA (17-18). Higher dose regimens were for PMB as 3, 6, and 12 mg/kg Q12h. Following recent

recommendations for dosing PMB, loading doses were used in all regimens to rapidly achieve steady-state (18).

Twelve milliliters of approximately 7 Log CFU/mL of *P. aeruginosa* were inoculated into the peripheral compartment of the hollow fiber cartridge of each experimental arm to mimic the total bacterial burden that may be found in patients with ventilator-associated pneumonia (19). For the five PMB regimens, an exposure-response relationship was observed between PMB doses and the amount of early killing of the parent *P. aeruginosa* isolate. With 5 hours of treatment, maximal killing of only 0.4 log CFU/mL was observed for the clinical regimen of 0.75 mg/kg Q12h and approximately 1.8 log CFU/mL for the supraclinical regimens of PMB 1.5 mg/kg Q12h and PMB 3 mg/kg Q12h. PMB 12 mg/kg Q12h achieved nearly 7 log CFU/mL of bacterial killing at this time point. However, by 24 hours of treatment all of the regimens had regrowth. The start of regrowth occurred later with higher PMB dosages, with the *total bacterial populations* in the treatment arms approximating those of the control arm between 1 and 4 days after the initiation of PMB infusions (Figure 1).

For all of the PMB regimens there was early isolation of *P. aeruginosa* colonies on PMB-containing CA-MHA agar plates (6 mg/L or 3 x the median geometric MIC of the parent isolate). For the clinical regimen of PMB 0.75 mg/kg Q12h, the density of the subpopulation of *P. aeruginosa* which grew on agars infused with 3x the geometric PMB MIC over the 10-day experiment were similar to those of the untreated control arm (Figures 2A and 2B), suggesting this regimen provided little or no drug pressure for resistance amplification. The increase in bacterial counts in the HFIM arms treated with PMB 1.5 mg/kg Q12h and higher dose regimens suggested the drug exposures

associated with those regimens did amplify the less-susceptible bacterial subpopulations (Figures 2C – 2F).

Dose-range results for PMB for the K. pneumoniae strain. Simulated PMB regimens for 0.75, 1.5, and 12 mg/kg Q12h were evaluated against a *bla*$_{KPC-3}$ harboring *K. pneumoniae* strain. The two lowest PMB regimens were to simulate the clinical and a slightly higher than clinical regimen while the PMB 12 mg/kg Q12h regimen was to determine if a supra-clinical dose could result in treatment success.

The quantitated starting inoculum added the each HFIM experimental arm was 12 mL of 7.16 log CFU/mL of the bacterium. The dose-range study outcomes with the *K. pneumoniae* strain (Figure 3) recapitulated the results of the *P. aeruginosa* HFIM study (Figure 1). A dose-response effect for bacterial killing was observed 5 hours after treatment initiation. The clinical regimen of 0.75 mg/kg Q12h produced only a 0.61 log CFU/mL reduction in the total bacterial density. The PMB 1.5 mg/kg Q12h regimen provided a 1.92 log CFU/g reduction and the 12 mg/kg Q12h regimen decreased the total bacterial population by 6.34 log CFU/mL. By the 24h time point all of the regimens showed some degree of regrowth. Between 1 and 4 days of treatment, the total bacterial densities for all of the PMB regimens were similar to those of the no-treatment control.

Consistent with the *P. aeruginosa* HFIM experiment, for the clinical PMB regimen of 0.75 mg/kg Q12h, the number of *K. pneumoniae* colonies that grew on agar supplemented with 3x the geometric MIC value of the parent isolate were similar to those of the no-treatment arm over the 10-day study. This suggested this regimen provided no or little drug pressure for resistance amplification. The PMB 1.5 and 12

mg/kg Q12h regimens resulted in increased bacterial densities on PMB-infused agars (Figure 4).

PMB susceptibility testing of colonies that grew on agar supplemented with 3x the geometric MIC of the parent P. aeruginosa isolate in the mutation frequency and HFIM experiments. A two-step algorithm for susceptibility testing was used on colonies which grew on PMB-supplemented agars in mutation frequency and HFIM studies to determine if these colonies expressed stable resistance or transient adaptive resistance to PMB (Figure 5).(20)

In Step I, is the traditional approach for susceptibility testing in which the "primary colonies" which grew on the 3xMIC PMB supplemented agars were *first expanded on drug-free agar*.(21) A portion of the mass of a colony which grew on drug-free agar underwent agar susceptibility testing using geometric 2-fold dilutions of PMB. A subset of colonies which had intermediate (MIC: 4 mg/L) or resistance PMB MICs of 4 mg/L and $\geq$ 8 mg/L, respectively, as defined by CLSI (22), were serially passaged on drug-free agar for 10 times. Susceptibility testing for PMB was simultaneously performed on the isolate before and after the ten serial passages to assess for the stability of the increased MIC value.

If the two MIC values are within 1 two-fold geometric dilution, then a higher-level stable resistance was identified. If after 10 passages on drug-free agar the MICs decrease by greater than 2 two-fold dilutions, then a higher-level "moderately stable" adaptive resistance was expressed. These isolates are categorized as "moderately stable" because two to ten passages on drug-free agars is required for the adaptive resistance to cease.

Step II of the susceptibility testing algorithm was performed only on "primary colonies" which grew on agars infused with 3x the geometric MIC of PMB in the mutation frequency and dose-range HFIM studies *and* had *WT PMB MIC values (± 1 two-fold geometric dilution)* when susceptibility testing was performed after the colonies were expanded on drug-free agar. Step II assessed whether these isolates expressed a transient lower-level adaptive resistance to PMB. In Step II, a portion of the remaining mass of a "primary colony" that grew on agar containing 3x the geometric MIC for PMB in the mutation frequency or HFIM studies was expanded on both drug-free agar and agar infused with 2x the geometric MIC for PMB of the parent isolate. Agar infused a lower multiple of MIC was used because many of the "primary colonies" did not grow when subcultured on new agar containing 3x the geometric MIC for PMB. The colonies which grew on both the drug-free agar and agar infused with 2x the geometric MIC for PMB were immediately subjected to agar dilution susceptibility testing employing serial arithmetic 0.5 mg/L increments of PMB (range: 0.25 to 5 mg/L). If the passaged "primary colony" grew on both agars and the MIC for the subculture grown on PMB-infused agar was at least two arithmetic dilutions higher than the MIC for the same colony passaged on drug-free agar, a transient adaptive resistance was identified. For this project, if the passaged "primary colony" grew only on drug-free agar and not on agar infused with PMB-infused agars, then adaptive resistance was not documented.

P. aeruginosa MICs from Step I of the susceptibility testing algorithm: The geometric PMB MICs that were identified after the colonies that grew on PMB-infused agars in the mutation frequency and HFIM studies were subcultured on *drug-free agar* are shown in Table 1. The distribution of geometric MIC values for colonies in the

mutation frequency study were in the susceptible and intermediate range. In the no-treatment HFIM arm, 50% of the *P. aeruginosa* colonies had wild-type geometric MIC values of 1-2 mg/L. Thirty-three percent of these isolates had an intermediate geometric MIC of 4 mg/L and 17% had geometric MIC values in the resistant range. For the PMB 0.75 mg/kg Q12h arm, the distribution of geometric MICs for colonies that grew on PMB-infused agar in the HFIM study, and had MICs determined after they were expanded on drug-free agar, was similar to the no-treatment control. This suggests this regimen provided no drug pressure for amplification of the less-susceptible subpopulations despite the observed regrowth. For the PMB 1.5 mg/kg Q12h regimen, 42% of the colonies that underwent susceptibility testing had wild-type geometric PMB MICs, while 58% had resistant MIC values. As the PMB dose regimens increased from 3 to 12 mg/kg Q12h, the bacteria that grew on PMB-infused agars had geometric PMB MICs restricted to the intermediate and resistant range and then only to the resistant range.

K. pneumoniae MICs from Step I of the susceptibility testing algorithm: The distribution of geometric MIC values for a subset these *K. pneumoniae* colonies are shown in Table 2. All of the *K. pneumoniae* colonies from the control arm and PMB 0.75 mg/kg Q12h regimen in which susceptibility testing was performed had geometric MIC values equivalent to the wild-type, parent isolate. As the dose in the PMB regimen increased from 0.75 to 1.5 and then to 12 mg/kg Q12h, a larger proportion of the *K. pneumoniae* colonies had reduced susceptibilities to this antibiotic.

Serial passage of P. aeruginosa and K. pneumoniae colonies with increased geometric PMB MICs in Step I of the susceptibility testing algorithm to assess for stable

221 *and adaptive resistances.* Twelve *P. aeruginosa* colonies and twelve *K. pneumoniae*
222 colonies that grew on PMB-infused agars as part of the mutation frequency and HFIM
223 studies, and had PMB MIC of 4 to 64 mg/L *after* they were expanded on drug-free agar,
224 were assessed for stability of their resistances to PMB. Geometric MICs were
225 determined for these colonies before and after they had undergone 10 passages on
226 drug-free agars. Ten *P. aeruginosa* and 11 *K. pneumoniae* colonies had MICs before
227 and after the serial passages on drug-free agars that were within one 2-fold geometric
228 dilution of each other. Since these colonies had MICs of 4 to 64 mg/L they expressed a
229 "higher-level *stable PMB resistance"* mechanism. Two *P. aeruginosa* with MICs of 8 and
230 32 mg/L and one *K. pneumoniae* colony with an MIC 16 mg/L had their MICs decrease
231 by ≥ 3 two-fold dilutions, back to wild-type values, after completing the serial passages.
232 Since these colonies required multiple passages on drug-free agar for their increased
233 MICs to return to wild-type values these colonies expressed *"higher-level, moderately*
234 *stable adaptive resistance"*. This terminology was used to differentiate this resistance
235 mechanism from the *"transient lower-level adaptive resistance"* that will be discussed in
236 the next subsection.

237 *Step II susceptibility testing - evaluation for transient lower-level adaptive*
238 *resistance to PMB in P. aeruginosa and K. pneumoniae:* Step II was performed only on
239 colonies that had wild-type (susceptible) MIC values in Step I of the susceptibility testing
240 algorithm (Tables 1 and 2). In Step I, these colonies grew on agar infused with 3x the
241 geometric PMB MIC of the parent isolate in the mutation frequency and HFIM studies
242 but had wild-type MIC values after one passage on drug-free agar. The methods for
243 Step II are described in Figure 5 and were used to determine if transient adaptive

resistance enabled these colonies to grow on agars supplemented with 3x the geometric MIC of PMB in the mutation frequency and HFIM studies. Transient adaptive resistance was identified if the arithmetic MIC for the colony expanded on agar containing 2x the geometric MIC for PMB was at least two 0.5 mg/L dilutions (or 1 mg/L) higher than the arithmetic MIC of the same colony that was expanded on drug-free agar. If a "primary colony" only grew on drug-free agar, transient resistance was not identified.

The shift in the distribution of arithmetic dilution (0.5 mg/L increments) PMB MIC values for susceptibility testing of "primary colonies" which were subcultured on drug-free agar and agar infused with 2x the geometric PMB MICs for the *P. aeruginosa* and *K. pneumoniae* strains are shown in Figures 6 and 7, respectively. The distribution of PMB MICs for colonies which were expanded on PMB-infused agars were higher than for colonies subcultured on drug-free agars. The change in MIC values met the criteria for transient adaptive resistance for a large proportion of the colonies examined. The largest increase in MICs between colonies subcultured on PMB-infused and drug-free agars was 2.5 mg/L. The highest MIC by arithmetic dilution susceptibility testing performed directly on colonies which were subcultured on agar infused with 2x the PMB MIC of the parent isolate was 4 mg/L for both *P. aeruginosa* and *K. pneumoniae*. To distinguish this mechanism of resistance from the "higher-level, moderately stable adaptive resistance" discussed in the preceding subsection, this resistance mechanism was termed *"transient low-level adaptive resistance"* because this adaptive resistance mechanism caps the increased MIC value after PMB exposure to 4 mg/L and, as shown

in Part I of the susceptibility testing algorithm, only one passage on drug-free agar was required for the heightened MICs to return to wild-type values.

PMB susceptibility testing of P. aeruginosa and K. pneumoniae that produced confluent growth on PMB-supplemented agar in the HFIM studies. To perform quantitative cultures on bacterial suspensions collected from the HFIM arms, serial 10-fold dilutions of the washed samples were plated on drug-free agar and agar supplemented with 3x the geometric PMB MIC of the parent isolate to characterize the effect of each PMB regimen on the total and less-susceptible bacterial populations, respectively. The number of colonies which grew on the drug-free agars were consistent with 10-fold dilutions of the bacterial suspensions. However, for the PMB 0.75 to 3.0 mg/kg Q12h regimens, the growth of serially-diluted samples on PMB-supplemented agars sometimes produced unexpected results: one dilution of the bacterial suspension may yield confluent growth on PMB-supplemented agar while the next sample in the 10-fold dilution series would yield less than 10 colonies on PMB-supplemented agar in contrast to the several hundred colonies that were expected to grow on this agar plate.

A 10 µl inoculating loop was used to passage a sample of the bacteria which grew confluently on the 3xMIC PMB-supplemented agar in the HFIM experiment onto fresh agar supplemented with 2x and 3x the geometric MIC of PMB and onto drug-free agar. The passaged bacteria did not grow on agar supplemented with either concentration of PMB but it did grow on antibiotic-free agars. Bacteria taken off of the PMB-supplemented agar plates with confluent growth and directly suspended in saline for susceptibility testing had wild-type MIC values when the study was performed using serial 0.5 mg/L arithmetic increments of PMB in agar. The MICs of the isolates that grew

as < 20 colonies on the PMB-supplemented agars in the HFIM study are described in the preceding section.

LC-MS/MS and bioassay measurements for total and bioactive concentrations of PMB in broth medium and centrifugation-derived P. aeruginosa pellets and supernatants from the HFIM study. In the *P. aeruginosa* dose-range study, broth samples were collected for PMB measurements from the central reservoir starting 48 hours before and continued for 72 hours after the HFIM systems were inoculated with bacteria. Bacterial samples were collected from the peripheral compartment of the HFIM at the 48 and 72h post-inoculation time points for PMB measurements. The bacterial suspensions were separated into supernatant and bacterial pellet fractions by centrifugation and the PMB content in the samples were measured. The *total PMB concentration* in a sample was measured by LC-MS/MS (lower limit of quantification or LLOQ of 0.25 mg/L) and the *biologically active component* was measured using a bioassay (LLOQ: 1.2 mg/L).

Total PMB concentrations in broth in the P. aeruginosa HFIM study as measured by LC-MS/MS and bioassay. Before a bacterium was inoculated into the HFIM arms, the PMB concentration-time profiles were well simulated when assessed by both assays. Both the total and bioactive PMB concentration-time profiles approximated the targeted PK profiles. Also, the assays showed that the total PMB concentrations consisted primarily of active drug (Figure 8).

Since the total and bioactive PMB concentrations approximated the targeted PK values in bacteria-free HFIM system, the deviation of these concentrations from the

targeted PK values after the inoculation of bacteria into the HFIM arms is the important parameter of interest.

After the *Pseudomonas* was inoculated into the HFIM, the first peak concentrations for *total* PMB that was measured in CA-MHB achieved the targeted values for the PMB 0.75 mg/kg Q12h and 1.5 mg/kg Q12h arms. But the total PMB concentrations in broth were below the targeted PK profiles beginning 4 hours after the bacteria were introduced to the HFIM system and the measured total PMB concentrations progressively decreased thereafter (Figure 9A). With the PMB 1.5 to 6 mg/kg Q12h regimens, the targeted PK *total* PMB concentration-time profiles were achieved longer. However, the measured PMB concentrations progressively decreased below the targeted values as the *Pseudomonas* regrowth passed through the treatment "failure window" (Figure 9B - 9E).

The "failure window" was defined *post-hoc* and began when bacterial regrowth had increased to approximate the pre-treatment (or stasis) bacterial inoculum of about 7 log CFU/mL and ended when the microbe first reached stationary phase growth of ~10 log CFU/mL.

Quantification of the bioactive concentrations of PMB in broth for the PMB 0.75 and 1.5 mg/kg Q12h regimens were limited by the LLOQ of the bioassay. For the clinical regimen, only the first peak concentration was measurable and its value achieved the targeted concentration. All subsequent peak levels were not detectable by bioassay (Figure 9A). For the slightly higher than clinical regimen of PMB 1.5 mg/kg Q12h, the bioactive PMB achieved the targeted concentration only for the first peak infusion after the bacteria were introduced into the HFIM. At all other time points and

before the onset of regrowth, the bioactive concentrations were below the targeted and total PMB concentrations in broth (Figure 9B). Differences between the total and biologically active concentrations in broth relative to the targeted values were also seen with the higher PMB regimens.

For each regimen, the decline in bioactive and total PMB concentrations relative to the targeted values began before the onset of regrowth (Figures 9 and 1, respectively). The measured total and biologically active PMB concentrations continued to decline as the *P. aeruginosa* entered and passed through the treatment "*failure window*." In the *P. aeruginosa* HFIM experiment, for the clinical regimen of 0.75 mg/kg Q12h the beginning of the "failure window" was assigned as 12 hours after the start of therapy. However, given the limited measurement time points, any time between 5 and 24 hours was equally valid. For the PMB 3 and 6 mg/kg Q12h regimens, the "failure window" began at the 24 hour time point and ended on day 2 or 3, depending of the PMB dose (Figures 9C – 9D). For the PMB 12 mg/kg Q12h regimen, a substantial reduction in the total PMB concentrations in broth was not observed, perhaps because sampling time points for PMB measurements ended 72 hours after the bacteria were inoculated into the HFIM systems and the "failure window" began sometime between days 3 and 4 and ended on day 4 of treatment (Figure 9E).

Total and bioactive PMB concentrations in the cell pellets and supernatants of centrifuged bacterial suspensions collected from the P. aeruginosa HFIM study. Total PMB concentrations were measured by LC-MS/MS in centrifugation-derived bacterial supernatant and cell pellet samples. For the PMB 0.75 and 1.5 mg/kg Q12h regimens, the total PMB concentrations in the bacterial pellets were substantially higher than the

broth concentrations at the 48-49 and 72-73 hour time points (Figures 9A and 9B). The total PMB concentrations were also higher in bacterial pellets at the 73 hour time point for the PMB 3 and 6 mg/kg Q12h regimens (Figures 9C – 9D). Except for the PMB 0.75 mg/kg Q12h arm, the total PMB concentrations in the bacterial supernatants were only slightly higher than those measured in broth medium. For example, in the PMB 6 mg/kg Q12h regimen, the total peak broth, bacterial supernatant, and bacterial pellet concentrations of PMB in samples collected at the 73 hour time point were 8.8, 10.4, and 30.1 mg/L, respectively. The targeted peak and trough broth concentrations for PMB were 12.2 and 4.7 mg/L, respectively.

The largest discordance was seen between the LC-MS/MS and bioassay-quantified total and bioactive PMB concentrations in the bacterial supernatant and pellet samples. For the PMB 0.75 mg/kg Q12h regimen, at the 73 hour the broth peak, bacterial supernatant and bacterial pellet samples had total PMB concentrations of 0.67, 3.91, and 4.45 mg/L, respectively. The corresponding bioactive concentrations in these specimens were less than the LLOQ of 1.2 mg/L. For the PMB 6 mg/kg Q12h regimen, the total PMB concentrations at broth peak and in the bacterial supernatant and pellet samples at the 73 hour time point were 8.8, 10.4, and 30.1 mg/L, respectively. The same samples had bioactive PMB concentrations of 4.6, <1.2, and <1.2 mg/L, respectively (Figure 9A - 9E).

To determine the size of *P. aeruginosa* components in the centrifugation-derived supernatants, a subset of samples were divided into two aliquots. One aliquot was passed through a 0.2 micron pore filter before the PMB concentrations in both the filtered and non-filtered samples were measured by LC-MS/MS. The concentrations in

the paired samples were similar. For example, a pre-filtered 24h supernatant sample had 5.14 mg/L of PMB as measured by LC-MS/MS while the post-filtered sample had 5.57 mg/L of drug. For a 169 hour sample, the pre- and post-filtered supernatants had total PMB concentrations of 13.8 and 13.9 mg/L, respectively. This suggests intact dead and live bacteria were not in the supernatants in substantial numbers.

PMB concentrations measured by LC-MS/MS and bioassay in broth medium and the supernatants and pellets of centrifuged K. pneumoniae suspensions. Media samples collected from the central reservoir and peripheral compartments of the HFIM arms for 48 hours before they were inoculated with *K. pneumoniae* were well-simulated as measured by LC-MS/MS and bioassay (data not shown). Additional broth media samples and supernatant and pellets of centrifuged bacterial suspensions were collected from each HFIM arm for 48 hours after the systems were inoculated with *K. pneumoniae* and between 168 and 173 hours to more fully characterize the total and bioactive PMB concentrations in these sites before and after bacterial regrowth.

After the HFIM arms were inoculated with *K. pneumoniae*, the concentrations of PMB in the central reservoir broth medium, as measured by LC-MS/MS and bioassay, progressively decreased (Figure 10) as the total bacterial population increased over the 10-day study (Figure 3).

Recapitulating the *P. aeruginosa* findings, the total PMB concentrations in the broth of the *K. pneumoniae* HFIM experiment steadily declined prior to entering the "failure window" and continued to be lower than the targeted concentrations as the bacteria passed through the "failure window". The bioactive PMB in broth decreased at

a faster rate, rapidly decreasing below the LLOQ of the bioassay of 1.2 mg/L (Figure 10).

For the clinical and slightly supraclinical regimens of PMB 0.75 and 1.5 mg/kg Q12h, the total PMB concentrations in the supernatant and pellet samples of the centrifuged *Klebsiella* suspensions, as measured by LC-MS/MS, steadily increased as bacterial regrowth entered and passed through the "failure window." The total PMB concentrations in the supernatant and bacterial pellets were above 7 mg/L while most of the total PMB concentrations measured in simultaneously collected broth medium were lower than the LLOQ of 0.25 mg/L for the LC-MS/MS assay. The total PMB concentrations were similar before and after supernatant samples were passed through 0.2 micron pore filters showing the supernatant consisted of primarily lysed bacterial fragments. For the PMB 12 mg/kg Q12h regimen, the total PMB concentrations, as measured by LC-MS/MS, were as high as 52.3 mg/L in the centrifuged bacterial pellet as the *K. pneumoniae* passed through the "failure window". In contrast, the total PMB concentrations in simultaneously collected supernatants approximated 3 mg/L. By bioassay, only a small fraction of the total PMB concentrations measured in broth and the bacterial pellets and supernatants were biologically active (Figure 10). The regrowth of *K. pneumoniae* with increased MICs to PMB confirmed that the drug was inactive in the bacterium.

Total PMB concentrations and bioactivity of PMB in a 24h-time-kill study for *P. aeruginosa* and MRSA. PMB does not have activity against Gram-positive bacteria and the bioactivity of PMB should not be affected by these bacteria. To show that the decrease in the bioactivity of PMB in the *P. aeruginosa* and *K. pneumoniae* HFIM

studies was not due to non-specific binding or degradation of PMB in CA-MHB, a time-kill study was performed in which the *P. aeruginosa* isolate used in the HFIM study (median geometric PMB MIC: 2 mg/L) and an MRSA isolate (PMB MIC: >256 mg/L) were exposed to PMB 4 mg/L for 24 hours. Additional arms consisted of each bacterium alone in CA-MHB and a microbe-free arm consisting of only PMB in broth.

PMB at 4 mg/L initially reduced the *P. aeruginosa* density by a maximum of 2.7 log CFU/mL. But this was followed by regrowth, recapitulating the HFIM study findings. The growth of the MRSA strain was not affected by PMB (Figure 11A). In the experimental arm in which *P. aeruginosa* was co-incubated with PMB, the bioactive PMB concentrations in the supernatant of centrifuged *P. aeruginosa* suspensions (as measured by bioassay) decreased faster than the total PMB concentrations that were measured by LC-MS/MS. The concentrations and bioactivities of PMB in the arms consisting of PMB alone and MSRA in combination with PMB were unchanged (Figure 11B). The total and bioactive PMB concentrations in broth declined before the amplification of the less-susceptible PMB subpopulations and regrowth of the *P. aeruginosa* isolate were observed (Figure 11C). These finding support the hypothesis that the decrease in total PMB as measured by LC-MS/MS and the rapid reduction in bioactive PMB in the HFIM studies with *P. aeruginosa* and *K. pneumoniae* was due to the drug interacting with these Gram-negative bacteria. The total and bioactive PMB concentrations were unchanged when the drug was incubated with MRSA or was incubated in a flask that contained only PMB and CA-MHB.

Discussion

With the rise in the bacterial isolates carrying multiple antibiotic resistance mechanisms PMB has become a drug of last resort, supplanting the carbapenem antimicrobials (23). PMB was originally licensed for clinical use by the US-FDA in June 1964 (24). Its approval pre-dated any modern developmental information on pharmacokinetics and pharmacodynamics. Funding agencies around the world recognized that additional preclinical information was sorely needed to optimize PMB for the treatment of serious infections cause by multidrug-resistant Gram-negative bacteria.

A number of laboratories have examined PMB in the HFIM (11-13). Rapid early bacterial killing was repeatedly seen with PMB. But a consistent observation was the ease and rapidity with which less-susceptible isolates subsequently arose in response to PMB treatment. As with these HFIM experiments, PMB underperformed in the clinic, leading many physicians to call for the use of PMB as part of combination antibiotic regimens (10). The presence of PMB hetero-resistant subpopulations within the larger wild-type bacterial population has been described *in vitro* by others (11, 15, 25). Moreover, emergence of resistance to polymyxins has been documented in patients treated with this antibiotic (26-28), although not with the frequency that HFIM experiments would predict. It was therefore our intent to study PMB in the HFIM to try to delineate the issues leading to early and substantial resistance emergence. To simplify the experiment, we chose to examine PMB as monotherapy.

Against *P. aeruginosa* and *K. pneumoniae* we studied a US-FDA licensed clinical dose regimen of PMB 0.75 mg/kg Q12h, a regimen proposed by an international consensus group of PMB 1.5 mg/kg Q12h (that is higher than the highest dose regimen of PMB 1.25 mg/kg Q12h that is licensed by the US-FDA), and additional dose

regimens as high as 12 mg/kg Q12h (17,18). Although nephrotoxic, the higher dosages were evaluated because Tam et al (13) reported that a PMB 10 mg/kg Q12h regimen prevented resistance amplification in a *P. aeruginosa* isolate in a 4-day HFIM study. In contradistinction to the PMB package insert,(17) a loading dose was used with each regimen with the intent of achieving steady-state PKs with the first dose as another approach to preventing treatment failure due to regrowth (18). As shown in Figures 1 and 3, there was a dose-effect for rapid early microbial kill for the *P. aeruginosa* and *K. pneumoniae* strains. The PMB 0.75 mg/kg Q12h and 1.5 mg/kg Q12h regimens produced a maximum killing of $\leq$ 0.62 and $\leq$ 1.92 log CFU/mL of *P. aeruginosa* and *K. pneumoniae* at 5 hours of treatment. The PMB 12 mg/kg Q12h regimen produced a maximum of ~6.92 log CFU/mL at this time point. However, the bacterial killing by all regimens was followed by bacterial regrowth.

The regrowth was due to three mechanisms of resistance. The first mechanism was a transient lower-level adaptive resistance. Isolates which expressed this resistance had MIC values of $\leq$ 4 mg/L that persisted as long as the isolate was exposed to PMB. However, with one passage on drug-free agar the PMB MIC for the isolates returned to wild-type values. The second mechanism was a higher-level resistance that was stable after 10 serial passages on antibiotic-free agar. *P. aeruginosa* isolates that expressed this resistance mechanism had MIC values for PMB of 4 to 64 mg/L while the *K. pneumoniae* isolates had MICs of 4 to 32 mg/L. The third mechanism is a higher-level moderately stable adaptive resistance that had PMB MICs of 8 to 32 mg/L in both pathogens. The adaptive resistance in these isolates was "moderately stable" because up to 10 passages on drug-free agar were required before

their MICs returned to wild-type values. Isolates which expressed a higher-level stable PMB resistance were mixed with those which expressed higher-level moderately stable adaptive resistance to PMB.

As shown in Figures 6 and 7, most of the colonies which grew on agar infused with 3x the geometric MIC for PMB in the mutation frequency and HFIM studies (and had PMB MICs in the susceptible range *after* passage on drug free agar) were isolates that expressed transient lower-level adaptive resistance. In the control arm and the PMB 0.75 mg/kg Q12h regimen, the majority of isolates that grew on PMB-supplemented agars expressed this transient resistance. With higher PMB dosages, the distribution of resistance mechanisms amplified included a mix of isolates that expressed transient lower-level adaptive and isolates with higher-level PMB resistances. The higher-level resistances in >90% of the *P. aeruginosa* isolates and in >95% of the *K. pneumoniae* isolates were stable after 10 serial passages on drug-free agar. The rest expressed a higher-level "moderately stable" adaptive resistance to PMB in that up to 10 passages was needed for the increased PMB MICs for these isolates to return to wild-type values. This compares with a report that one in four *P. aeruginosa* isolates with higher-level resistance to PMB had a reduction in MICs after 20 passages (13). The highest dose regimens of PMB 6 and 12 mg/kg Q12h for *P. aeruginosa* and the regimen of PMB 12 mg/kg Q12h for *K. pneumoniae*, prevented the emergence of isolates that expressed transient lower-level adaptive resistance. Only isolates with higher-level stable resistance and higher-level "moderately stable" adaptive resistance were amplified.

The traditional antibiotic susceptibility testing method, using serial two-fold geometric dilutions of PMB in agar, readily identified isolates that had higher-level MICs to PMB due to stable resistance or moderately stable adaptive resistance mechanisms. However, the susceptibility testing method had to be modified to identify and further characterize isolates that expressed transient lower-level adaptive resistance. In the traditional antibiotic susceptibility testing method, MICs are determined after colonies are cultured on drug-free agar (21). To identify isolates that expressed transient lower-level adaptive resistance, we used a previously employed method (20). Colonies which grew on agars infused with 3x the geometric PMB MIC of the parent isolate in the mutation frequency and HFIM studies were passaged on drug-free agar and agar infused with 2x the geometric MIC value of the parent isolate. This concentration of PMB in agar was used because the colonies did not grow when passaged on agar infused with 3x the geometric MIC for PMB. Further, agar dilution susceptibility testing was performed using arithmetic 0.5 mg/L increments of PMB since most of the isolates that expressed transient lower-level adaptive resistance had MICs that were within one 2-fold dilution of the parent isolate. Since CLSI (21) considers, “the acceptable reproducibility of the test is within one twofold dilution of the endpoint”, use of the traditional 2-fold geometric dilution series would not have identified many of the isolates which expressed transient lower-level adaptive resistance to PMB.

Amplification of lower- and higher-level adaptive resistance and stable higher-level resistance mechanisms were central to PMB treatment failure. Another important factor is the mechanism of action of PMB itself. PMB acts by binding to membrane-bound lipopolysaccharide A (LPS) to displace the cations associated with the outer

membrane of Gram-negative bacteria. This destabilizes membrane function and integrity, leading to bacterial death (1,2). The ~10^8 total bacteria placed into each HFIM arm was consistent with the high densities of bacteria that can be found in ventilator-associated pneumonia (19). All of the PMB regimens were well-simulated in the CA-MHB of all HFIM arms before they were inoculated with a bacterium. For all of the PMB regimens evaluated, the bioactive concentrations of PMB in the broth medium quickly decreased below the targeted PK profiles soon after the *P. aeruginosa* and *K. pneumoniae* isolates were introduced into the HFIM arms and before regrowth was documented (Figures 9 and 10). Total and bioactive PMB also decreased in the broth of the time-kill study, where more frequent samplings showed that the decrease in both the total and bioactive PMB in broth occurred before the emergence of less-susceptible isolates and regrowth were documented. The total PMB concentrations in broth that were measured by LC-MS/MS also decreased in HFIM arms that received lower dose regimens for PMB. Overall, the largest reductions in the total and bioactive PMB concentrations in broth medium were observed as the bacteria entered and passed through the treatment "failure window." The "failure window" was defined *post hoc* to begin when the bacterial regrowth reached the stasis concentration of ~ 7 log CFU/mL and ended when the bacteria approximated the 10 log CFU/mL density of the control arm.

For the *P. aeruginosa* HFIM study, the decline in PMB concentrations in the broth medium with treatment failure was coupled with a small to moderate increase in the total PMB concentrations measured in the supernatants of centrifuged bacterial suspensions and a larger increase in total PMB in the bacterium. But bioactive PMB

was not detected in the supernatant nor in the bacteria. The regrowth of the bacteria confirmed that the total PMB concentrations measured in the *P. aeruginosa* were biologically inactive. Others have reported resistance to PMB was mediated through modifications in LPS structure that results in decreased PMB binding (3-5). In contrast, the current study found total PMB to accumulate in the PMB-resistant isolates and, to a lesser extent, in bacterial fragments.

The total and bioactive PMB profiles measured in the broth medium in the *K. pneumoniae* HFIM study mirrored those observed in the *P. aeruginosa* experiment. In both studies, the decline in bioactive PMB concentrations preceded regrowth and treatment failure. The only difference between the experiments was the amount of total PMB measured in the supernatants of centrifuged bacterial suspensions. In the *P. aeruginosa* HFIM study, the total PMB measured in supernatants were lower than those measured in the bacteria. However, for the PMB 0.75 mg/kg Q12h and PMB 1.5 mg/kg Q12h regimens, the supernatants and bacteria in the *K. pneumoniae* HFIM study had similar, high concentrations of PMB. There was no measurable bioactive PMB in the supernatant and bacterial samples. The concentrations of total PMB in the supernatant samples were not affected by filtration with a 0.22 micron pore filter. This suggests the PMB in the supernatant was bound to bacterial membrane fragments and possibly free LPS that was shed from the *K. pneumoniae* isolate (5).

A limitation of this project is the small number of bacterial strains that formed the basis for our conclusions. However, the two strains evaluated were of different bacterial species and the dose-related outcomes are remarkably similar, providing cross validation of the study findings. Further, the LLOQ of the bioassay made it impossible

to quantitate the biologically-active concentrations of PMB in many of the broth, bacterial pellets and supernatant samples. The bioassay measurements did correlate well with the broth concentrations of PMB quantitated by LC-MS/MS in the HFIM arms dosed with PMB *before* they were inoculated with bacteria (Figure 8). The two assays also had similar measured PMB peak values for the first dose of PMB administered to the HFIM arm after they were inoculated with bacteria. Importantly, the LC-MS/MS method and bioassay showed large differences in the total and biologically active PMB concentrations in the broth, bacterial pellets, and supernatant samples. Another limitation is that this study did not characterize the molecular basis for bacterial regrowth and PMB treatment failure. However, we were able to phenotypically identify three resistance mechanisms that contributed to PMB treatment failure. They included: (i) a transient lower-level adaptive resistance that emerged with the lower PMB dose regimens that had PMB MICs capped at 4 mg/L, (ii) a higher-level, moderately stable adaptive resistance with MICs of 4 – 64 mg/L, and (iii) a higher-level stable resistance also with MICs of 4 – 64 mg/L. The higher-level resistances were amplified with higher PMB dosed regimens. It is likely that different characterized and yet to be characterized resistance mechanisms that alter the binding of PMB to the LPS of *P. aeruginosa* and *K. pneumoniae*, alone and in combination with inducible efflux pumps, and other mechanisms for altered membrane physiology interplay to produce the different phenotypic resistances to PMB that were identified in this project (4-6, 39-43). Reduction in bioactive PMB in broth relative to the targeted values and in centrifuged-derived bacterial supernatants and pellets also contributed to treatment failure for the *P. aeruginosa* and *K. pneumoniae* isolates.

In summary, PMB dose regimens as high as 12 mg/kg Q12h provided a dose-response killing of *P. aeruginosa* and *K. pneumoniae*. However, all the regimens examined ultimately failed with regrowth. The regrowth was due to isolates that expressed transient, lower-level adaptive resistance; a higher-level moderately stable adaptive resistance; and/or a stable higher-level resistance. Two important questions remain. When PMB is combined with drugs of other classes, do the findings described in this project carry over to those scenarios? Perhaps most importantly, will these findings carry over to preclinical animal systems, particularly with high burden infections in a defined compartment, such as is seen in *in vivo* pneumonia models?

Materials and Methods

Microbes. The high β-lactamase overexpressing *Pseudomonas aeruginosa* PAO1 isolate was kindly provided by Drs. Karen Bush and Ann-Marie Queenan (at the time of the gift, employed by Johnson and Johnson). It had an MIC of 1 mg/L to meropenem. The *bla*$_{KPC-3}$ harboring *K. pneumoniae* strain 1088 was from our collection. It had an MIC of 64 mg/L to meropenem and >512 mg/L for cefepime. For each experiment, bacteria from a frozen stock were grown overnight on blood agar at 35°C, ambient air. A few colonies were propagated to mid-logarithmic phase growth in CA-MHB and adjusted to the desired concentration with broth medium. Quantitative cultures were performed on the bacterial suspension, which was used immediately. MRSA ATCC 33591 was grown to mid-log phase growth in CA-MHB and was diluted to the desired concentration for use in a time-kill study with the *P. aeruginosa* strain to compare the bioactivity of PMB when exposed to a Gram-positive and Gram-negative bacterium.

629 ***Antibiotic.*** A single lot of polymyxin B sulfate for injection (Lot CD711, Fresenius Kabi,
630 Lake Zurich, IL) was purchased from Curascript SD (Lake Mary, FL) and was used for
631 all the studies. It was also used to generate the standard curves for the LC-MS/MS and
632 biological assays. Drug solutions were prepared per manufacturer's instructions and
633 were used immediately.

634 ***In vitro susceptibility testing.*** The susceptibility of the bacterial strains to PMB were
635 determined using the microdilution broth method described by the Clinical and
636 Laboratory Standards Institute in CA-MHB (21) and on cation-adjusted Mueller-Hinton
637 agar (CA-MHA – Becton Dickinson). Serial two-fold geometric drug dilutions and 0.5
638 arithmetic increments of drug concentrations were used. The MICs were read after the
639 cultures had incubated for 16 – 20 hours at 35°C, ambient air. For the geometric and
640 arithmetic dilution methods, the MIC was defined as the lowest concentration of drug
641 that resulted in no visible growth.

642 ***Mutation frequency assessments.*** The microbes were grown to late logarithmic
643 phase growth in CA-MHB. The bacteria were quantitatively cultured on drug-free CA-
644 MHA to enumerate the total bacterial burden. Four mL of the bacterial suspensions
645 were also quantitatively cultured onto agar supplemented with 3x the geometric MIC
646 value of PMB for that parent bacterium. After 48 hours of incubation, colonies on the
647 drug-free and antibiotic-supplemented agars were enumerated. The mutation
648 frequency values were calculated by dividing the bacterial density enumerated on PMB-
649 supplemented agar by the number of colonies that grew on drug-free agar.

650 ***Dose-range effect of PMB for the P. aeruginosa and K. pneumoniae isolates.*** The
651 HFIM is used to evaluate the impact of simulated fluctuating concentration-time profiles

of different antibiotic exposures on the amount of bacterial killing. It is also used to identify the lowest dosage or exposure of an antibiotic that minimizes the amplification of the pre-existing bacterial subpopulations that were less-susceptible to the administered drug (29-33).

The studies were performed using hollow fiber cartridges purchased for FiberCell Systems (Frederick, MD). HFIM experimental arms were inoculated with 12 ml of approximately 7 log CFU/mL of the AmpC overexpressing *P. aeruginosa* isolate or the *bla*$_{KPC-3}$ harboring *K. pneumoniae*.

For the *P. aeruginosa* study, one HFIM arm served as a no-treatment control. The remaining 5 arms aimed to simulate the free plasma concentration-time profiles and exposures for the clinically-prescribed regimen of PMB 0.75 mg/kg Q12h and the supra-clinical regimens of 1.5, 3, 6, and 12 mg/kg Q12h. The HFIM dose-range study using the *K. pneumoniae* strain included a no-treatment control and experimental arms in which the free (non-protein bound) plasma PK profiles for PMB 0.75. 1.5, and 12 mg/kg Q12h were simulated. Loading doses were administered with the intent to rapidly achieve steady-state PK profiles (18). Each dosing of PMB was infused over 1 hour. The intended simulated half-life was 8 hours. The intended PK parameters for the five PMB regimens were calculated based on the assumption that AUC exposures for this antibiotic were dose proportional. The intended steady-state PK-PD parameter values for the PMB regimens for the dose-range study with the *P. aeruginosa* and *K. pneumoniae* isolates are shown in Tables 3 and 4, respectively.

The antibiotic was administered for 2 days *before* the bacteria were inoculated into the HFIM systems. Broth samples collected over this timespan for PMB

measurements by LC-MS/MS and bioassay showed that the targeted PK profiles were well simulated in the HFIM arms prior to the introduction of bacteria (Figure 8). After a bacterium was inoculated into the HFIM arms, PMB treatment was continued for 10 days. Additional broth samples were collected from the central reservoirs for 2 to 3 days after bacteria were added to the HFIM arms (depending on the experiment) and the concentrations of PMB in those samples were measured by LC-MS/MS and bioassay to quantify the impact the bacteria had on the total and bioactive PMB concentration-time profiles that were achieved.

For both HFIM studies, aliquots of bacterial specimens were collected from the experimental arms at baseline (time = 0h) and at 0.17, 1, 2, 3, 4, 6, 8 and 10 days after a microbe was introduced to the HFIM systems. The bacteria were washed twice with normal saline and then quantitatively cultured onto drug-free agar to evaluate the effect of each regimen on the total bacterial population. A sample of the washed bacterial suspensions was also quantitatively cultured on CA-MHA supplemented with 3x the geometric MIC for PMB of the respective parent isolate to characterize the effect of each regimen on the killing or amplification of the less-susceptible bacterial subpopulations. For the *P. aeruginosa* HFIM experiment, additional bacterial suspensions were collected at 48, 49, 71 and 73h after the bacterium was inoculated into the HFIM arms. For the *K. pneumoniae* HFIM study, additional broth and bacterial suspensions were collected 169 – 173 hours after the bacterium was introduced into the HFIM systems. The *P. aeruginosa* and *K. pneumoniae* suspensions were centrifuged and separated into bacterial pellets and the supernatants. The total PMB concentrations in the bacterial pellets and supernatants were assayed by LC-MS/MS. The biologically

active concentrations of PMB in these samples were quantified using a bioassay (see below for detailed methods for the assays).

PMB susceptibility testing, including serial passage studies to assess for transient, reversible adaptive resistance. The two-step susceptibility testing algorithm was described in the Results section. In Step I, a subset of colonies that grew on agar supplemented with 3x the geometric MIC of the parent strain were subcultured on drug-free agar and MICs were performed on the resultant colonies (Figure 5). The distribution of the PMB MICs that were categorized as susceptible, intermediate, and resistant were reported in Tables 1 and 2 for the *P. aeruginosa* and *K. pneumoniae* colonies, respectively.

The stability of the resistance mechanisms was assessed in a subset of isolates which had intermediate and resistant PMB MIC values. Twelve *P. aeruginosa* isolates with PMB MICs of 4 to 64 mg/L and 12 *K. pneumoniae* isolates which had PMB MICs of 4 to 32 mg/L were serially passaged for 10 times on drug-free agar. Agar geometric dilution susceptibility studies were performed on the colonies that did not and did undergo the serial passages. Colonies which had geometric MICs before and after the serial passages that were within one 2-fold dilution of each other were deemed to express a stable PMB resistance mechanism. Colonies which had MIC differences that were $\geq$ 2-fold dilutions were deemed to express a "moderately stable" adaptive resistance. The term "moderately stable" was applied to differentiate this adaptive resistance from the transient lower-level adaptive resistance described in Figure 5, in which the higher MICs identified in isolates that grew on PMB-supplemented agars reverted to wild-type values after one passage on drug-free agar.

Colonies reported in Tables 1 and 2 that grew on PMB-supplemented agars in the mutation frequency and HFIM studies and had wild-type MIC values after they were subcultured once on drug-free agar, were assessed for transient adaptive resistance following the methods described in Step II in Figure 5.

A PMB time-kill study for *P. aeruginosa* and MRSA. A time-kill study was performed to characterize the effect of 4 mg/L of PMB on the killing of the *P. aeruginosa* high AmpC hyperproducing strain (MIC: 2 mg/L) and MRSA ATCC 33591 (PMB MIC: >256 mg/L) and to quantify by LC-MS/MS and bioassay the effect of each bacterium on the total and bioactive concentrations of PMB. Growth controls included the *P. aeruginosa* and MRSA isolates incubated in CA-MHB without PMB. Another control arm had PMB 4 mg/L in CA-MHB without bacteria. Bacterial samples were collected from the applicable experimental arms after 0, 1, 3, 5, and 24h of incubation at 35°C, ambient air. The bacterial suspensions were washed by centrifugation. Quantitative cultures were performed on drug-free agar after the bacteria were resuspended in sterile normal saline. Supernatants of centrifuged bacterial suspensions were assayed for PMB total concentrations and bioactivities by LC-MS/MS and bioassay, respectively. The *P. aeruginosa* culture that was exposed to PMB was also quantitatively cultured on agar infused with 3x the geometric MIC of the parent strain to evaluate for resistance emergence.

PMB LC-MS/MS Assay. The methods of Cheah et al. and Covelli et al. (34, 35) were used, with modifications. CA-MHB media samples and the bacterial pellets and supernatants from centrifuged bacterial suspensions were collected from the central reservoir and peripheral compartment, respectively. The samples were stored at -80°C

744 until analysis. After thawing at room temperature, 0.02 mL of each sample was
745 processed using 0.005 mL of internal standard (pretomanid 1 mg/L in water) followed by
746 0.2 mL of 1% formic acid in acetonitrile. Samples were vortexed well and centrifuged for
747 10 minutes. The resulting sample supernatant was transferred to an LC-MS vial and
748 5µL was used as injection volume for analysis. The standard curves and quality control
749 samples were made from the same vial of pharmaceutical-grade PMB that was used in
750 the PK-PD studies.

751 Determination of PMB was performed using LC-MS/MS consisting of a
752 Prominence HPLC (Shimadzu) and an API5000 triple quadrupole mass spectrometer
753 (ABSciex). Chromatography was performed using a Kinetex C18 50 x 3mm, 2.6µ
754 (Phenomenex) HPLC column at 40°C with a run time of 7 minutes. Mobile phases
755 consisted of 0.5% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a
756 flow rate of 0.500mL/min in gradient mode. The mass spectrometer was operated in
757 positive ion mode using the Turbo Ion Spray (TIS) probe interface. Multiple reaction
758 monitoring (MRM) m/z 602.581/101.1 $[M+1]^{2+}$ (quantifier) and MRM m/z 602.581/73.8
759 $[M+1]^{2+}$ (qualifier) was used for PMB; and MRM m/z 360.06/175.1 for internal standard
760 pretomanid.

761 Linearity for PMB using a quadratic regression with a weighting of $1/x^2$, with a
762 dynamic range of 0.250 to 40.0 mg/L, was demonstrated for each calibration curve over
763 2 separate runs with a correlation coefficient (R) of ≥0.9978 and coefficient of
764 determination (R^2) of ≥0.9956. Within-run as well as between-run accuracies for each
765 calibration curve were within ±9.4% of the nominal concentrations and <4.6% for the
766 respective coefficients of variation of the mean values. Calibration curve precision

within-run ranged from 0.8% to 3% and between-run 2.2% to 4.5%. Performance of quality control samples within-run as well as between-run accuracies were within ±10.8% of the nominal concentrations and <12.5% for the respective coefficients of variation of the mean values. Quality precision within-run ranged from 3% to 12.4% and between-run 5.4% to 9.2%. Calculated concentrations were performed using Analyst software v 1.6 (AB Sciex).

PMB Bioassay for central and peripheral compartment samples. In initial studies, the LLOQ of a bioassay using *E. coli* ATCC 22952 was 4 mg/L. Since the intended peak, mean, and trough concentrations of PMB to be simulated in the HFIM for the lowest dosage regimen (PMB 0.75 mg/kg Q12h) were 1.5, 1, and 0.58 mg/L, respectively, a more sensitive bioassay was needed. The low sensitivity of PMB bioassays, in general, was due to: the large size of the molecule, which slows the diffusion of the drug through agarose, and the interaction of this positively charged antibiotic with the negative charge of the sulfides on the agarose (36-38). With consideration of these factors and to improve the sensitivity of the assay, 0.1 mL of serial two-fold dilutions of PMB standards (range 0.625 to 40 mg/L) and CA-MHB, supernatant and bacterial pellet samples collected from HFIM arms were pipetted into 200 µl hollow tubular Peni cylinders (BioLogics, Inc., Manassa, VA) which were placed onto the surface of solidified CA-MHA (supplement Figure S1). The agar was made using 80% of the medium powder specified by the manufacturer. The PMB standards and the samples collected from the HFIM arms that were dispensed into the Peni cylinders were allowed to diffuse into the agar over 24 hours at room temperature. The Peni cylinders were removed from the surface of the agars and *E. coli* ATCC25922

(agar PMB MIC: 0.25 mg/L) was streaked onto the surface of the agars. The agar plates were incubated for 18h at 35°C, ambient air before the zones of inhibition were measured with calipers. The assay was performed twice for each sample and the results were averaged. The lower limit of sensitivity of the modified bioassay was 1.2 mg/L. The standard curve was linear from 1.2 to 40 mg/L (R^2 values of >0.994). The intraday and interday CV% for the 40 mg/L concentration is 3.7% and 5.8%, respectively. For the 1.2 mg/L concentration, the intraday and interday CV% values are 4.5% and 7.3%, respectively.

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973

974 **Figure 1:** Effect of five PMB regimens on the killing of the total population of a *P.*
975 *aeruginosa* PA01 isolate that overexpressed an AmpC β-lactamase. The median
976 geometric MIC value for PMB was 2 mg/L (geometric MIC range: 1 – 2 mg/L).

977

978 **Figure 2:** Effect of five PMB regimens on the total *P. aeruginosa* PAO1 populations
979 (blue diamonds) and the subpopulations which grew on CA-MHA supplemented with 3x
980 the geometric median PMB MIC (red squares) for the parent isolate. The geometric
981 mean PMB MIC for the microbe was 2 mg/L and the antibiotic-supplemented agar
982 contained 6 mg/L of PMB.

983

984 **Figure 3.** Dose-range effect of PMB on the total population of *K. pneumoniae*. The
985 geometric and arithmetic MIC value for PMB were both 1 mg/L.

986

987 **Figure 4.** Effect of individual PMB regimens on the total and less-susceptible *K.*
988 *pneumoniae* populations. The concentration of antibiotic added to the "PMB 3xMIC"
989 agar was 3 mg/L, which corresponds to 3x the mean geometric MIC of 1 mg/L for the
990 parent isolate.

991

992 **Figure 5.** Two step algorithm for PMB susceptibility testing performed on "primary
993 colonies" which grew on agar infused with 3x the geometric MIC of PMB of the parent
994 bacterium in the mutation frequency and HFIM dose-range experiments.

995

Figure 6. Only the *P. aeruginosa* colonies which grew on PMB-infused agars in the mutation frequency and HFIM studies and had wild-type MIC values after they were passaged on drug-free agar (Table 1), were evaluated for transient lower-level adaptive resistance to PMB. This was accomplished employing Step II of the antibiotic susceptibility algorithm outlined in Figure 5. The distribution of arithmetic MICs for *P. aeruginosa* colonies subcultured on fresh PMB-supplemented agar (red bars) were higher than the distribution of arithmetic MICs that were performed on the same colonies after they were passaged on drug-free agar (blue bars). The second column in the table reports the number of "primary colonies" that grew on both PMB-infused and drug free agars and the number of these colonies that had at least a two dilution (or $\geq$ 1 mg/L) difference in MIC values for PMB for susceptibility testing that was simultaneously performed on colonies which grew on PMB-containing and drug-free agars. The third column describes the MIC values for the colonies that were passaged and grew on drug-free and PMB-infused agars that did not meet the definition for transient adaptive resistance.

Figure 7. Only the *K. pneumoniae* colonies which grew on PMB-infused agars in the mutation frequency and HFIM studies and had wild-type MIC values after they were passaged on drug-free agar (Table 2), were evaluated for transient lower-level adaptive resistance to PMB. See Figure 6 for explanation of the column titles in Figure 7. The distribution of arithmetic MICs for *K. pneumoniae* colonies subcultured on fresh PMB-supplemented agar (red bars) were higher than the distribution of arithmetic MICs that

were performed on the same colonies after they were passaged on drug-free agar (blue bars).

Figure 8. Examples of the LC-MS/MS and bioassay measurements of PMB in hollow fiber arms *before* they were inoculated with *P. aeruginosa:* (A) PMB 0.75 mg/kg Q12h, (B) PMB 1.5 mg/kg Q12h, and (C) PMB 3 mg/kg Q12h regimens. The PK profiles for PMB were well-simulated when evaluated by both drug assays. For the PMB 0.75 mg/kg Q12h regimen, many of the targeted PMB are below the LLOQ of 1.2 mg/L of the bioassay.

Figure 9: PMB PK measurements for the first 173 hours *after* the HFIM arms were inoculated with *P. aeruginosa*. PMB measurements (mg/L) in the broth medium collected from the central reservoir and in the supernatant and bacterial pellet obtained after centrifuging the bacterial suspension collected from the peripheral compartment are shown. The solid curves are the intended concentration-time profiles for simulation. The symbols are the measured PMB concentrations in broth collected from the central reservoir and the centrifuged bacterial pellet and respective supernatants as determined by LC-MS/MS or bioassay. The no treatment control and is not shown.

Figure 10. PMB PK measurements (mg/L) for the first 172 hours *after* HFIM arms were inoculated with a *bla*$_{KPC-3}$ harboring *K. pneumoniae* strain. PMB measurements in the broth medium collected from the central reservoir and in the supernatant and bacterial pellet obtained after centrifuging the bacterial suspension collected from the peripheral

compartment are shown. The solid curves are the intended concentration-time profiles for simulation. The symbols are the measured PMB concentrations in media collected from the central reservoir and the centrifuged bacterial pellet and respective supernatants as determined by LC-MS/MS or bioassay. "Arm A" was the no-treatment control arm and is not shown.

Figure 11. Time-kill study for PMB at 2x MIC for the *P. aeruginosa* (PA) strain (MIC: 2 mg/L). The same PMB exposure had no effect on the growth of an MRSA strain which had a PMB MIC of > 256 mg/L (A). The concentrations of PMB measured by LC-MS/MS and bioassay in supernatants of centrifuged *P. aeruginosa* and MRSA suspensions and in bacteria-free broth medium (B). Relationship between PMB concentrations in broth supernatant and total and less-susceptible PA populations (C). LLOQ: lower limit of quantification.

1054 **Table 1:** Effect of PMB exposures on the distribution of MICs for a subset of *P.*
1055 *aeruginosa* colonies that grew on agars infused with 3x the geometric PMB MIC of the
1056 parent isolate from the respective mutation frequency and HFIM experiments. The
1057 colonies were expanded on *drug-free agar* before susceptibility studies were performed
1058 using serial geometric dilutions of PMB. The MIC breakpoints were defined by CLSI
1059 (22). For these susceptibility studies, the median geometric PMB MIC value for the
1060 parent *P. aeruginosa* isolate was 2 mg/L.

Isolate Source	Number of Isolates Tested	Geometric Dilution MIC Range (mg/L)	% Susceptible (≤ 2 mg/L)	% Intermediate (4 mg/L)	% Resistant (≥ 8 mg/L)
Mutation Frequency Plates	3	0.5 – 4	66%	33%	0%
			2 Isolates	1 Isolate	0 Isolates
No Treatment Control	12	1 – 32	50%	33%	17%
			6 Isolates	4 Isolates	2 Isolates
0.75 mg/kg Q12h	12	1 – 64	75%	17%	8%
			9 Isolates	2 Isolates	1 Isolates
1.5 mg/kg Q12h	12	1 – 64	42%	0%	58%
			5 Isolates	0 Isolates	7 Isolates
3 mg/kg Q12h	10	4 – 64	0%	20%	80%
			0 Isolates	2 Isolates	8 Isolates
6 mg/kg Q12h	9	16 – 64	0%	0%	100%
			0 Isolates	0 Isolates	9 Isolates
12 mg/kg Q12h	9	16 – 64	0%	0%	100%
			0 Isolates	0 Isolates	9 Isolates

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1062

Table 2: Effect of different PMB regimens on the distribution of geometric (2-fold) dilution MICs determined on a subset of *K. pneumoniae* colonies recovered from agar infused with 3x the geometric MIC of PMB that were part of the mutation frequency and HFIM dose-range studies. The geometric (2-fold) dilution MICs shown on this table were performed *after* the colonies that grew on agars supplemented with 3x the geometric PMB MIC of the parent isolate were expanded on drug-free agar. CLSI and the U.S. Food and Drug Administration have not established PMB breakpoint values for susceptible, intermediate, and resistant to *K. pneumoniae*. For this project the breakpoints for *P. aeruginosa* were used. The median geometric MIC value for PMB for the *K. pneumoniae* isolate inoculated into the HFIM experimental arms was 1 mg/L.

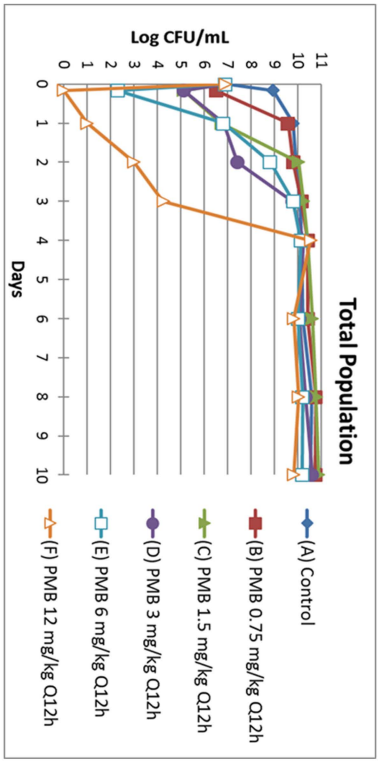
Isolate Source	Number of Isolates	Geometric Dilution MIC Range (mg/L)	% Susceptible (≤ 2 mg/L)	% Intermediate (4 mg/L)	% Resistant (≥ 8 mg/L)
Mutation Frequency Plates	4	1 – 2	100%	0%	0%
			4 Isolates	0 Isolate	0 Isolates
No Treatment Control	24	1 – 2	100%	0%	0%
			24 Isolates	0 Isolates	0 Isolates
0.75 mg/kg Q12h	24	1 – 2	100%	0%	0%
			24 Isolates	0 Isolates	0 Isolates
1.5 mg/kg Q12h	24	1 – 8	79%	17%	4%
			19 Isolates	4 Isolates	1 Isolates
12 mg/kg Q12h	15	4 – 32	0%	47%	53%
			0 Isolates	7 Isolates	8 Isolates

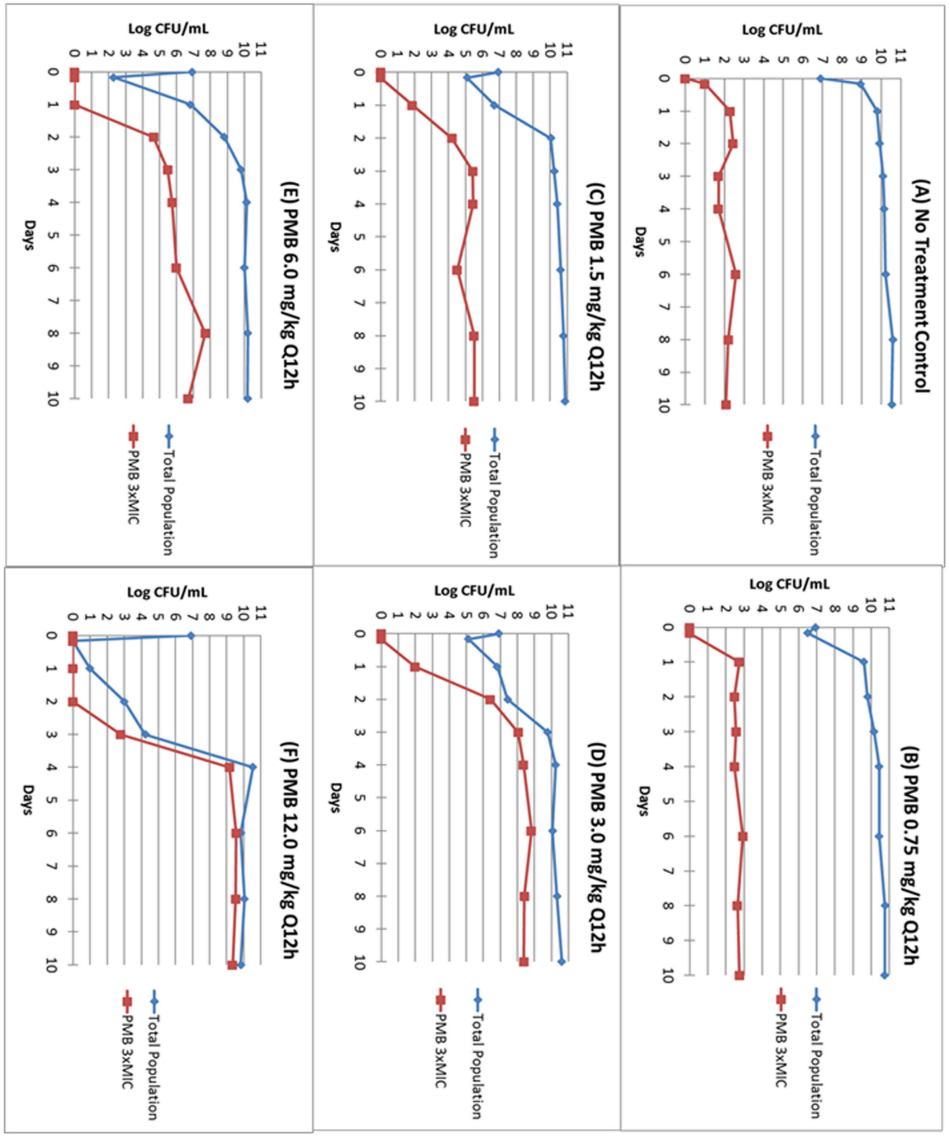
Table 3. Intended PK-PD parameters for simulation in the HFIM for the *P. aeruginosa* isolate. The geometric PMB MIC of 2 mg/L was used to calculate the pharmacodynamic parameter values in the table. These PK-PD parameters were well-simulated *before* the HFIM were inoculated with the bacterium (Figure 8). Figure 9 shows the targeted and measured PMB PKs achieved *after* the *P. aeruginosa* strain was inoculated into the HFIM systems.

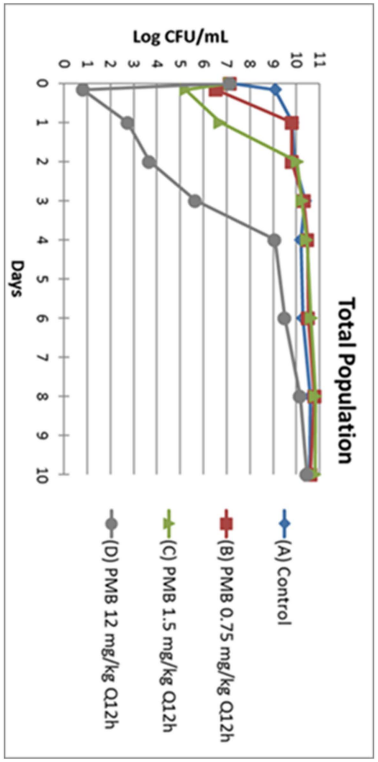
Treatment Arm	AUC/MIC	Cmax/MIC	Trough/MIC	Time > MIC (h)	% Time above MIC
(A) No treatment Control	0.00	0.00	0.00	0	0%
(B) PMB 0.75 mg/kg Q12h	11.64	0.77	0.29	0	0%
(C) PMB 1.5 mg/kg Q12h	23.28	1.53	0.59	10.5	44%
(D) PMB 3 mg/kg Q12h	46.56	3.06	1.18	24	100%
(E) PMB 6 mg/kg Q12h	93.12	6.12	2.36	24	100%
(F) PMB 12 mg/kg Q12h	186.25	12.24	4.72	24	100%

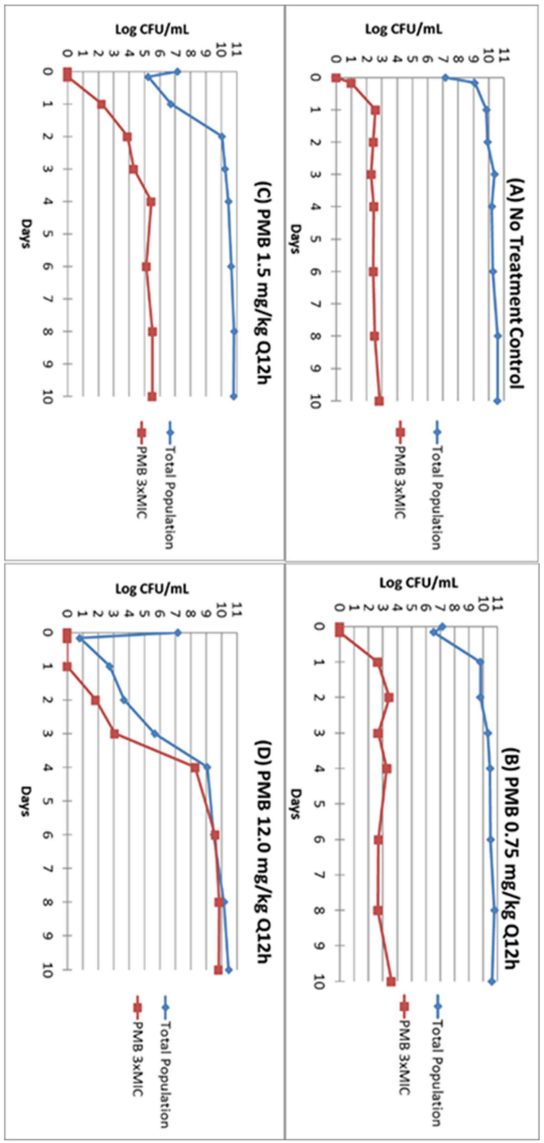
Table 4. Intended PK-PD parameters for simulation in the PMB HFIM study with the *K. pneumoniae* strain. The geometric PMB MIC value of 1 mg/L was used to calculate the pharmacodynamic parameter values in the table. These PK-PD parameters were well-simulated *before* the HFIM were inoculated with the bacterium (data not shown). Figure 10 shows the targeted and measured PMB concentration-time curves achieved after *K. pneumoniae* was inoculated into the HFIM cartridges.

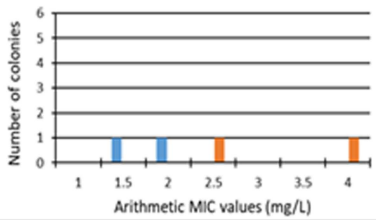
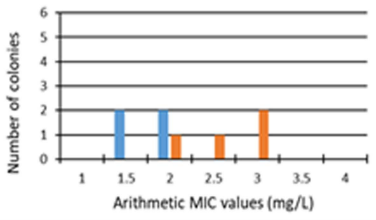
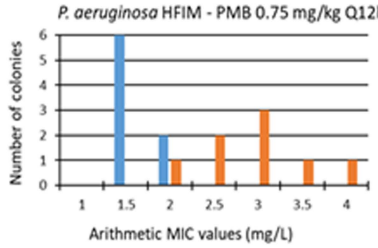
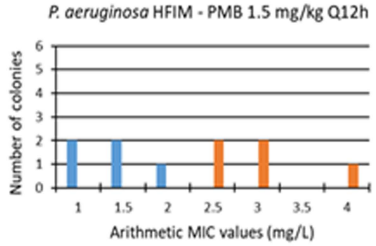
Treatment Arm	AUC/MIC	Cmax/MIC	Trough/MIC	Time > MIC (h)	% Time above MIC
(A) No treatment Control	0.00	0.00	0.00	0	0%
(B) PMB 0.75 mg/kg Q12h	23.28	1.53	0.59	0	0%
(C) PMB 1.5 mg/kg Q12h	46.56	3.06	1.18	10.5	44%
(D) PMB 12 mg/kg Q12h	372.49	24.48	9.44	24	100%

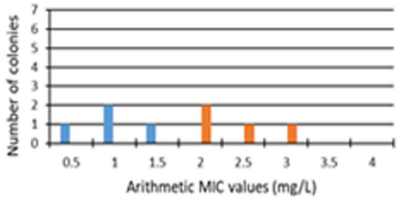
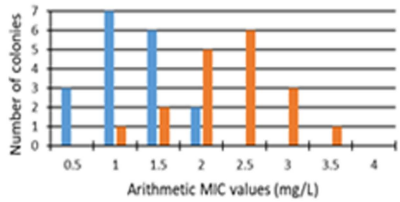
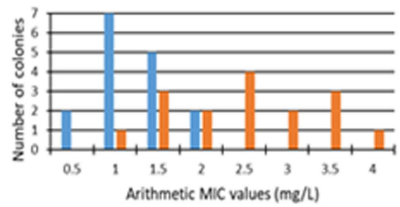
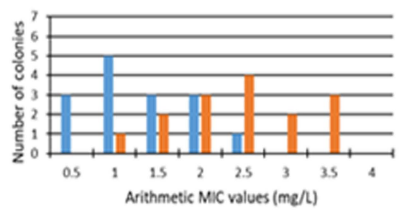








MIC distribution for the same colonies after they were passaged and grew on drug-free agar (blue bars) and on PMB-infused agar (red bars)	No. of passaged colonies that grew on both PMB-infused and drug-free agars. (No. of CFUs expressing transient lower-level adaptive resistance)	Arithmetic MICs for CFUs that grew on both PMB-infused and drug-free agars that did not meet the definition for transient adaptive resistance (MIC after passage on drug-free agar → MIC after passage on PMB-infused agar)
P. aeruginosa - Mutation frequency study  	2 of 2 CFUs grew on both agars. (2 of 2 expressed lower-level transient adaptive resistance)	Not applicable
P. aeruginosa HFIM Study - No treatment  	4 of 6 colonies grew on both agars (3 of 4 CFUs expressed transient lower-level adaptive resistance)	1.5 mg/L → 2.0 mg/L for 1 CFU
P. aeruginosa HFIM - PMB 0.75 mg/kg Q12h  	8 of 9 CFUs grew on both agars (7 of 8 colonies expressed transient lower-level adaptive resistance}	1.5 mg/L → 2.0 mg/L for 1 CFU
P. aeruginosa HFIM - PMB 1.5 mg/kg Q12h  	5 of 5 CFUs grew on both agars (5 of 5 CFUs expressed transient lower-level adaptive resistance)	Not applicable

MIC distribution for the same colonies after they were passaged and grew on drug-free agar (blue bars) and on PMB-infused agar (red bars)	No. of passaged colonies that grew on both PMB-infused and drug-free agars. (No. of CFUs expressing transient lower-level adaptive resistance)	Arithmetic MICs for CFUs that grew on both PMB-infused and drug-free agars that did not meet the definition for transient adaptive resistance (MIC after passage on drug-free agar → MIC after passage on PMB-infused agar)
K. pneumoniae - Mutation frequency study  	4 of 4 CFUs grew on both agars (3 of 4 CFUs expressed transient lower-level adaptive resistance)	1.5 mg/L → 2 mg/L for 1 CFU
K. pneumoniae HFIM Study - No Treatment  	18 of 24 grew on both agars (16 of 18 CFUs expressed transient lower-level adaptive resistance)	0.5 mg/L → 1.0 mg/L for 1 CFU 1.5 mg/L → 2.0 mg/L for 1 CFU
K. pneumoniae HFIM - PMB 0.75 mg/kg Q12h  	16 of 24 CFUs grew on both agars (12 of 16 CFUs expressed transient lower-level adaptive resistance)	0.5 mg/L → 1.0 mg/L for 1 CFU 1.0 mg/L → 1.5 mg/L for 2 CFUs 1.5 mg/L → 2.0 mg/L for 1 CFU
K. pneumoniae HFIM - PMB 1.5 mg/kg Q12h  	15 of 24 CFU grew on both agars (11 of 15 CFUs expressed transient lower-level adaptive resistance)	0.5 mg/L → 1.0 mg/L for 1 CFU 1.0 mg/L → 1.5 mg/L for 1 CFU 1.5 mg/L → 1.5 mg/L for 1 CFU 1.5 mg/L → 2.0 mg/L for 1 CFU