Evaluating the Killing Dynamics of Polymyxin B and Meropenem as Monotherapy and Combination Therapy Against Hypermutable *Pseudomonas aeruginosa*

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ABSTRACT

Introduction

Conserving the activity of the remaining viable last line antibiotic agents for the treatment of highly resistant *Pseudomonas aeruginosa* (PSA) is a critical challenge and data regarding treatment that is effective against hypermutant PSA is limited. To help overcome this challenge, the objective of this study is to perform phenotypic characterization of 33 PSA clinical isolates from cystic fibrosis (CF) patients, and evaluate the killing dynamics of monotherapy and combination therapy against hypermutable PSA.

Methods

Phenotypic characterization was performed to detect hypermutators and bacterial susceptibilities for colistin (COL), polymyxin B (PMB), and meropenem (MEM) were evaluated. Static time–kill experiments were performed to determine the rate and extent of bacterial killing using PMB and MEM as monotherapy and combination therapy against hypermutatant and non-hypermutatant strains, PSA 203 and PSA 205, respectively.

Results

6 out of the 33 (18%) PSA clinical isolates obtained from CF patients were determined to be hypermutators. Time-kill results demonstrated poor sustained killing of PMB monotherapy for both isolates. MEM 10 mg/L and MEM 40 mg/L concentrations in combination with PMB 0.5 mg/L and 1 mg/L resulted in regrowth at 8 h for both PSA 203 and PSA 205 isolates, while combination of PMB 2 mg/L and 4 mg/L resulted in sustained bactericidal killing.

Conclusions

PMB based combination therapy resulted in superior pharmacodynamic activity compared to monotherapy with either drug, which is consistent with previous *in vitro* studies that have evaluated polymyxins in combination with carbapenems against highly resistant Gram-negative bacteria in non-CF patients.

INTRODUCTION

Cystic fibrosis (CF) is a progressive, genetic disease that causes persistent lung infections and limits the ability to breathe over time.[1] CF is caused by mutations in the gene encoding the CF transmembrane regulator, which disrupts electrolyte secretion, leading to a viscous mucus that causes the lungs defense mechanisms to be less effective, hence making CF patients more susceptible to chronic lung bacterial infections.[2] *Pseudomonas aeruginosa* (PSA), a ubiquitous, opportunistic, Gram-negative pathogen, is the major cause of morbidity and mortality in CF patients.[3] In fact, by the age of 20, about 80% of CF patients are chronically infected by PSA.[4] PSA presents a serious therapeutic challenge, as it can develop resistance to multiple classes of antibiotics either through horizontal gene transfer or through mutational processes that alter gene expression.[5] The most common bacterial resistance mechanisms are demonstrated in **Figure 1.** In particular, hypermutant PSA strains are those with an increased mutation frequency due to mutations in mismatch repair (MMR) genes, as shown in **Figure 1c**.[6]

In a pivotal study by Oliver et al. (2000) it was found that 37% of CF patients chronically infected with PSA were colonized by hypermutable strains versus the absence of hypermutators in acute pseudomonas infections of non-CF patients, illustrating the high prevalence and important role of mutators in this patient population.[3] Furthermore, resistance rates of PSA isolates from CF patients have been found to be higher than those found in other settings, including isolates from patients in ICUs.[7] Many studies have demonstrated the link between mutators in CF infections and enhanced antimicrobial resistance development.[8-10] However, data regarding treatment that is effective against hypermutant PSA is limited, and conserving the activity of the remaining viable last line antibiotic agents for the treatment of highly resistant pathogens is a serious challenge.

Multi drug resistant (MDR) PSA have developed resistance to widely-used carbapenems, such as imipenem (IPM) and meropenem (MEM), by producing beta-lactamases (see **Figure 1a**), leading to carbapenemase resistant Enterobacteriaceae or CRE.[11] This is alarming because carbapenems are widely used for the treatment of PSA infections.[12] Another class of antibiotics, polymyxins (colistin (COL) and polymyxin B (PMB)), have historically been reserved as last-line for the treatment of multi-resistant Gram negative bacterial infections.[13,14] However, polymyxins were re-introduced in the 1990s because of CRE and because of the few antimicrobial agents being in development which target MDR Gram-negative bacteria.[13,14] Unlike carbapenems which target the bacterial cell wall, polymyxins target the bacterial cell membrane. The polycationic peptide ring of the polymyxins interacts with the anionic lipopolysaccharide (LPS) molecules in the outer membrane of Gram-negative bacteria, thereby displacing the calcium and magnesium cations that stabilize the LPS molecules.[15] This results in an increase in cell-envelope permeability, leakage of cell contents, and consequently cell death.[15] However, one of the biggest hindrances of PMB being used clinically is its nephrotoxicity.[16,17] Another concern is the re-emergence of resistance with usage of polymyxin monotherapy. Specifically, monotherapy with these agents is unlikely to generate reliably efficacious plasma concentrations, and has been shown to regrow even with concentrations greatly exceeding those achievable clinically.[18] Therefore, polymyxin combination therapy has been increasingly used clinically against MDR gram-negative bacteria.[19]

The objective of this present study is to evaluate the *in vitro* killing dynamics of PMB and MEM as monotherapy and combination therapy against PSA isolates obtained from CF patients. Phenotypical characterization was done to identify hypermutators and static time-kill experiments were performed to evaluate the pharmacodynamics of monotherapy and combination therapy against determined hypermutatant and non-hypermutant strains.

METHODS

Test system for phenotypic characterization

Rifampicin (Rif) is a potent and broad spectrum antibiotic against bacterial pathogens stemming from its inhibition of the bacterial RNA polymerase by binding in a pocket of the RNA polymerase beta subunit.[20] The rpoB gene encodes for the beta subunit of RNA polymerase in bacteria.[21] Thus, mutations to this rpoB gene leads to the phenotype of rifampicin resistance (Rif(r)).[21] This is a common protocol used as a test system for studying mutational processes.[21] RpoB/Rif(r) is an accurate and easy to employ detection system, and offers the advantage of allowing analysis of the MMR genes.[22]

Phenotypic characterization

33 strains of PSA obtained from CF patients were utilized in this study. All isolates were stored at -80°C and subcultured on Mueller-Hinton agar (MHA) 24 hours prior to each experiment. Bacteria from the overnight culture were targeted to a concentration of 10^8 CFU/mL and subcultured into 3 ml of cation-adjusted Mueller-Hinton broth (MHB). Subsequently, 100 µL from this suspension, as well as from serial dilutions, were seeded onto non-drug MHA plates and 300 µg/mL prepared rifampicin MHA plates. In all cases, the isolates were originally susceptible to such concentrations of rifampicin. Plates were incubated for 36 hours at 37°C. Colony counting was performed using ProtoCOL HR automated bacterial colony counter (Synbiosis, Frederick, MD). Mutation frequencies were calculated by dividing the CFU/mL on rifampicin plates over the CFU/mL on non-drug agar plates. The PSA standard laboratory strain, PAO1, was the negative control strain because the genome sequence for this strain is known.

According to Oliver et al 2000, among CF patients nonmutator and mutator groups were distinguished with a mean mutation frequency of $2.9 \pm 2.5 \times 10^{-8}$ and $3.2 \pm 2.5 \times 10^{-6}$ respectively. $2.4 \pm 2.1 \times 10^{-8}$ was the mean mutation frequency found in non-CF patients.[3] From the 33 CF isolates, a strain was considered a mutator strain if the corresponding mutation frequency was $> 2.9 \pm 2.5 \times 10^{-8}$.

Susceptibilities:

Minimum inhibitory concentrations (MICs) of bacterial isolates were determined in triplicate by the microdilution method on 96 well plates using cation-adjusted Mueller-Hinton broth (MHB) against colistin (COL), polymyxin B (PMB), and meropenem (MEM). Bacteria from the overnight culture were targeted to a concentration of 10^6 CFU/mL and subcultured into 3 ml of MHB. Standard procedures followed and resistance breakpoints used were those of the Clinical and Laboratory Standards Institute or CLSI (formerly NCCLS).[23] MICs were read after 18-24 hours (24 hours for slow-growing strains) of incubation at 37°C.

Static time-kill:

Static time–kill experiments were performed over 24 hours to evaluate the rate and extent of killing of PMB (0.5, 1, 2, 4, 8, 16, and 64 mg/L) and MEM (10, 40, 80, 120 mg/L) alone, and PMB and MEM combination therapy against two *Pseudomonas aeruginosa* isolates determined to be hypermutators, with varying susceptibilities (PSA 203 and PSA 205). Bacteria from an overnight culture was targeted to a concentration of 10^6 CFU/mL. Samples were collected at the 0, 2, 4, 6, 8, and 24-hour time points to obtain bacterial quantification. 50 µL of bacterial samples were plated onto MHA using a Whitley Automated Spiral Plater II (Don Whitely Scientific, West Yorkshire, UK). Colony counting was performed in plates after 24 hours of incubation at 37°C using ProtoCOL HR automated bacterial colony counter (Synbiosis, Frederick, MD).

Concentrations used were based on what is used clinically. The PMB concentrations 0.5, 1, 2, and 4 mg/L were based on clinically achievable unbound plasma concentrations, where a study found

average steady-state plasma concentration (Css,avg) to be 2.79 ± 0.90 mg/L (range, 0.68–4.88 mg/L) in critically ill patients following intravenous (IV) administration.[24] The MEM concentrations 10, 40, and 80 mg/L were based on clinically achievable unbound plasma concentrations, where a study found plasma concentrations to be 30.2 ± 10.7 µg/ mL with 1 g and 67.1 ± 31.0 µg/mL with 2 g in critically ill patients following a 3-h extended infusion.[25] PMB 8 mg/L and 16 mg/L and MEM 120 mg/L concentrations were used to determine the behavior of the bacteria at very high concentrations.

Pharmacodynamic analysis:
Plots of colony count (log10 CFU/mL) versus time were constructed to evaluate the killing activity of the various individual and combination therapies. Bactericidal activity was defined as a ≥3 log_{10} CFU/mL reduction from baseline after 24 hours, bacteriostatic activity was defined as a ≤ 3 log_{10} CFU/mL reduction from baseline after 24 hours, and growth was any positive change at 24 hours. For combination, synergy was defined as a ≥2 log_{10} CFU/mL reduction at 24 hours and additivity as ≥1 to <2 log10 CFU/mL reduction at 24 hours, when compared to the most active agent in monotherapy.

RESULTS

Characterization of the clinical isolates:

6 out of the 33 (18%) PSA clinical isolates from CF patients were determined to be hypermutators. MICs of hypermutators against PMB, COL, and MEM are shown in **Table 1.** One out of six of the determined hypermutators was found to be have a MIC susceptibility of 0.5 mg/L for all three antibiotics tested (PSA 203). One out of the remaining 27 of the determined non-hypermutators was found to be resistant to all three antibiotics tested (PSA 205).

Polymyxin B and meropenem monotherapy:

The time-kill curves for PMB and MEM monotherapy against PSA 205 and PSA 203 are shown in **Figure 2**. For PSA 205 PMB monotherapy, the clinically relevant PMB concentrations of 0.5, 1, 2 and 4 mg/L resulted in continued growth at 24 hours. The higher PMB concentrations of 16 mg/L (2 X MIC) and 64 mg/L (8 X MIC) resulted in bactericidal killing with a ≥3 $\log_{10}$ CFU/mL reduction from baseline at 8 hours. However, the initial killing was not sustained, with substantial bacterial re-growth found at 24 hours. For PSA 203 PMB monotherapy, bactericidal killing was observed for all PMB clinically relevant concentrations as soon as 4 hours, but positive growth with steep slopes at 24hours, similarly to PSA 205. For PSA 205 MEM monotherapy, bacterial killing activity was very similar for all MEM concentrations of 10 mg/L, 40 mg/L, 80 mg/L, and 120 mg/L for the first 8 hours. After 24 hours, bactericidal killing activity with a ≥3 $\log_{10}$ CFU/mL reduction from baseline was found for 2/4 of the concentrations. However, bactericidal activity was not seen for the first 8 hours. For PSA 203 MEM monotherapy, bactericidal activity was seen as soon as 2 hours. Also, MEM concentrations of 40 mg/L, 80 mg/L, and 120 mg/L overlapped in the bacterial killing behavior. No re-growth was seen at 24 hours.

Polymyxin B and meropenem combination therapy:

The time-kill curves for PMB and MEM combination therapy against PSA 205 and PSA 203 are shown in **Figure 2**. For PSA 205 PMB and MEM combination therapy, synergy was determined for regimens of PMB 2 mg/L + MEM 10 mg/L, PMB 4 mg/L + MEM 10 mg/L, PMB 2 mg/L + MEM 40 mg/L, and PMB 4 mg/L + MEM 40 mg/L with ≥2 $\log_{10}$ CFU/mL reduction at 24 hours, when compared to the most active agent in monotherapy. However, PMB 2 mg/L + MEM 40 mg/L, and PMB 4 mg/L + MEM 40 mg/L regimens demonstrated no growth earlier on at 6 hours, compared to PMB 2 mg/L + MEM 10 mg/L. For PSA 203 PMB and MEM combination therapy, synergy was determined for regimens of PMB 1 mg/L + MEM 10 mg/L, PMB 2 mg/L + MEM 10 mg/L, PMB 4 mg/L + MEM 10 mg/L, PMB 1 mg/L + MEM 40 mg/L, PMB 2 mg/L + MEM 40 mg/L, and PMB 4 mg/L + MEM 40 mg/L with ≥2 $\log_{10}$ CFU/mL reduction at 24 hours, when compared to the most active agent in monotherapy. PMB 0.5 mg/L + MEM 10 mg/L and PMB 0.5 mg/L + MEM 40 mg/L did not result in sustained bactericidal killing, illustrating re-growth at 8 hours.

Point-based analysis:

A summary of changes in bacterial density (log10 CFU/mL) at 4, 8 and 24 h compared with the initial inoculum (0 h) for polymyxin B (PMB) in combination with meropenem (MEM) is shown in **Table 2.** PMB 0.5 mg/L and 1 mg/L concentrations in combination with MEM 10 mg/L and MEM 40 mg/L concentrations resulted in regrowth at the 8 h for both PSA 203 and PSA 205 isolates. In contrast, PMB 2 mg/L and 4 mg/L concentrations in combination with MEM 10 mg/L and MEM 40 mg/L concentrations resulted in sustained bactericidal killing.

DISCUSSION

Antibiotic-resistant PSA isolates have shown to be associated with increased morbidity, mortality, length of hospital stay, and increased overall cost of treating the infection.[5] It has been established that there is a high prevalence of hypermutators in PSA in the lungs of CF patients.[3] Thus, in the past decade, there has been increasing interest in the role of hypermutators in antibiotic resistance and the importance of mutation in bacterial adaptation.[26] In this current study, 18% of the 33 PSA from CF patients were determined to be hypermutators. This finding supports that there is a high prevalence of hypermutators in patients of this population infected with PSA. Given that PSA is a highly versatile bacterium capable of occupying a variety of environmental niches, it appears that the highly stressful and fluctuating environments of CF patients, compared to other environments, promote increased mutation rates and the selection of mutator strains via the bacteria's need to rapidly adapt to the heterogeneous and changing environments in the lungs. This is supported by prior literature.[3,4]

Because of the link of hypermutators to increased antibiotic resistance, mutators are a significant clinical concern.[27,28] In this current study using PSA hypermutant isolates, the time-kill results demonstrated poor sustained killing of PMB monotherapy, illustrating continued growth for clinically relevant PMB concentrations, and re-growth at 24 hours for higher PMB concentrations that initially demonstrated early bactericidal killing. The insufficiency of PMB monotherapy is supported by other *in vitro* pharmacodynamics studies.[18,19] In addition, dose-related nephrotoxicity is a serious clinical concern for polymyxin therapy.[18,21] Thus, neither PMB monotherapy nor PMB dose escalation would be clinically viable approaches to suppressing the emergence of resistance of these resistant hypermutators, especially in the CF patient population of severe infections and an impaired immune system.

The time-kill results of PMB and MEM combination demonstrated positive *in vitro* synergy against selected hypermutator and non-hypermutator PSA isolates from CF patients. Particularly, higher PMB concentrations (PMB 2 & 4 mg/L) in combination with higher MEM concentrations (MEM 40 mg/L) demonstrated sustained bactericidal activity earlier on compared to in combination with lower MEM concentration of MEM 10 mg/L. The synergy observed may be due to the mechanistic synergy resulting from using antibiotics with differing mechanisms of action acting on different molecular targets. The bactericidal activity of PMB results in increased permeability of the outer membrane, allowing for the enhanced entry of MEM and therefore increased binding to the penicillin-binding proteins, which are bacterial enzymes necessary in the final steps of bacterial cell wall synthesis.[29] Furthermore, the loss in cell wall stability caused by MEM binding allows for enhanced binding of PMB as well, increasing the pharmacodynamics activity.[29] PMB based combination therapy resulted in superior PD activity compared to monotherapy with either drug, which is consistent with previous *in vitro* studies that have evaluated polymyxins in combination with carbapenems against highly resistant Gram-negative bacteria in non-CF patients.[15,30,31] Furthermore, combination therapy enables a lower dose of each antibiotic to be used, reducing nephrotoxicity as an adverse effect, which is particularly a concern with PMB.[31]

One limitation of this study is that two strains were evaluated and the PSA 203 and PSA 205 clinical isolates may not be representative of all PSA hypermutators and non-hypermutators. Second, even though the stability of the antibiotics cannot be trusted beyond 24 hours, 24 hours may not be enough time to notice differences between the emergences of resistance among the antibiotic regimens. Third, the heterogeneity of highly resistant bacteria such as hypermutators makes it challenging to come up with conclusive statements. It should be recognized that results from these *in vitro* studies might not be directly applicable to clinical practice. This is due to the fact that the interactions between the infecting bacteria and drug at the specific site of infection in the context of the host immune response will be

different, as the static time-kill setup does not take the host immune response into consideration. Also, antibiotic resistance most likely develops on a different time scale in clinical practice. Furthermore, *in vitro* studies do not allow one to observe toxicity, which is highly clinically relevant with polymyxin combination therapy.

The combination of a carbapenem and a polymyxin against PSA is supported *in vitro* by high synergy and bactericidal activity, which results in reduced emergence of resistance. Using PMB based combination therapy against hypermutant PSA isolates requires further investigation via dynamic *in vitro* infection models like the Hollow fiber infection model, that can simulate 'humanized' exposure for these antibiotics and allow for observation of killing patterns over a longer duration of time to better evaluate the impact of these regimens on the emergence of resistance. Also, other drug combinations against PSA hypermutator isolates can be tested in the future, such as with more novel drugs like ceftolozane/tazobactam and cefepime/zidebactam. Another future approach is using one of the CFTR modulators, such as ivacaftor (Kalydeco), in combination with these antibiotic therapies to see if bacteria would behave differently in a less harsh environment, since this drug helps restore the water content of the mucus. Based on the current study, polymyxin combination therapy requires further investigation in patients in order to fully define their therapeutic role, and multi-center, randomized trials are needed to better understand the benefits of polymyxin combination therapy. Data with *in vitro* pharmacodynamic and animal models more closely embodying human pharmacokinetics and the immune system are also needed. Additionally, future genetic testing may help with describing bacterial behavior and identifying differences between hypermutators and non-hypermutators.

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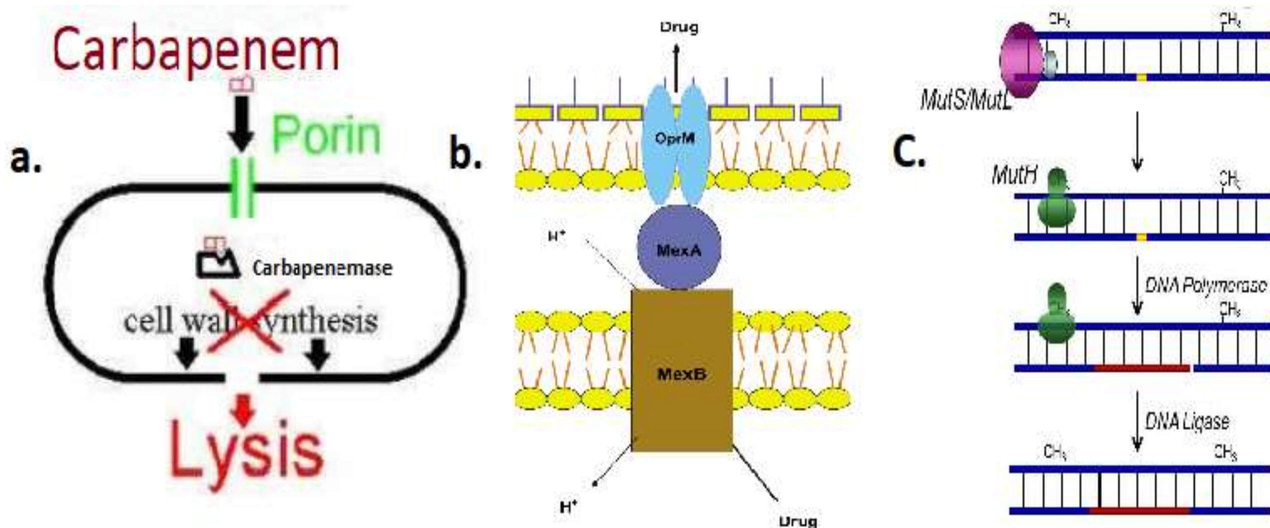

Figure 1. **a.** Carbapenemase resistance mechanism: Normally, carbapenems (i.e. meropenem) exert its action by interfering with cell wall synthesis, and thus causing bacterial lysis or disintegration. Carbapenem being inactivated by carbapenemase, an enzyme synthesized by the bacterial cell, prevents this process.[32] **b.** Example of the main efflux pump in *Pseudomonas aeruginosa*: Mex-AB-OprM lies across the cytoplasmic membrane and outer membrane, kicking out the drug from the cell in an energy-dependent manner by using a proton gradient [33] **c.** DNA MMR schematic with role of MutS/MutL and MutH: Process where MutS/MutL and MutH repair bacterial DNA mismatch that occurs during DNA replication or homologous recombination of bacteria. Inactivation of this process leads to an increase in mutation rate.[6]

*Note: Figures are adapted from publications which are designated as superscripts.

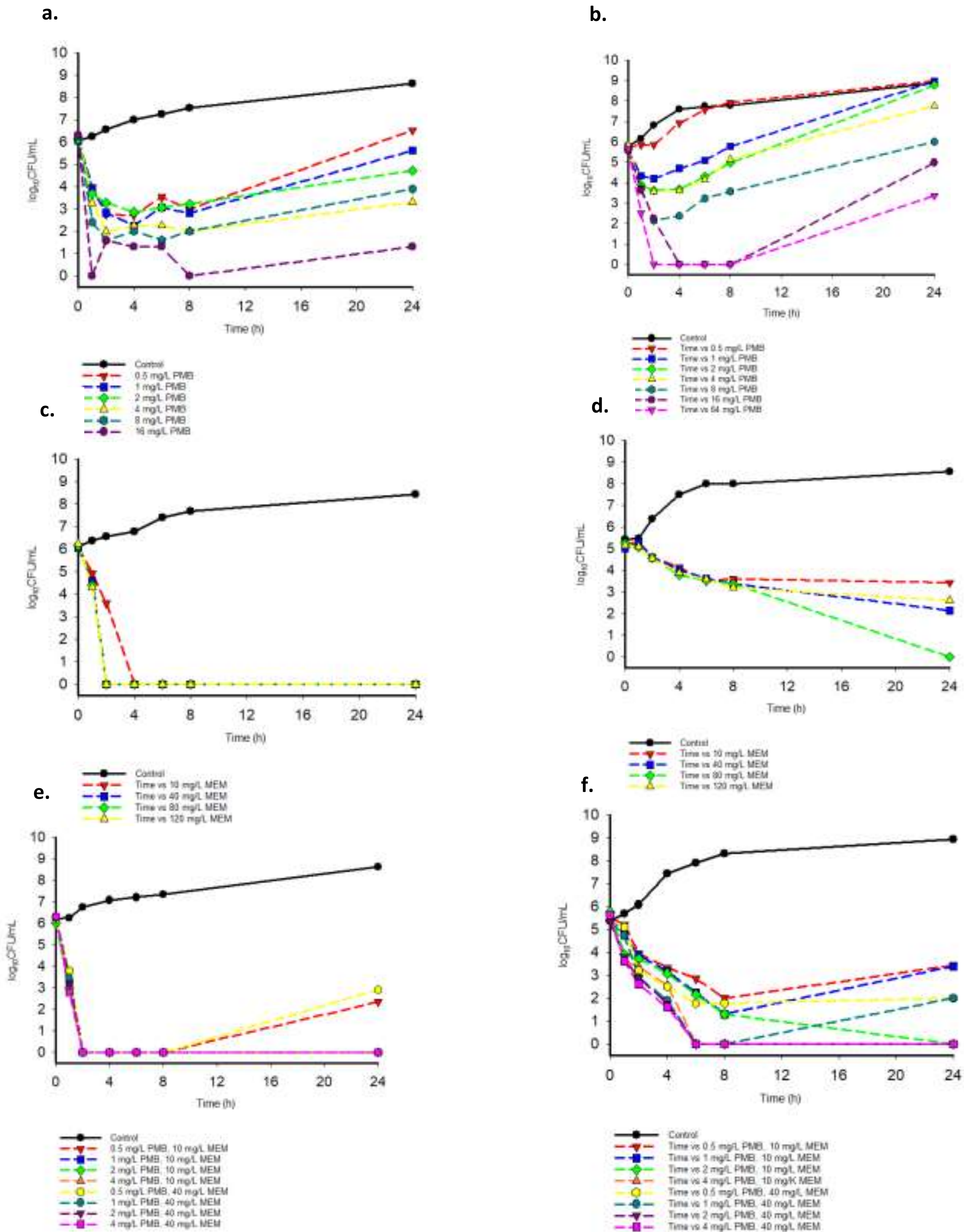

Figure 2. Time–kill curves with polymyxin B (PMB) and meropenem (MEM) alone and in combination against an initial inoculum of 10^6 CFU/mL of clinical isolates, hypermutator PSA 203 and non-hypermutator PSA 205. PSA 203 and PSA 205 over 24 hours: **a.** PSA 203 PMB Monotherapy (MIC 0.5 mg/L); **b.** PSA 205 PMB Monotherapy (MIC 8 mg/L); **c.** PSA 203 MEM Monotherapy (MIC 0.5 mg/L); **d.** PSA 205 MEM Monotherapy (MIC 16 mg/L); **e.** PSA 203 PMB and MEM Combination; **f.** PSA 205 PMB and MEM Combination.

Table 1. Antibiotic minimum inhibitory concentrations (MICs) of 6 *Pseudomonas aeruginosa* clinical isolates determined to be hypermutators.

Isolate	MIC (mg/L)[a.]		
	PMB	COL	MEM
PSA 200	1 **S**	1-2 **S**	0.5 **S**
PSA 203	<0.5 **S**	<0.5 **S**	<0.5 **S**
PSA 204	<0.5 **S**	<0.5 **S**	16 **R**
PSA 209	1 **S**	<0.5 **S**	32 **R**
PSA 220	<0.5 **S**	1 **S**	8 **R**
PSA 221	1 **S**	<0.5 **S**	16 **R**

PMB, polymyxin B; COL, colistin; MEM, meropenem; S, susceptible; I, intermediate; R, resistant; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute.

a. The isolates were classified as susceptible, intermediate or resistant based on the 2015 EUCAST and CLSI breakpoints against Enterobacteriaceae.[23]

Table 2. Summary of changes in bacterial density (log10 CFU/mL) at 4, 8 and 24 h compared with the initial inoculum (0 h) for polymyxin B (PMB) in combination with meropenem (MEM)

			Change in log_{10} CFU/mL							
			MEM at 10 mg/L plus PMB at:				**MEM at 40 mg/L plus PMB at:**			
Strain	Time (h)	Control	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L
PSA203	**2**	0.518	-6.25	-6.24	-6.02	-6.18	-6.17	-6.26	-6.26	-6.31
	4	0.849	-6.25	-6.24	-6.02	-6.18	-6.17	-6.26	-6.26	-6.31
	8	1.11	-6.25	-6.24	-6.02	-6.18	-6.17	-6.26	-6.26	-6.31
	24	2.39	-3.91	-6.24	-6.02	-6.18	-3.26	-6.26	-6.26	-6.31
PSA205	**2**	0.699	-1.56	-1.74	-2.04	-2.09	-2.24	-2.47	-2.46	-3.02
	4	2.07	-2.17	-2.43	-2.69	-2.89	-2.94	-3.48	-3.56	-4.02
	8	2.92	-3.51	-4.3	-4.46	-5.45	-3.67	-5.38	-5.34	-5.62
	24	3.55	-2.07	-2.21	-5.76	-5.45	-3.45	-3.38	-5.34	-5.62

ADDENDUM

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Conflicts of Interest
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