



Polymyxin B in combination with meropenem against carbapenemase-producing *Klebsiella pneumoniae*: pharmacodynamics and morphological changes

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ABSTRACT

Combination therapy provides a useful therapeutic approach to overcome resistance until new antibiotics become available. In this study, the pharmacodynamics, including the morphological effects, of polymyxin B (PMB) and meropenem alone and in combination against KPC-producing *Klebsiella pneumoniae* clinical isolates was examined. Ten clinical isolates were obtained from patients undergoing treatment for mediastinitis. KPCs were identified and MICs were measured using microbroth dilution. Time–kill studies were conducted over 24 h with PMB (0.5–16 mg/L) and meropenem (20–120 mg/L) alone or in combination against an initial inoculum of ca. 10⁶ CFU/mL. Scanning electron microscopy (SEM) was employed to analyse changes in bacterial morphology after treatment, and the log change method was used to quantify the pharmacodynamic effect. All isolates harboured the *bla*_{KPC-2} gene and were resistant to meropenem (MICs ≥8 mg/L). Clinically relevant PMB concentrations (0.5, 1.0 and 2.0 mg/L) in combination with meropenem were synergistic against all isolates except BRKP28 (polymyxin- and meropenem-resistant, both MICs >128 mg/L). All PMB and meropenem concentrations in combination were bactericidal against polymyxin-susceptible isolates with meropenem MICs ≤16 mg/L. SEM revealed extensive morphological changes following treatment with PMB in combination with meropenem compared with the changes observed with each individual agent. Additionally, morphological changes decreased with increasing resistance profiles of the isolate, i.e. increasing meropenem MIC. These antimicrobial effects may not only be a summation of the effects due to each antibiotic but also a result of differential action that likely inhibits protective mechanisms in bacteria.

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1. Introduction

The incidence of carbapenem-resistant Enterobacteriaceae (CRE), particularly *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae*, has increased over the past decade, and infections caused by these bacteria are associated with significantly high rates of treatment failure and mortality [1,2]. Carbapenemase-encoding genes are easily transferred by plasmid-mediated conjugation to

other susceptible strains, thereby conferring carbapenem resistance to other organisms [3]. The US Centers for Disease Control and Prevention (CDC) estimates that CRE are responsible for ca. 9300 (ca. 6.6%) of the estimated 140,000 healthcare-associated Enterobacteriaceae infections in the USA each year [4].

The increasing burden of CRE infections, the high rate of treatment failure, the emergence of resistance during treatment [5,6] and the dearth of novel antimicrobial agents highlight the urgency to investigate effective approaches for treating these infections. Polymyxins [polymyxin B (PMB) and polymyxin E (colistin)] are rapidly bactericidal against KPC-producing *K. pneumoniae* [7]. It is believed that interactions between polymyxins and the lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria increase membrane permeability and the loss of intracellular contents, ultimately resulting in cell death [8]. However, concerns for the rapid emergence of resistance to polymyxins during treatment as well as

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dose-limiting nephrotoxicity favour the clinical use of combination therapy for treatment of CRE infections [9]. Furthermore, improved outcomes have been reported from select prospective clinical studies of polymyxin-based combination therapy for multidrug-resistant (MDR) *Acinetobacter baumannii* infections [10].

However, the role of polymyxin-based combinations in the treatment of infections caused by KPC-producing *K. pneumoniae* has not been well studied. The role of carbapenems as part of combination therapy against isolates with varying degrees of susceptibility needs to be explored in order to optimise dosage regimens. Meropenem, a broad-spectrum carbapenem, is active against Enterobacteriaceae owing to its high binding affinity for penicillin-binding protein 2 (PBP2), PBP3 and PBP4, which inhibits cell wall formation and facilitates bacterial cell lysis [11]. The objective of this study was to evaluate the pharmacodynamics of PMB in combination with meropenem against MDR KPC-producing *K. pneumoniae* with a range of susceptibility for both antibiotics and to assess the impact of this combination on bacterial morphology using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Bacterial strains and antibiotics

Ten *K. pneumoniae* clinical isolates were obtained from ten different patients during outbreaks of KPC infections that occurred between June 2009 and June 2013 at Instituto Dante Pazzanese de Cardiologia (Sao Paulo, Brazil), a tertiary hospital specialising in cardiovascular surgery (Table 1) [12]. All isolates from this single cardiac treatment facility were stored at -80°C and were subcultured onto Mueller–Hinton agar plates before each experiment.

Fresh stock solutions of PMB and meropenem were prepared prior to each experiment by dissolving polymyxin B sulphate powder (Sigma-Aldrich, St Louis, MO; lot #WXB4470V) and meropenem (AK Scientific, Union City, CA; lot #LC24337) in water and normal saline, respectively, and filter-sterilising through a $0.20\ \mu\text{m}$ syringe filter (Corning Inc., Corning, NY).

2.2. *K. pneumoniae* genotyping

DNA was isolated from bacterial isolates using an E.Z.N.A.[®] Bacterial DNA Kit (Omega Bio-tek, Norcross, GA). Seven primer sets for β -lactamases of Ambler classes A (GES and KPC), B (NDM, VIM and IMP) and D (OXA-48 and OXA-40) [13] as well as one set of primers

for the *mgrB* gene [14] were used in the PCR for characterisation of the isolates (Supplementary Table S1). Q5 Hi-Fidelity Taq DNA Polymerase (NEB, Ipswich, MA) was used in PCR reactions according to the manufacturer's instructions. Reactions were carried out in an Eppendorf Mastercycler[®] (Eppendorf, Hamburg, Germany) and were analysed by agarose gel electrophoresis. PCR product sequencing was performed at the sequencing facility at the Roswell Park Cancer Institute (Buffalo, NY). The National Center for Biotechnology Information (NCBI) website was used for nucleotide and deduced protein sequence analysis (<http://www.ncbi.nlm.nih.gov>). Insertion sequences (IS) were analysed using the ISFinder website (<http://www-is.biotoul.fr>).

2.3. Bacterial isolates and antimicrobial susceptibility testing

The susceptibility of each isolate was determined for the antibiotics listed in Table 1 using the broth microdilution method in cation-adjusted ($25.0\ \text{mg/L}\ \text{Ca}^{2+}$ and $12.5\ \text{mg/L}\ \text{Mg}^{2+}$) Mueller–Hinton broth (Becton Dickinson & Co., Franklin Lakes, NJ), according to Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. The minimum inhibitory concentration (MIC) breakpoints for meropenem and colistin were defined according to the CLSI [15] and European Committee on Antimicrobial Susceptibility Testing (EUCAST) [16], respectively.

2.4. Time–kill studies

Static time–kill kinetics were examined to determine the rate and extent of bacterial killing in the absence (growth control) and presence of PMB and meropenem as monotherapy and in combination against the isolates. PMB concentrations of 0.5, 1, 2, 4, 8, 16, 64, 128 and $256\ \text{mg/L}$ and meropenem concentrations of 10, 20, 40, 60 and $120\ \text{mg/L}$ and a 5×4 concentration array of PMB (0.5, 1, 2, 4 and $16\ \text{mg/L}$) in combination with meropenem (20, 40, 60 and $120\ \text{mg/L}$) were evaluated against an initial inoculum of ca. $10^6\ \text{CFU/mL}$. Antibiotic(s) was added to the bacterial suspension in log growth phase. Bacterial samples obtained at 0, 1, 2, 4, 6, 8 and 24 h were diluted with normal saline and the appropriate dilution of bacterial cell suspension ($50\ \mu\text{L}$) was spirally plated on Mueller–Hinton agar using a Whitley automatic spiral plater (Don Whitley Scientific Ltd., Shipley, UK). Bacteria were quantified using a ProtoCOL HR automated bacterial colony counter (Synbiosis, Frederick, MD) following 24 h of incubation at 37°C . The lower limit of quantification was $2.0\ \log_{10}\ \text{CFU/mL}$.

Table 1

Antibiotic minimum inhibitory concentrations (MICs) and genotyping results of ten multidrug-resistant KPC-2-producing *Klebsiella pneumoniae* strains isolated from mediastinitis patients.

Isolate	MIC (mg/L) ^a														KPC-2 carbapenemase
	PMB	CST	MEM	AMK	ATM	CAZ	CHL	CIP	FOF	GEN	MIN	RIF ^b	TGC ^c	TMP	
BRKP30	0.5 S	<0.5 S	8 R	4 S	64 R	>256 R	128 R	128 R	16 S	1 S	4 S	32	1 S	>256 R	+
BRKP20	<0.5 S	0.5 S	16 R	4 S	64 R	>256 R	32 R	128 R	16 S	<0.5 S	8 I	32	1 S	>256 R	+
BRKP22	<0.5 S	<0.5 S	16 R	1 S	64 R	16 R	64 R	128 R	16 S	<0.5 S	32 R	32	2	>256 R	+
BRKP31	<0.5 S	<0.5 S	16 R	<0.5 S	64 R	64 R	32 R	64 R	8 S	32 R	2 S	32	1 S	>256 R	+
BRKP21	0.5 S	0.5 S	16 R	2 S	64 R	>256 R	128 R	128 R	16 S	<0.5 S	8 I	32	2	>256 R	+
BRKP27	1 S	1 S	16 R	2 S	64 R	32 R	32 R	64 R	8 S	64 R	4 S	32	2	>256 R	+
BRKP67	8 R	16 R	64 R	4 S	32 R	128 R	128 R	128 R	64 R	128 R	32 R	32	8 R	>256 R	+
BRKP76	<0.5 S	<0.5 S	64 R	2 S	>256 R	32 R	>256 R	64 R	32 S	128 R	1 S	64	<0.5 S	4 S	+
BRKP61	<0.5 S	0.5 S	128 R	8 S	32 R	32 R	32 R	128 R	256 R	<0.5 S	1 S	32	<0.5 S	>256 R	+
BRKP28	>128 R	>128 R	256 R	2 S	32 R	128 R	32 R	64 R	128 I	64 R	2 S	16	1 S	>256 R	+

PMB, polymyxin B; CST, colistin; MEM, meropenem; AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; FOF, fosfomicin; GEN, gentamicin; MIN, minocycline; RIF, rifampicin; TGC, tigecycline; TMP, trimethoprim; 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.

^b Neither the CLSI nor EUCAST has defined rifampicin breakpoints for Gram-negative organisms.

^c EUCAST has defined breakpoint concentrations of tigecycline for susceptible and resistant, but no information has been provided for intermediate.

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