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Carbapenem-associated multidrug-resistant *Acinetobacter baumannii* are sensitised by aztreonam in combination with polyamines

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Abstract

Carbapenem-associated multidrug-resistant *Acinetobacter baumannii* (MDR-Ab) are common among clinical isolates worldwide and are a major therapeutic challenge. Previously it was shown that exogenous polyamines (spermine and spermidine) enhanced susceptibility to β -lactams but induced resistance to polymyxins in *Pseudomonas aeruginosa*. This study aimed to explore the possible availability of exogenous polyamines in treating carbapenem-associated MDR-Ab. The effects of polyamines on the growth rate of *A. baumannii*, minimal inhibitory concentrations (MICs) of antibiotics, and time–kill and checkerboard assays were determined. Roles of lipopolysaccharide (LPS) and β -lactamase activity of *A. baumannii* were also assessed for the polyamine effects. Growth of *A. baumannii* was unaffected at 4 mM spermine and 16 mM spermidine alone, but was significantly inhibited by a subinhibitory concentration of aztreonam (AZT) (8 μ g/mL) and those concentrations of the polyamines. MICs to AZT alone (128 μ g/mL) were reduced to the range 0.25–8 μ g/mL in combination with polyamines in all carbapenem-associated MDR-Ab. MICs to penicillins, but not to ceftazidime and meropenem, were also significantly reduced, whilst MICs to other antibiotics, including polymyxin B, were unaffected in combination with polyamines for all tested *A. baumannii*. Polyamine effects on AZT were strongly synergistic with bactericidal activity and were retained at concentrations of 5 mM MgCl₂ (or CaCl₂) or 200 mM NaCl. Roles of LPS and β -lactamase in the polyamine effects were excluded. Overall results suggest that AZT in combination with polyamines may be useful for the treatment of carbapenem-associated MDR-Ab.

Keywords

Polyamines; β -Lactam susceptibility; *Acinetobacter baumannii*

1. Introduction

Acinetobacter baumannii is a strictly aerobic, non-fermenting, Gram-negative coccobacillus. This bacterium has been proven to be a major cause of healthcare-associated infections worldwide, including bacteraemia, ventilator-associated pneumonia, urinary tract infections,

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meningitis and wound infection, particularly in patients admitted to intensive care units [1]. Infections with drug-susceptible *A. baumannii* strains are commonly treated either with the combination ampicillin/sulbactam or with a carbapenem. However, isolates resistant to β -lactam antibiotics have been increasingly reported worldwide. Currently, most clinical *A. baumannii* isolates resistant to carbapenems are usually resistant to all classes of β -lactams as well as to other classes of antibiotics [1]. Infection with carbapenem-associated multidrug-resistant *Acinetobacter baumannii* (MDR-Ab) strains is the most troublesome to treat in patients [1,2].

Polyamines (spermine and spermidine) are low-molecular-weight natural compounds with positively charged amine groups existing at millimolar levels in all living cells. Cellular contents of the polyamines are regulated by biosynthesis, degradation and transport [3]. Polyamines interact with negatively charged macromolecules and are involved in the regulation of physical and chemical properties of membranes, nucleic acid structures, and the functions and modulation of enzyme activities [4]. We previously reported polyamine effects on the antibiotic susceptibility of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* [5,6]. Exogenous polyamines enhanced susceptibility to some antibiotics, particularly β -lactams, whilst they increased resistance to polymyxins in *P. aeruginosa*. The aim of this study was to investigate polyamine availability in treating carbapenem-associated MDR-Ab. The results revealed that all tested carbapenem-associated MDR-Ab were sensitised by aztreonam (AZT) in combination with exogenous polyamines, but were not affected by polymyxin B (PMB), ciprofloxacin or gentamicin.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Acinetobacter baumannii ATCC 19606 was obtained from the American Type Culture Collection (Manassas, VA), and antibiotic-susceptible *A. baumannii* A118 was obtained from Ramirez et al. [7]. Clinical isolates of OXA-type carbapenemase-producing MDR-Ab AB0057 (*bla*_{OXA-23}) [8], AB017 (*bla*_{OXA-23}) [9], AB1204 (*bla*_{OXA-24}) [10], KB037 (*IS*_{Aba1}–*bla*_{OXA-51}) [11] and AB047 (*bla*_{OXA-58}) [12] as well as non-enzymatic carbapenem-resistant isolate (KB305) [11] were obtained from the corresponding authors. All bacterial strains were routinely grown on Luria–Bertani agar plates or broth at 37 °C. Growth curve measurements were performed as described previously [5]. All antibiotics, polyamines and other chemicals were purchased from Sigma (St Louis, MO) and appropriate concentrations were supplemented in the culture media when needed.

2.2. Antibiotic susceptibility testing

Minimal inhibitory concentrations (MICs) were determined by the standard broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Serial two-fold dilutions of antibiotics were performed in Mueller–Hinton broth (pH 7.0) supplemented with spermine, spermidine, MgSO₄, CaCl₂ or NaCl when needed. MIC measurements were repeated and were confirmed by three independent experiments.

2.3. Time–kill assay and checkerboard assay

Time–kill and checkerboard assays were performed as previously described [5]. Synergy was defined as a fractional inhibitory concentration index (FICI) of ≤ 0.5 , indifference as an FICI of >0.5 but ≤ 4 , and antagonism as an FICI of >4 [13].

3. Results and discussion

3.1. Effect of polyamines on the growth rate of *Acinetobacter baumannii*

The effect of polyamines on the growth rate of *A. baumannii* ATCC 19606 was determined in the presence of exogenous polyamines. The results revealed that growth was unaffected at 4 mM spermine and 16 mM spermidine but was inhibited at 8 mM and 32 mM, respectively, compared with the growth rate without polyamines. Polyamine effects on the growth rate of strain ATCC 19606 and the carbapenemase-producing MDR-Ab AB0057 was also determined in the presence of subinhibitory concentrations of both AZT and the polyamines. The growth rate of both strains of *A. baumannii* was unaffected at 8 µg/mL AZT compared with growth without any antibiotic. However, the growth rate of both strains was significantly inhibited with the same subinhibitory concentration of AZT in combination with polyamines (4 mM spermine and 16 mM spermidine) (Fig. 1). Similar growth inhibition at the same subinhibitory concentration of carbenicillin (8 µg/mL) in combination with the same concentration of polyamines was also observed in *A. baumannii* ATCC 19606.

Exogenous polyamines alone inhibited the growth rate of *A. baumannii* at 8 mM and 32 mM of spermine and spermidine, respectively, suggesting that the polyamines may have antimicrobial activity. The antimicrobial activity of polyamines had been studied until early 1990 and was not pursued possibly because of suspected toxicity to human cells at high concentrations [14]. Growth of *A. baumannii* ATCC 19606 was not inhibited by the subinhibitory concentration of AZT with polyamines until the early exponential growth phase, whilst the growth rate of carbapenemase-producing MDR-Ab AB0057 was significantly inhibited by the same condition after the lag phase. These results indicate that, although the polyamine effects appeared to be strain-specific, the polyamines at concentrations not affecting cellular growth were sufficient to inhibit *A. baumannii* growth in the presence of a subinhibitory concentration of AZT (or carbenicillin). Overall results suggest that exogenous polyamines enhance susceptibility to AZT and carbenicillin in *A. baumannii*.

3.2. Effect of polyamines on antibiotic susceptibility

MIC measurements were performed to examine the effects of polyamines on the antibiotic susceptibility of carbapenem-associated MDR-Ab strains, including antibiotic-susceptible strains (ATCC 19606 and A118). MICs to all tested antibiotics, including β-lactams but not PMB, were much higher in the carbapenem-associated MDR-Ab strains than the antibiotic-susceptible strains (Table 1). MICs to AZT in combination with polyamines (1 mM or 4 mM spermine and 8 mM or 16 mM spermidine) were significantly reduced from 32 µg/mL up to 0.25 µg/mL compared with those of the β-lactam alone both in carbapenem-associated MDR-Ab and antibiotic-susceptible strains. MICs to penicillins (carbenicillin, piperacillin and ticarcillin) in combination with polyamines were also significantly reduced, but the MICs in the carbapenem-associated MDR-Ab strains mostly remained in the resistant range (MICs > 16 µg/mL) (Table 1). MICs to ceftazidime or meropenem in combination with polyamines were slightly or moderately reduced, and MICs to other antibiotics (PMB, ciprofloxacin and gentamicin) were essentially unchanged compared with those of the antibiotics alone in all tested *A. baumannii* strains. The difference of polyamine concentrations (1 mM or 4 mM spermine and 8 mM or 16 mM spermidine) did not significantly affect antibiotic susceptibility (Table 1).

To assess whether the polyamine effects on β-lactam susceptibility were retained at physiological concentrations of cationic ions, MICs to AZT (or carbenicillin) in combination with polyamines were determined in the presence of cationic ions (magnesium, calcium and

sodium ions). Results revealed that MICs to AZT (or carbenicillin) in combination with polyamines were unchanged in the presence of 5 mM MgCl₂ (or CaCl₂) and 200 mM NaCl both in strains AB0057 and ATCC 19606. These results suggest that the polyamine effects on β -lactam susceptibility are retained at physiological cationic concentrations.

MIC measurement revealed that all carbapenem-associated MDR-Ab strains were resistant to all tested β -lactams compared with the antibiotic-susceptible strains ATCC 19606 and A118. However, all of them had significantly reduced MICs to AZT in combination with the exogenous polyamines (MICs from 128 to 8 μ g/mL). Recently, a susceptible phenotype to most antibiotics was reported for *A. baumannii* A118 [7] and this strain showed an AZT MIC of 32 μ g/mL. Based on these observations, clinical isolates of carbapenem-associated MDR-Ab may be sensitised by AZT in combination with polyamines. Importantly, the polyamine effects on AZT were retained at physiological concentrations of cationic ions. These results indicate that AZT in combination with polyamines may be applicable to treat patients infected with carbapenem-associated MDR-Ab.

3.3. Bactericidal effect of β -lactam antibiotics in combination with polyamines

The bactericidal effect of AZT in combination with polyamines was examined by time-kill assays of the carbapenemase-producing MDR-Ab strain AB0057 and the strain ATCC 19606. Cell numbers of the strain AB0057 increased (or were unchanged) in the presence of AZT (10 μ g/mL) alone or with spermine (4 mM) alone, similar to that shown in the control without AZT or spermine. However, cell numbers in the presence of both AZT (10 μ g/mL) and spermine (4 mM) dramatically decreased up to >99% within 12 h (Fig. 2B). Similarly, cell numbers of the strain ATCC 19606 were increased (or unchanged) in the presence of AZT (8 μ g/mL) alone or spermine (4 mM) alone, similar to that shown in the control without AZT or spermine, but cell numbers in the presence of both AZT (8 μ g/mL) and spermine (4 mM) decreased 100% within 12 h (Fig. 2A). The bactericidal effect of carbenicillin in combination with spermine was also examined by time-kill assay of strain ATCC 19606 and resulted in similar bactericidal effects as that of the strain AB0057 (data not shown). These results suggest that the polyamines are associated with bactericidal effects of β -lactam antibiotics on *A. baumannii*.

3.4. Synergistic effect of polyamines on β -lactam antibiotics

Polyamines inhibit the growth of *A. baumannii* in combination with subinhibitory concentration of AZT or carbenicillin more significantly than polyamines alone, and MIC levels of spermine or spermidine were 1024 μ g/mL in *A. baumannii* strains ATCC 19606, A118 and AB0057. To test polyamine interactions with β -lactam antibiotics, the FICI was determined by a checkerboard assay as described previously [5]. FICI levels for spermine and AZT were <0.062 in the three strains, lower than the FICI of 0.5 suggested for a synergistic effect [13], indicating that polyamines have a strong synergistic effect on β -lactam antibiotics. Polyamines are cationic compounds and possibly interact with the negatively charged cell wall, which may enhance β -lactam activity.

3.5. Roles of lipopolysaccharide (LPS) and β -lactamase in polyamine effects

The molecular mechanism of the polyamine effects on β -lactam susceptibility is currently unknown. Polyamines are positively charged compounds and possibly interact with the negatively charged outer membrane of *A. baumannii*. LPS is a major component of the outer membrane of Gram-negative bacteria and is associated with membrane permeability [14]. *Acinetobacter baumannii* lacking LPS has been developed by polymyxin selection pressure with the acquisition of polymyxin resistance; polymyxin-resistant strains also harboured mutant alleles of the LPS biosynthesis gene *lpxA* [15]. To understand the role(s) of LPS in the polyamine effects on β -lactam susceptibility, *A. baumannii* strains ATCC 19606 and

AB0057 were used to develop PMB-resistant strains as previously described [15]. PMB-resistant strains were readily produced by PMB selective pressure (MICs from 0.5 µg/mL to 128 µg/mL), but their MICs to β-lactam antibiotics were significantly reduced (MICs from 1024 µg/mL up to 0.06 µg/mL) compared with their parental strains. Susceptibility to β-lactam antibiotics in polymyxin (colistin)-resistant clinical isolates was also reported by other investigators [15]. DNA sequence determination of the LPS biosynthesis gene *lpxA* revealed that the resistant strain ATCC 19606R harboured a frameshift mutation from the 198th amino acid owing to deletion of two nucleotides at positions 594 and 595 (TT), and the resistant strain AB0057R harboured a missense mutation from Gly (at position 142) to Ala owing to a nucleotide substitution of G425→C. PMB susceptibility of the two PMB-resistant strains was fully complemented by an intact *lpxA* gene cloned from PMB-susceptible strain ATCC 19606. These results were similar to a previous report [15] suggesting that PMB-resistant strains are caused by lack of LPS.

The two PMB-resistant strains were used to assess polyamine effects on β-lactam and PMB susceptibility as done above. Results revealed that β-lactam susceptibility for the two PMB-resistant strains was further increased 4–32-fold in the presence of polyamines, whilst PMB susceptibility was unchanged. These results suggest that LPS may not be a major player in the polyamine effects on β-lactam susceptibility. Since LPS is in the outer leaflet of the outer membrane and is likely involved in membrane permeability, these results also suggest that changes in membrane permeability may not be associated with the polyamine effects on β-lactam susceptibility. This is consistent with the previous observation that outer membrane permeability was excluded from the polyamine effects in *P. aeruginosa* [6].

All *A. baumannii* strains harbour chromosomally encoded AmpC cephalosporinases [1] that may be involved in the polyamine effects on β-lactam susceptibility. To test this possibility, total β-lactamase activities from *A. baumannii* ATCC 19606 grown in the presence or absence of 1 mM spermine were measured as described previously [6]. Results revealed no significant difference (950 nmol/mg/min vs. 920 nmol/mg/min) between cells grown in the presence and absence of spermine. This result was similar to that of *P. aeruginosa* [6], suggesting that β-lactamase activity itself might not be involved in the polyamine effects on β-lactam susceptibility. Molecular details for the polyamine effects on β-lactam susceptibility are unknown in any bacterial species; we are currently trying to elucidate the molecular details of polyamine effects in *A. baumannii*.

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Fig. 1.

Growth rate of *Acinetobacter baumannii* in the presence or absence of exogenous polyamines in combination with a subinhibitory concentration of aztreonam (AZT). (A) Growth rate of *A. baumannii* ATCC 19606: ◆, no antibiotic or polyamines; ■, 8 µg/mL AZT alone; ▲, 8 µg/mL AZT and 4 mM spermine; ●, 8 µg/mL AZT and 16 mM spermidine. (B) Growth rate of carbapenemase-producing multidrug-resistant *A. baumannii* AB0057: ◆, no antibiotic or polyamines; ■, 8 µg/mL AZT alone; ▲, 8 µg/mL AZT and 2 mM spermine; ●, 8 µg/mL AZT and 16 mM spermidine. Error bars at each data point are the standard deviation.

Fig. 2.

Time-kill effect on *Acinetobacter baumannii* in the presence of aztreonam (AZT) alone or AZT with spermine. (A) Time-kill effect on *A. baumannii* ATCC 19606: ◆, no antibiotic or polyamines; ■, 8 µg/mL AZT alone; ▲, 4 mM spermine alone; ●, 8 µg/mL AZT and 4 mM spermine. (B) Time-kill effect on carbapenemase-producing multidrug-resistant *A. baumannii* AB0057: ◆, no antibiotic or polyamines; ■, 10 µg/mL AZT alone; ▲, 4 mM spermine alone; ●, 10 µg/mL AZT and 4 mM spermine. Error bars at each data point are the standard deviation.

Table 1
Antibiotic susceptibility of *Acinetobacter baumannii* in the presence of polyamines

Antibiotic/polyamine ^a	MIC (μg/mL) ^b							
	ATCC 19606	A118	KB305	AB0057 (<i>bla</i> _{OXA-23})	AB017 (<i>bla</i> _{OXA-23})	AB1204 (<i>bla</i> _{OXA-24})	KB037 (<i>ISAba1-bla</i> _{OXA-51})	AB047 (<i>bla</i> _{OXA-58})
Aztreonam								
None	32	32	256	256	512	128	256	512
Spermine (1 mM)	4	4	8	4	8	4	8	0.25
Spermine (4 mM)	2	1	1	2	4	0.5	4	0.25
Spermidine (8 mM)	4	4	16	8	8	4	8	4
Spermidine (16 mM)	4	2	4	4	4	4	4	4
Carbenicillin								
None	64	64	1024	1024	1024	1024	1024	1024
Spermine (1 mM)	8	4	256	256	32	256	128	2
Spermine (4 mM)	4	4	32	128	16	16	16	0.5
Spermidine (8 mM)	2	4	256	256	256	512	128	64
Spermidine (16 mM)	4	4	32	128	128	512	128	16
Piperacillin								
None	32	16	256	1024	512	1024	512	512
Spermine (1 mM)	4	2	64	256	32	256	128	32
Spermine (4 mM)	4	1	16	128	16	64	32	8
Spermidine (8 mM)	4	4	64	256	32	256	128	32
Spermidine (16 mM)	2	4	16	128	16	128	32	16
Ticarcillin								
None	64	32	1024	1024	1024	1024	1024	1024
Spermine (1 mM)	4	8	128	256	512	1024	256	256
Spermine (4 mM)	2	1	64	128	256	512	128	64
Spermidine (8 mM)	4	4	128	256	512	1024	256	256
Spermidine (16 mM)	2	4	64	128	512	512	64	128
Ceftazidime								
None	16	8	1024	1024	128	1024	64	32
Spermine (1 mM)	8	0.5	256	256	32	1024	64	4

Antibiotic/polyamine ^a	MIC (μg/mL) ^b								
		ATCC 19606	A118	KB305	AB0057 (<i>bla_{OXA-23}</i>)	AB017 (<i>bla_{OXA-23}</i>)	AB1204 (<i>bla_{OXA-24}</i>)	KB037 (<i>ISAba1-bla_{OXA-51}</i>)	AB047 (<i>bla_{OXA-58}</i>)
Spermine (4 mM)	8	0.5	0.5	64	256	16	256	64	4
Spermidine (8 mM)	8	1	1	128	256	64	1024	64	8
Spermidine (16 mM)	8	1	1	64	256	32	256	64	8
Meropenem									
None	0.5		0.25	8	32	32	128	16	8
Spermine (1 mM)	0.5		0.25	8	32	16	64	4	0.25
Spermine (4 mM)	0.5		0.25	2	16	4	32	4	0.25
Spermidine (8 mM)	0.5		0.25	4	32	16	64	16	1
Spermidine (16 mM)	0.5		0.25	4	16	8	32	8	0.5
Polymyxin B									
None	0.5		1	1	1	0.5	0.5	0.5	2
Spermine (1 mM)	1		0.5	0.5	0.5	0.5	0.5	0.5	2
Spermine (4 mM)	1		0.5	0.5	0.5	0.5	0.5	0.5	1
Spermidine (8 mM)	0.5		1	0.5	1	0.5	0.5	0.5	2
Spermidine (16 mM)	1		0.5	1	0.5	0.5	0.5	0.5	1
Ciprofloxacin									
None	1		0.25	64	128	64	64	256	16
Spermine (1 mM)	1		0.25	128	128	64	64	256	8
Spermine (4 mM)	1		0.25	128	128	64	128	256	8
Spermidine (8 mM)	1		0.25	128	128	64	64	256	16
Spermidine (16 mM)	1		0.25	128	128	128	64	256	16
Gentamicin									
None	2		1	32	256	32	512	64	64
Spermine (1 mM)	2		0.5	32	128	32	256	64	64
Spermine (4 mM)	1		0.5	64	128	32	256	128	64
Spermidine (8 mM)	1		1	32	128	32	256	128	64
Spermidine (16 mM)	2		1	32	128	32	256	128	64

MIC, minimal inhibitory concentration.

^aMICs of spermine or spermidine were 1024 μg/mL in all *A. baumannii* isolates.

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^bMIC measurement was repeated three times with identical results in Mueller–Hinton broth (pH 7.0) supplemented with the indicated antibiotics and polyamines.