

In vitro and in vivo Pharmacodynamics of Colistin and Aztreonam Alone and in Combination against Multidrug-Resistant *Pseudomonas aeruginosa*

Yuka Yamagishi^{a, b} Mao Hagihara^{a–c} Hideo Kato^{b, c} Jun Hirai^a
Naoya Nishiyama^{a, b} Yusuke Koizumi^{a, b} Daisuke Sakanashi^b
Hiroyuki Suematsu^b Hazuki Nakai^a Hiroshige Mikamo^{a, b}

Departments of ^aClinical Infectious Diseases, ^bInfection Control and Prevention and ^cPharmacy, Aichi Medical University Hospital, Nagakute, Japan

Key Words

Colistin · Aztreonam · Combination therapy · Multidrug-resistant *Pseudomonas aeruginosa*

Abstract

Background: Reports of *Pseudomonas aeruginosa* with high antimicrobial resistance have steadily emerged, threatening the utility of a mainstay in antipseudomonal therapy. This study evaluated the antimicrobial activities of various combination therapies against *P. aeruginosa* with high antimicrobial resistance, including multidrug-resistant *P. aeruginosa* (MDRP) using an in vitro and in vivo study. **Methods:** We evaluated 24 combination therapies, including colistin, aztreonam, meropenem, ceftazidime, ciprofloxacin, amikacin, rifampicin, arbekacin and piperacillin against 15 MDRP isolates detected at Aichi Medical University Hospital with the break-point checkerboard method. Based on the results of the in vitro study, we evaluated antimicrobial activity against highly antimicrobial-resistant *P. aeruginosa* with an in vivo murine thigh infection model. **Results:** The combination regimens including colistin and aztreonam showed higher antimicrobial activity against the 15 MDRP isolates. In the in vivo study, the high-dose colistin monotherapy (16 mg/kg

every 12 h) achieved greater log₁₀ CFU changes than the normal-dose colistin regimen (8 mg/kg every 12 h) against 5 *P. aeruginosa* isolates, including 2 MDRP isolates ($p < 0.05$). Aztreonam monotherapy (400 mg every 8 h) yielded bacterial densities similar to untreated control mice for the MDRP isolate evaluated. The combination therapy with a higher dose of colistin had superior antimicrobial activity against 5 *P. aeruginosa* with colistin (MIC 0.5 µg/ml) and aztreonam (MIC ≥128 µg/ml) than colistin monotherapy. **Conclusion:** The data suggest that the combination treatment of colistin and aztreonam could be the most useful for treating highly resistant *P. aeruginosa* with a higher susceptibility to colistin, including MDRP infections.

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Introduction

Pseudomonas aeruginosa is a clinically significant Gram-negative rod and is an important cause of hospital-acquired infection and multidrug-resistant *P. aeruginosa* (MDRP) infections. MDRP are defined as having resistance to 3 groups of antimicrobials, carbapenems, aminoglycosides, and fluoroquinolones, and has become a com-

mon occurrence in many geographic regions worldwide [1–6]. The increased incidence of MDRP has threatened the utility of the current antipseudomonal therapy.

In clinical settings, the choice of a definitive antimicrobial therapy is typically guided by the antimicrobial susceptibility profile of the infecting organism. However, limited antimicrobial choices make it more difficult to treat MDRP infections. Antimicrobial combination therapy is often used to improve clinical efficacy in patients where a given therapy is thought to have limitations when used alone. A break-point checkerboard plate is used to evaluate the effect of antimicrobial combination therapy and was previously shown to be effective clinically for MDRP infections [1, 3].

However, a limitation of previous *in vitro* studies is that only single-drug concentrations of the combination were explored. Thus, these *in vitro* studies neglected the importance of antimicrobial exposures. Therefore, we evaluated the *in vivo* efficacy of the effective combination therapy using *in vitro* studies of *P. aeruginosa*, including MDRP.

Material and Methods

Microorganisms

P. aeruginosa isolates were collected from clinical isolates between November 2012 and October 2015 at Aichi Medical University Hospital (995 beds), Aichi, Japan. Fifteen MDRP isolates were evaluated as part of the *in vitro* study.

For the *in vivo* studies, we used 2 clinical MDRP isolates that were used in the *in vitro* study, and 3 clinical *P. aeruginosa* isolates that were highly resistant to several tested antimicrobials (table 1) to evaluate whether the selected combination therapy would be effective not only for MDRP, but also against *P. aeruginosa* isolates with high antimicrobial resistance. The study was reviewed and approved by the Aichi Medical University Hospital Institutional Animal Care and Use Committee.

Susceptibility Testing

The MIC values of the antimicrobial agents, including colistin, were determined by the E-test according to the manufacturer's specifications (bioMérieux, Durham, NC, USA) or the microdilution method defined by the Clinical and Laboratory Standards Institute (CLSI) guidelines [7]. MDRP isolates are defined as having resistance to 3 antimicrobial drugs in the following 3 groups: carbapenems (MIC of imipenem ≥ 16 $\mu\text{g/ml}$), aminoglycosides (MIC of amikacin ≥ 32 $\mu\text{g/ml}$), and fluoroquinolones (MIC of ciprofloxacin ≥ 4 $\mu\text{g/ml}$).

Break-Point Checkerboard Plate

A break-point checkerboard plate (Eiken Chemical, Tokyo, Japan) is used to evaluate the effect of a combination therapy with reference to the breakpoint concentration. Since the breakpoint of arbekacin against *P. aeruginosa* was not defined, the gentamicin

Table 1. Scoring of the combination effects for each antimicrobial combination against all 15 MDRP isolates

Antimicrobial	A	CPFX	CPFX	CPFX	CPFX	CPFX
	B	MEPM	CAZ	AZT	PIPC	AMK
Average		0.47	0.80	0.33	0.60	0.60
SD		1.25	1.47	0.90	1.30	1.30
Antimicrobial	A	AMK	AMK	AMK	AMK	AMK
	B	MEPM	CAZ	AZT	PIPC	RFP
Average		0.73	1.47	0.80	0.67	0.87
SD		1.33	1.77	1.52	1.23	1.30
Antimicrobial	A	ABK	ABK	ABK	ABK	ABK
	B	MEPM	CAZ	AZT	CPFX	RFP
Average		0.80	1.27	0.80	0.60	0.53
SD		1.52	1.87	1.47	1.40	1.41
Antimicrobial	A	CL	CL	CL	CL	CL
	B	MEPM	CAZ	AZT	CPFX	RFP
Average		3.80	3.80	3.87	3.33	3.73
SD		0.41	0.56	0.52	0.90	1.03
Antimicrobial	A	RFP	RFP	RFP	RFP	RFP
	B	MEPM	CAZ	AZT	CPFX	
Average		0.27	0.73	0.13	0.13	
SD		0.70	1.44	0.52	0.52	

CPFX = Ciprofloxacin; MEPM = meropenem; CAZ = ceftazidime; AZT = aztreonam; PIPC = piperacillin; AMK = amikacin; ABK = arbekacin; RFP = rifampicin; CL = colistin.

criterion was used as an alternative. It allows for simultaneous evaluation of the effect of combination antimicrobial therapy using 9 clinically important agents (colistin, aztreonam, piperacillin, meropenem, ceftazidime, amikacin, arbekacin, rifampicin, and ciprofloxacin) on a single plate [1, 3]. The concentrations ($\mu\text{g/ml}$) of each agent were the following: colistin (2, 1), aztreonam (32, 8), piperacillin (32, 16), meropenem (8, 4), ceftazidime (16, 8), amikacin (16, 8), arbekacin (2, 1), rifampicin (4, 2), and ciprofloxacin (2, 1).

Combination effects were scored and evaluated as previously reported [4]. Scoring was performed using a 0–4 scale to evaluate the combination effects with reference to the breakpoint concentration established by the break-point checkerboard plate. A score of 4 (inhibited bacterial growth in the following combination: antimicrobial A [susceptible] + antimicrobial B [susceptible]) indicated the most promising combined effect, while a score of 0 (bacterial growth not inhibited even by the following combination: antimicrobial A [intermediate] + antimicrobial B [intermediate]) indicated that there was no combination effect. Based on the results of the *in vitro* study, we evaluated the most effective combination therapy in an *in vivo* murine thigh infection model with *P. aeruginosa* with high resistance to several antimicrobials, including MDRP.

Metallo- β -Lactamase Production

Quick Chaser Iae IMP[®] (Mizuho Medy Co. Ltd, Tokyo, Japan) was used to detect the production of IMP-type metallo- β -lactamase

Table 2. Characteristics of the *P. aeruginosa* isolates

Isolates	IMP	AMK	CPFX	CL	AZT	PIPC	MEPM	CAZ	ABK	RFP	
AMU 14-6735	>32	64	>32	0.5	>256	>64	>8	>16	>2	>4	(MDRP)
AMU 13-4632	>32	64	>32	1	64	>64	>8	>16	≤1	>4	(MDRP)
AMU 14-1860	8	64	>32	0.5	128	>64	>8	>16	2	>4	
AMU 14-0763	>32	4	4	0.5	>256	≤16	>8	8	>2	>4	
AMU 13-9592	>8	16	>2	0.5	64	64	>8	>16	>2	>4	(MBL+)

IMP = Imipenem; AMK = amikacin; CPFX = ciprofloxacin; CL = colistin; AZT = aztreonam; PIPC = piperacillin; MEPM = meropenem; CAZ = ceftazidime; ABK = arbekacin; RFP = rifampicin; MBL+ = metallo-β-lactamase positive.

and aminoglycoside 6'-N-acetyl-transferase (AAC(6')-Iae), which is an aminoglycoside-modifying enzyme [8, 9].

Animals

Pathogen-free female ICR mice weighing approximately 22 g were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). In this study, we used a previously described mouse thigh infection model [10, 11].

Antimicrobial Agents

Commercially available colistin methanesulfonate (JHP Pharmaceuticals, LLC, Rochester, MI, USA) and aztreonam (Eisai Inc., Tokyo, Japan) were used for all of the in vivo studies. Each drug was reconstituted and diluted with normal saline to achieve the desired concentration immediately prior to administration.

Neutropenic Thigh Infection Model

The mouse thigh infection model [10–12] was adopted to examine the relationship between antimicrobial exposure and reduction in the density of *P. aeruginosa* in the thigh muscles of neutropenic mice. Three hours prior to the initiation of antimicrobial therapy, each thigh was inoculated intramuscularly with 0.1 ml of a solution containing approximately 10⁸ CFU/ml of the test isolate. The concentration of the bacterial inoculum was confirmed by quantitative culture. The thighs were harvested after 24 h of antimicrobial therapy, and the treatment efficacy was calculated as the change in bacterial density 24 h after the start of antimicrobial treatment (0 h).

We used the antimicrobial agents for the treatment, 3 h post-inoculation (0 h), at an interval of every 12 h (q12h) for colistin and every 8 h (q8h) for aztreonam, by intraperitoneal injection and subcutaneous injection. The antimicrobial dosing regimens were 400 mg q8h for aztreonam and either 8 or 16 mg/kg (q6h or q12h) for colistin [9, 10]. At the stated doses of these antimicrobial agents, the concentrations of these drugs after the antimicrobial exposure in mice were similar to those in humans following intravenous regimens of 2 g q8h for aztreonam and either 2.5 or 5 mg/kg (q6h or q12h), with a 30-min infusion for colistin.

The sample size for this part of the study was calculated as follows. For typical antimicrobial agents, the optimal dosing regimen usually produces approximately a 2–3 log₁₀ CFU decrease in bacterial density with a %CV of 5 to obtain an observed mean that deviates from the true mean by no more than 1 SD using a two-sided 95% confidence interval with 80% probability; n = 6 data points were required.

Statistical Analysis

The statistical analyses were performed using the Wilcoxon signed-rank test. A p value <0.05 was considered significant.

Results

Break-Point Checkerboard Plate

The scoring of the combination effects for each antimicrobial combination against all 15 MDRP isolates is shown in table 1. The combination effects were scored and evaluated as described above. The combinations including colistin showed higher scores than the other combinations (table 1). Among some combination therapies including colistin, the combination of colistin and aztreonam had the highest score against 15 MDRP isolates (mean score 3.82). The combination effect scores for the combination therapies including colistin were: colistin + aztreonam, mean score 3.87; colistin + meropenem, 3.80; colistin + ceftazidime, 3.80; colistin + rifampicin, 3.73, and colistin + ciprofloxacin, 3.33.

Bacterial Isolates

The performances of several antimicrobials against 5 *P. aeruginosa* isolates are shown in table 2. The 5 *P. aeruginosa* isolates used in this study were highly resistant to aztreonam (MIC 64 to >254 μg/ml). On the other hand, they were susceptible to colistin, while 1 of the 5 isolates showed a different MIC (0.5 and 1 μg/ml). Based on the criteria used, 2 out of the 5 *P. aeruginosa* isolates were MDRP.

Metallo-β-Lactamase Production

Four of the 5 *P. aeruginosa* isolates used in the in vivo study did not produce IMP-type metallo-β-lactamase or AAC(6')-Iae. On the other hand, the AMU 13-9502 isolate produced metallo-β-lactamase.

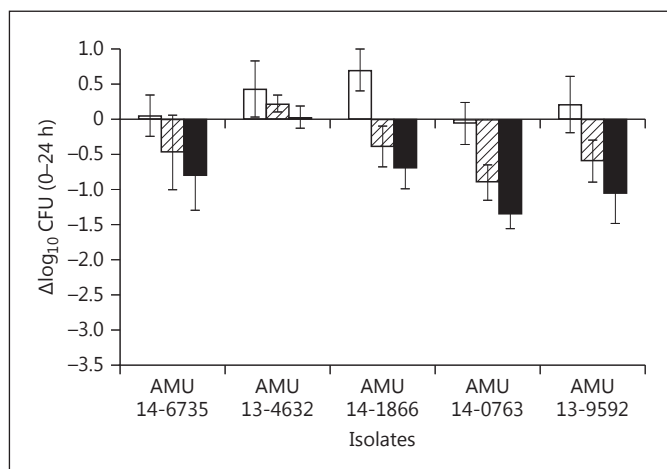


Fig. 1. Change in the \log_{10} CFU (mean \pm SD) for simulated human regimens of colistin and combination therapy of colistin and aztreonam against 5 *P. aeruginosa* isolates in the neutropenic murine thigh infection model. □: Colistin 8 mg/kg/12 h; ▨: colistin 8 mg/kg/6 h; ■: colistin 16 mg/kg/12 h.

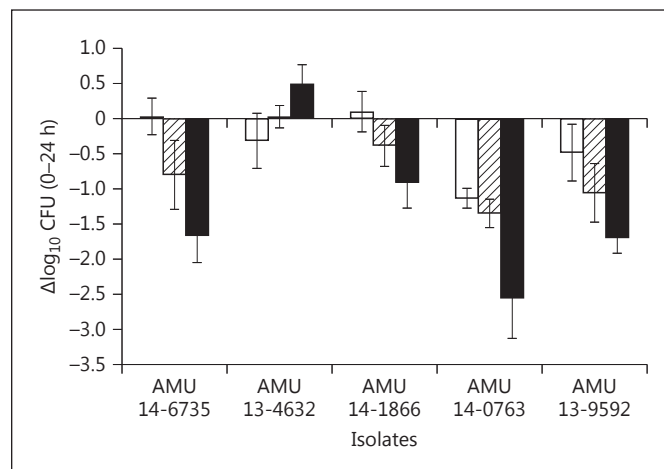


Fig. 2. Change in the \log_{10} CFU (mean \pm SD) for simulated human regimens of colistin, aztreonam, and combination therapy against 5 *P. aeruginosa* isolates in the neutropenic murine thigh infection model. □: Aztreonam 400 mg/8 h; ▨: colistin 16 mg/kg/12 h; ■: colistin 16 mg/kg/12 h + aztreonam 400 mg/8 h.

In vivo Efficacy

The bacterial densities in the thighs of control mice at 0 h ranged from 5.66 to 6.39 \log_{10} CFU. The isolates grew to 7.56–9.28 \log_{10} CFU after 24 h in the untreated control animals.

The results showed that treatment with high-dose colistin monotherapy (16 mg/kg q12h) achieved greater \log_{10} CFU changes than the normal dosage regimen (8 mg/kg q12h) against all *P. aeruginosa* isolates, including 2 MDRP isolates (AMU 14-6735, AMU 13-4632, AMU 14-1866, AMU 14-0763, and AMU 13-9592; $p < 0.05$). Furthermore, the high-dose regimen achieved greater \log_{10} CFU changes than the more frequent dosage regimen (8 mg/kg q6h), though the difference between the 2 regimens was not significant (fig. 1).

Aztreonam monotherapy yielded bacterial densities similar to those in the untreated control animals against the MDRP isolates evaluated (7.5–8.3 \log_{10} CFU; fig. 2). The colistin monotherapy (colistin MIC of 1 $\mu\text{g}/\text{ml}$) also yielded bacterial densities similar to those in the untreated animals against the MDRP isolate. The combination therapy including colistin (16 mg/kg q12h) with aztreonam was statistically superior to the colistin monotherapy against MDRP isolates (AMU 14-6735; -1.67 ± 0.39 vs. -0.80 ± 0.49 , $p < 0.05$; fig. 2). Additionally, the combination therapy group had greater antimicrobial activity than the colistin monotherapy group (16 mg/kg q12h) against all but 1 of the *P. aeruginosa* isolates tested (fig. 2).

Discussion

Reports of *P. aeruginosa* with high antimicrobial resistance, including MDRP, have steadily increased. This threatens the utility of current antipseudomonal therapy. *P. aeruginosa* infections due to MDRP are increasingly problematic because of the limited therapeutic options available. There are few effective drugs for MDRP infections, and colistin is still a key antimicrobial agent [2]. In fact, the combination therapy including colistin showed more enhanced antimicrobial efficacy against 15 MDRP isolates with the break-point checkerboard method in this study (table 2). The results were partially consistent with a previous study [1–4]. However, we thought that the high scores of the combination therapy including colistin may only reflect the colistin-sensitive strains among the 15 MDRP isolates. In fact, colistin monotherapy showed higher antimicrobial activity against *P. aeruginosa* with high antimicrobial resistance, including MDRP. However, one of the major problems of colistin therapy is the appearance of resistant mutants. Hence, in general, colistin is used as a combination therapy with other antimicrobials in the clinical setting. In our study, among the combination therapies including colistin, aztreonam showed the highest score in the in vitro study. Therefore, our data suggest that the combination of aztreonam with colistin is one of the most potent treatment candidates for antimicrobial therapy against *P. aeruginosa* with high an-

timicrobial resistance, including MDRP, if the isolates have a high susceptibility to colistin.

In vitro and in vivo models, the ratio of the area under the unbound concentration-time curve to the MIC (fAUC/MIC) has been shown to be the PK-PD index that best predicts bacterial killing by colistin [13–16]. In the Japanese practical guide for the appropriate use of colistin, it is recommended to use 1.25–2.5 mg/kg q12h, and some experts recommend using a loading dose (2 times the maintenance dose) at the start of treatment [17]. Consistent with a previous study, our results revealed that colistin monotherapy had dose-dependent antimicrobial activity [18] (fig. 1). The dose of colistin was selected to simulate the area under the curve in the concentration-time plot for estimation of the antimicrobial content in the plasma observed following an intravenous regimen of 2.5 and 5 mg/kg of colistin q12h in humans [19] and an intravenous regimen of 2 g of aztreonam q8h [20]. Hence, although colistin is generally used at 2.5 mg/kg q12h for infectious treatment, our study suggested that a higher dose of colistin (5 mg/kg/12 h) would be more effective than that of the normal dosage regimen (fig. 1).

Aztreonam has demonstrated low toxicity against a number of infections. It has been used in combination therapy and enhances the antimicrobial activity against several types of bacteria [3]. It is unique compared to other β -lactams because it resists hydrolysis by Ambler class B metallo- β -lactamases [21, 22]. Our results from the break-point checkerboard method indicate that combination therapy using colistin and aztreonam could be useful for the treatment of MDRP infections (table 1). Moreover, according to the results of the in vivo study, the combination therapy of aztreonam with colistin was also useful for treating *P. aeruginosa* with high resistance to several antimicrobials, including MDRP, when the isolates had a colistin MIC of 0.5 μ g/ml (fig. 2).

Previous in vitro studies have shown that combination therapies of colistin and aminoglycosides have the most enhanced antimicrobial activity [1–4]. Our in vivo study did not aim to evaluate the combination therapy of colistin with aminoglycosides, which was more effective against metallo- β -lactamase-producing isolates than against metallo- β -lactamase-negative isolates [3]. However, few studies have evaluated the antimicrobial activity of combination therapies including colistin, with the exception of combination studies of colistin and aminoglycosides. Regarding metallo- β -lactamase production, there are a few reports that consider the differences in the effectiveness of combination regimens between metallo- β -lactamase-producing isolates and negative MDRP iso-

lates. Nakamura et al. [3] showed that aztreonam with aminoglycosides was effective against MDRP and, remarkably, it was more effective against metallo- β -lactamase-positive isolates than against metallo- β -lactamase-negative isolates. Metallo- β -lactamase-positive MDRP isolates accounted for approximately 40–60% of all MDRP isolates [23], and we did not confirm whether these isolates produce other types of metallo- β -lactamase, such as VIM or OXA-48. Hence, our in vitro break-point checkerboard study with 15 MDRP isolates could possibly include metallo- β -lactamase-positive and negative isolates. Consequently, the combination therapy with colistin and aztreonam showed the highest antimicrobial activity against the 15 MDRP isolates among all combination therapies in the in vitro study, and the combination therapy with humanized dosage regimens showed enhanced antimicrobial activity against *P. aeruginosa* isolates, including MDRP and metallo- β -lactamase-producing isolates, compared with colistin monotherapy (fig. 2). Although we evaluated efficacy against 4 non-IMP-type metallo- β -lactamase-producing *P. aeruginosa* isolates and 1 IMP-type metallo- β -lactamase-producing *P. aeruginosa* isolate in an in vivo study, our results suggested that the combination therapy of colistin and aztreonam could become a potent candidate against highly resistant *P. aeruginosa* infections, including MDRP infection. In future studies, it will be necessary to determine how the antimicrobial activity against metallo- β -lactamase-positive and negative isolates could change.

Our study has some limitations, including the lack of molecular characterization and fingerprinting of the *P. aeruginosa* isolates. The combination therapy with aztreonam and aminoglycosides showed differing antimicrobial activity between metallo- β -lactamase-positive and metallo- β -lactamase-negative isolates [4]. Metallo- β -lactamase-producing isolates commonly carry additional β -lactamases, which may include ESBLs, AmpC enzymes, and serine carbapenemases that can inactivate aztreonam. Finally, one MDRP isolate (AMU 13-46320) appeared to show no antimicrobial efficacy to the combination therapy of colistin and aztreonam in the in vivo study. We expected that a lower susceptibility of colistin (MIC \geq 1 μ g/ml) and/or molecular characterization of the isolate might induce a reduction of antimicrobial activity. Hence, epidemiological typing could provide additional insight into the resistance mechanisms of *P. aeruginosa* with high resistance to several antimicrobials, including MDRP.

In conclusion, our study showed that colistin and aztreonam could be a promising combination regimen against *P. aeruginosa* with high antimicrobial resistance,

including MDRP isolates, when the isolates had a colistin susceptibility of less than 1.0 µg/ml. We expect that this finding could drive therapeutic selections, while further investigation of the translational relevance of the utility of this combination therapy in patients is needed. Additionally, it is possible that the genotype associated with the MIC may play additional roles in predicting therapeutic outcomes.

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Disclosure Statement

We have no conflicts of interest to declare.

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