

ORIGINAL ARTICLE

In vitro and *in vivo* activities of the diazabicyclooctane OP0595 against AmpC-derepressed *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a common cause for healthcare-associated infections, which have been historically treated by antipseudomonal β -lactam agents in the clinical setting. However, *P. aeruginosa* has evolved to overcome these β -lactam agents via multiple endogenous resistance mechanisms, including derepression of the chromosomal cephalosporinase (AmpC). In this article, we investigated the effective concentration of OP0595 for combination with piperacillin, cefepime or meropenem in *in vitro* susceptibility tests, and the antibacterial activity of cefepime in combination with OP0595 in both *in vitro* time-kill studies and *in vivo* murine thigh infection model study with AmpC-derepressed *P. aeruginosa*. The sufficient combinational concentration of OP0595 was a $4 \mu\text{g ml}^{-1}$ with all these three β -lactam agents. OP0595 increased the antibacterial activity of cefepime in both *in vitro* and *in vivo* studies against all strains tested. Taken together, OP0595 is the diazabicyclooctane serine β -lactamase inhibitor with activity against AmpC-derepressed *P. aeruginosa* and its combinational use with a β -lactam agent will provide a new approach for the treatment of *P. aeruginosa* infections.

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INTRODUCTION

Pseudomonas aeruginosa is a common cause for healthcare-associated infections including pneumonia, bloodstream infections, urinary tract infections, and surgical site infections.¹ To treat *P. aeruginosa* infections, antipseudomonal β -lactam agents such as piperacillin, ceftazidime, cefepime, imipenem and meropenem have been administered in the clinical setting. However, *P. aeruginosa* strains have overcome against these β -lactam agents by means of endogenous multiple resistance mechanisms, including derepression of the chromosomal cephalosporinase (AmpC), upregulation of efflux systems, and loss of an outer membrane porin protein. These mechanisms may function in composite and are able to resist all antipseudomonal β -lactam agents.^{2–4}

As a result, new approaches to the treatment of infection are hoped, and diazabicyclooctanes including avibactam, relebactam (MK-7655) and OP0595 have been researched.⁵ Diazabicyclooctanes exhibit strong inhibitory activity against both Class A β -lactamases including KPC types and Class C AmpC enzymes of the Ambler classification, and are expected as a new class of β -lactamase inhibitors.^{6,7} Avibactam and relebactam have determined ceftazidime and imipenem as an initial partner β -lactam agent, respectively. Avibactam and relebactam increase the antibacterial activity of partner β -lactam agents against serine β -lactamases producing strains.^{8–11}

OP0595 acts in three ways: (i) as a β -lactamase inhibitor, (ii) as an antibacterial agent against Enterobacteriaceae, and (iii) as

an ‘enhancer’ of the activity of various β -lactam agents differing from avibactam and relebactam.⁶ However, OP0595 does not show antibacterial activity or enhancer activity toward *P. aeruginosa* and works only as a β -lactamase inhibitor in this case.¹²

As a first approach to characterize the efficacy of OP0595 against *P. aeruginosa*, here its antibacterial activity in combination with β -lactam agents was evaluated to determine the effective concentration of OP0595 for combinatorial use. Then OP0595 was tested in both *in vitro* time-kill studies and an *in vivo* murine thigh infection model study. In the latter studies, cefepime was used as a partner β -lactam agent for OP0595.

MATERIALS AND METHODS

Compounds

OP0595 was synthesized by Meiji Seika Pharma Co., Ltd. (Tokyo, Japan) and used as anhydride.⁶ Antibacterial compounds were obtained from the following sources: piperacillin from Sigma-Aldrich (St Louis, MO, USA); meropenem from United States Pharmacopeial Convention (Rockville, MO, USA); and cefepime from United States Pharmacopeial Convention and GlaxoSmithKline K.K. (Tokyo, Japan).

Bacterial strains

The three strains of AmpC-derepressed *P. aeruginosa* (MSC17689, MSC17715 and MSC17716) are clinical isolates from Japan and were obtained from the Antimicrobial Susceptibility Surveillance Research Group (Osaka, Japan). The expressions of AmpC under a non-inducible condition were detected by

real-time quantitative RT-PCR using the previous method.¹³ The levels of AmpC in all strains were over 1.5 times higher than that in wild-type *P. aeruginosa* ATCC 27853 (ATCC, Manassas, VA, USA) and these strains were regarded as AmpC-derepressed strains.¹⁴ The strain of *P. aeruginosa* ATCC 27853 was used for quality control.

Susceptibility testing

The MIC of each compound was determined by broth microdilution according to CLSI guidelines.^{15,16} The inoculum size was $\sim 5 \times 10^4$ CFU per well. The MIC was determined as the lowest concentration to inhibit the visible growth after incubation at 35 °C for 18 h.

Time-kill experiments

Bactericidal activity was determined with the time-kill method, standardized by CLSI guidelines as follows.¹⁷ Well-isolated bacterial colonies were added to cation-adjusted Mueller–Hinton broth (CAMHB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and cultured for ~ 16 h at 35 °C. The cultured bacterial suspensions were diluted with CAMHB and cultured for 2 h, while being shaken at 100 r.p.m. at 35 °C. An aliquot of 4.9 ml of bacterial suspension was added to a test tube and mixed with 0.1 ml of the test compound solution. The suspension was then cultured at 35 °C with shaking at 100 r.p.m. At time points of 0, 2, 4, 6 and 24 h after addition of the test compound, a sample was collected from the culture medium, serially diluted, spread on a Mueller–Hinton agar (MHA; Becton, Dickinson and Company) plate, and cultured at 35 °C. The number of colonies growing on the plate was counted after ~ 24 h. The detection limit was set at $< 1.3 \log_{10}$ CFU ml⁻¹; if no colonies were detected, the value of $1.3 \log_{10}$ CFU ml⁻¹ was adopted. A bactericidal effect was estimated as a decrease of $\geq 3 \log_{10}$ CFU ml⁻¹ relative to the control sample obtained at 0 h.

Thigh infection model in neutropenic mice

All animal studies and protocols were approved by the Animal Experiment Management Committee of the Pharmaceutical Research Center, and were based on Guidelines on the Management of Animal Experiments established by the Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd. Four-week-old, specific-pathogen-free, male Crj:CD1 (ICR) mice (Charles River Laboratories Japan, Inc., Kanagawa, Japan) weighing 18–22 g were used for all the tests. These mice were used because a murine model of thigh infection with *P. aeruginosa* has been established in this strain.¹⁸ The mice were kept in an animal room under controlled conditions (temperature, 21–25 °C; humidity, 50–70%; lighting hours, 07:00–19:00), and allowed to acclimatize for 1 week before the study. During the acclimation and study periods, feed (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and water were available *ad libitum*.

The neutropenic mice were fabricated by i.p. administration of cyclophosphamide (Sigma-Aldrich) 4 days before infection (150 mg kg^{-1} of body weight) and 1 day before infection (100 mg kg^{-1} of body weight). Six mice per group were infected with *P. aeruginosa* by an injection into the thigh. The mice were treated by s.c. administration of the test compound at 1, 3 and 5 h after infection, and euthanized 24 h after infection by cervical dislocation. The thigh was then removed and homogenized. Each homogenate was diluted 10-fold serially with physiological saline, and an aliquot of each initial homogenate and dilution series was smeared onto a plate of MHA, which was then cultured at 35 °C. The number of colonies grown on the plate was counted after ~ 22 h. The detection limit was set at $< 2.15 \log_{10}$ CFU per thigh; if no colonies were detected in the initial homogenate, the value of $2.15 \log_{10}$ CFU per thigh was adopted. The data were expressed as the mean \pm s.d. \log_{10} CFU per thigh. The number of viable cells was compared between the vehicle and administration groups by using Steel's test.

RESULTS

Concentration dependency of *in vitro* antibacterial activity

To determine the effective concentration of OP0595 for use with β -lactam agents, the MICs of piperacillin, cefepime and meropenem in combination with multiple concentrations of OP0595 were determined against three strains of AmpC-derepressed *P. aeruginosa*,

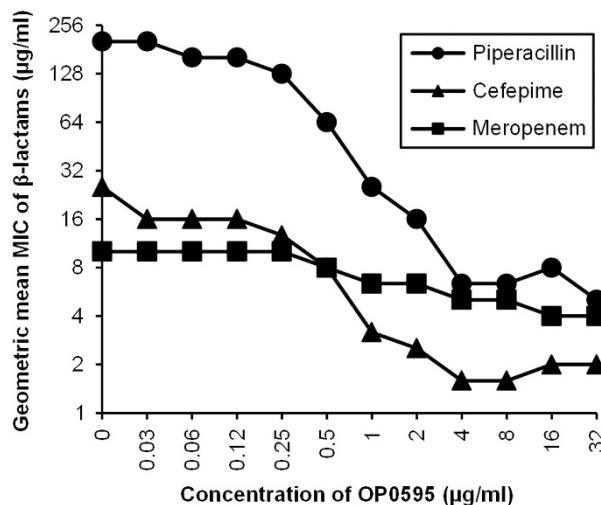


Figure 1 Antimicrobial activity of β -lactam agents in combination with various concentrations of OP0595 against three strains of AmpC-derepressed *P. aeruginosa*. A MIC of $> 128 \mu\text{g ml}^{-1}$ was counted as $256 \mu\text{g ml}^{-1}$ when calculating the geometric mean.

and the geometric mean MICs were plotted (Figure 1). For all β -lactam agents, the geometric mean MICs decreased according to the increase of the OP0595 concentration. The MIC attained a plateau at $4 \mu\text{g ml}^{-1}$ of OP0595 in all cases, and the MICs of cefepime-OP0595 were lowest among the three β -lactam agents tested. Collectively, these results suggest that a $4 \mu\text{g ml}^{-1}$ of OP0595 is sufficient to show the effective MIC reduction *in vitro* activities against AmpC-derepressed *P. aeruginosa*, and one of the optimal partner β -lactam agents for OP0595 is cefepime.

Bactericidal activity against AmpC-derepressed *P. aeruginosa*

To assess the detailed combinational activity of OP0595 against the antibacterial activity of cefepime, bactericidal activity was tested using three strains of AmpC-derepressed *P. aeruginosa*.

OP0595 did not show the antibacterial activity at $16 \mu\text{g ml}^{-1}$ and cefepime showed weak antibacterial activity against these strains (Table 1). On the other hand, Cefepime-OP0595 showed strong antibacterial activity against them and all the MICs were susceptible concentrations according to the CLSI guidelines.¹⁵ In this study, two concentrations of OP0595 were used to evaluate the capacity of its β -lactamase inhibitory activity. The data in Figure 1 indicated that the applicable combinatorial concentration of OP0595 was $4 \mu\text{g ml}^{-1}$; therefore, the concentrations of OP0595 were set at 1 and $4 \mu\text{g ml}^{-1}$ to compare an insufficient concentration with the applicable concentration. The concentrations of cefepime were set at 8 and $16 \mu\text{g ml}^{-1}$, which are the respective susceptible and intermediate concentrations against *P. aeruginosa* according to the CLSI guidelines. The time-kill curves are shown in Figure 2.

An aliquot of $16 \mu\text{g ml}^{-1}$ of cefepime did not decrease the viable cell counts of all the test strains. On the other hand, $16 \mu\text{g ml}^{-1}$ of cefepime combined with OP0595 decreased the viable cell counts. In particular, the strong bactericidal effect was observed at $4 \mu\text{g ml}^{-1}$ of OP0595 in combination with $16 \mu\text{g ml}^{-1}$ of cefepime. All test strains decreased their viable cell counts by $3 \log_{10}$ CFU ml⁻¹ after 24 h from the counts at 0 h. By contrast, cefepime alone, OP0595 alone and cefepime-OP0595 combination (16 and $1 \mu\text{g ml}^{-1}$, respectively) did not show a bactericidal effect against any of the test strains. These

results suggested that the bactericidal effect of cefepime was restored by OP0595 that acted as an AmpC inhibitor.

Efficacy in *in vivo* model

To assess the efficacy of OP0595 in combination with cefepime *in vivo*, we carried out a study in a neutropenic murine thigh infection model (Figure 3). The strain tested was AmpC-derepressed *P. aeruginosa* MSC17715, which had been checked to increase in the neutropenic murine thighs. Administration of cefepime alone or OP0595 alone showed the bacterial count in the murine thigh similar to that in the

vehicle group. In contrast, combinational administration of cefepime-OP0595 decreased the bacterial count to 2–4 log₁₀ CFU per thigh (*P*<0.05). These results suggest that OP0595 works as an AmpC inhibitor and OP0595 alone has no antibacterial activity *in vivo*.

DISCUSSION

In this study we have demonstrated that, when used in combination with cefepime, the diazabicyclooctane molecule OP0595 has potency against AmpC-derepressed *P. aeruginosa* strains in both *in vitro* time-kill studies and *in vivo* infection model study.

Recently, the concentrations of β-lactamase inhibitors have been tested at 4 μg ml⁻¹ in combinational studies of ceftolozane–tazobactam, ceftazidime–avibactam, and imipenem–relebactam, among other combinations.^{5,9,19} Against CTX-M-15-positive *Escherichia coli* and KPC-positive *K. pneumoniae* strains, the optimal concentration of OP0595 in combination with piperacillin, cefepime or meropenem was 4 μg ml⁻¹.²⁰ Similar results were obtained by our *in vitro* tests and the sufficient combinational concentration of OP0595 was also 4 μg ml⁻¹ against AmpC-derepressed *P. aeruginosa*. Interestingly, OP0595 did not affect the antibacterial activity of piperacillin, cefepime and meropenem against *P. aeruginosa* ATCC 27853, which expresses wild-type levels of AmpC (data not shown),¹⁴ indicating that OP0595 functions as an AmpC inhibitor.

Table 1 *In vitro* antibacterial activity of cefepime and OP0595 against AmpC-derepressed *P. aeruginosa*

Organism	MIC (μg ml ⁻¹)		
	OP0595	Cefepime	Cefepime-OP0595 ^a
<i>P. aeruginosa</i> MSC17689	>16	>64	2
<i>P. aeruginosa</i> MSC17715	>16	32	2
<i>P. aeruginosa</i> MSC17716	>16	16	2

^aThe combined concentration of OP0595 was fixed at 4 μg ml⁻¹.

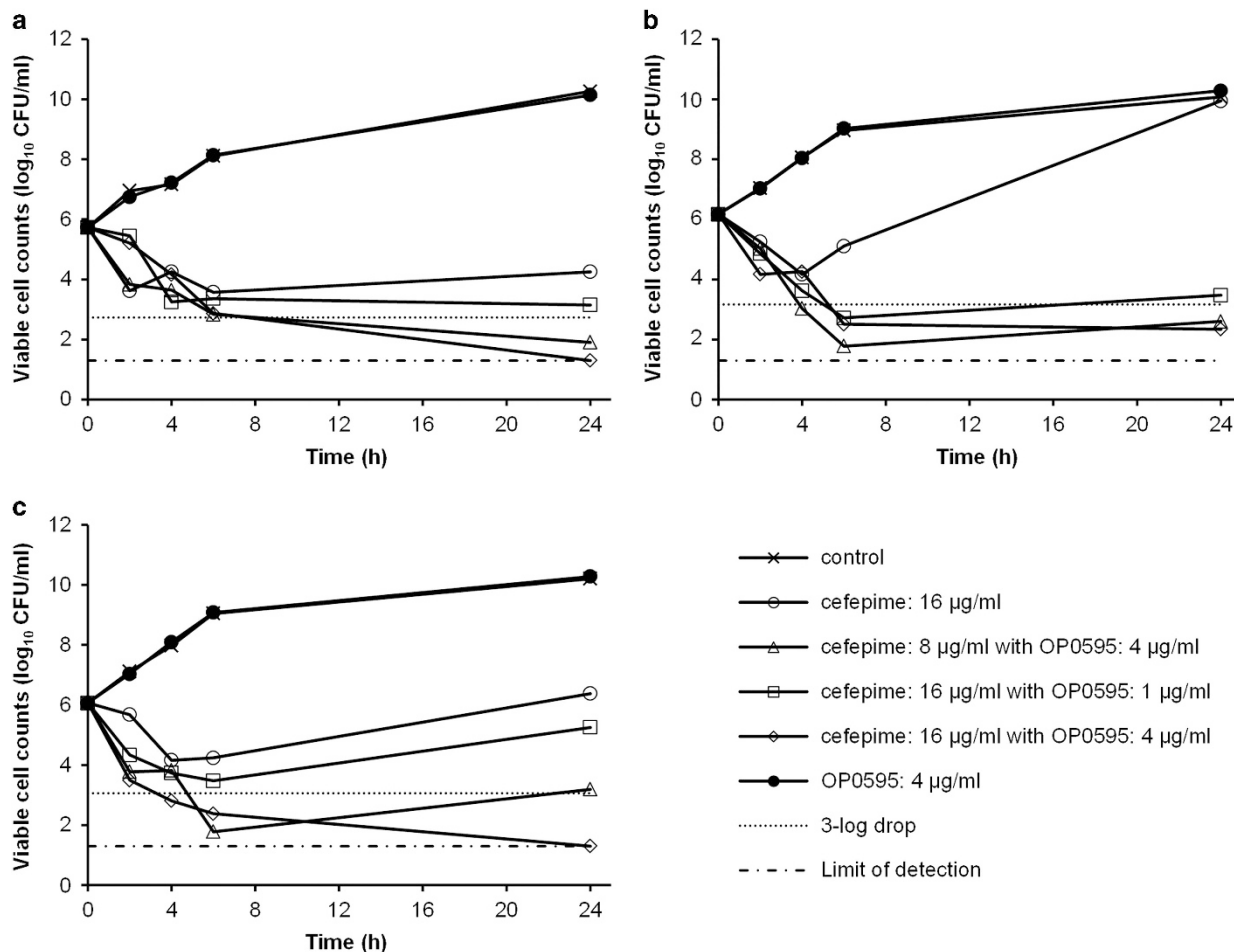


Figure 2 Time-kill curve of a combination of OP0595 and cefepime against AmpC-derepressed *P. aeruginosa*. (a) *P. aeruginosa* MSC17698; (b) *P. aeruginosa* MSC17715; (c) *P. aeruginosa* MSC17716. The detection limit was 1.30 log₁₀ CFU ml⁻¹. FEP, cefepime.

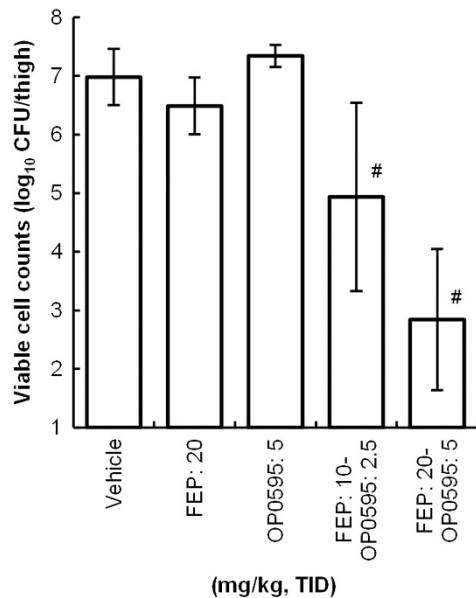


Figure 3 Comparison of the efficacy between sole and combined administration of β -lactam agents in a neutropenic murine model of thigh infection with AmpC-derepressed *P. aeruginosa* MSC17715. Six mice per group were rendered neutropenic, and 5.36 log₁₀ CFU per mouse of *P. aeruginosa* MSC17715 was injected into the thigh, followed by s.c. administration of the test compounds at 1, 3 and 5 h after infection. The mean log₁₀ CFU per thigh recovered from the infected thigh after 24 h is shown; error bars represent the s.d. A *P*-value of <0.05 indicates significance relative to the untreated control (vehicle) and is indicated by a hash (#). FEP, cefepime; TID, three times a day.

OP0595 reduced the MICs of piperacillin and cefepime, but weakly reduced the MIC of meropenem. Piperacillin and cefepime are easily hydrolyzed by AmpC, but meropenem is stable against AmpC activity.²¹ This is consistent with the results shown in Figure 1. OP0595 inhibits serine-carbapenemase such as KPC, and the meropenem-OP0595 combination is expected to show strong antibacterial activity against KPC-positive *P. aeruginosa* strains.

To predict the efficacy of OP0595 in humans, *in vitro* and *in vivo* pharmacokinetic/pharmacodynamic (PK/PD) studies, such as an *in vitro* hollow-fiber infection model study using human PK and a murine thigh infection model study using murine PK, are needed. In the case of Enterobacteriaceae, OP0595 works not only as a β -lactamase inhibitor but also as an antibacterial agent and β -lactam enhancer; by contrast, OP0595 works only as a β -lactamase inhibitor against AmpC-derepressed *P. aeruginosa*, and thus calculations of the PK/PD parameters of OP0595 will be simplified. Currently, combinational PK/PD studies with β -lactam agent and β -lactamase inhibitor, such as ceftolozane-tazobactam, ceftaloline-avibactam and aztreonam-avibactam, have been performed with *in vitro* and *in vivo* models.^{18,22–24} For example, an *in vitro* hollow-fiber model was investigated to detect the effect of β -lactamase inhibitory activity. They used the human PK value of a β -lactam agent and the stationary concentration of a β -lactamase inhibitor. The effects of OP0595 might be clarified in similar studies using AmpC-derepressed *P. aeruginosa* to establish detailed information for clinical development.

In conclusion, OP0595 has been shown to work as a β -lactamase inhibitor against *P. aeruginosa* and increased the antibacterial activities of cefepime in both *in vitro* time-kill studies and an *in vivo* infection

model study. These data present that OP0595 has a potential as a new β -lactamase inhibitor to treat *P. aeruginosa* infections.

CONFLICT OF INTEREST

AM, KY, TT, TA, TF, YS, TI: relevant shareholdings-Meiji Holdings, amounting to <10% of portfolio value. All authors are employees of Meiji Seika Pharma Co., Ltd.

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