

ORIGINAL

A *Pseudomonas aeruginosa* Quorum-Sensing autoinducer analog enhances the activity of antibiotics against resistant strains

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Abstract : In this study, we have investigated the effects of the newly synthesized analog of *Pseudomonas aeruginosa* quorum-sensing autoinducer named AIA-1 (autoinducer analog) against antibiotic-resistant bacteria. *In vitro* susceptibility and killing assays for *P. aeruginosa* PAO1Δ*oprD* mutant and clinical isolates were performed by using antibiotics and AIA-1. In an *in vivo* assay, a luminescent carbapenem-resistant strain derived from PAO1Δ*oprD* was injected into neutropenic ICR mice and bioluminescence images were acquired after the treatment with antibiotics and AIA-1. Additionally, we investigated the effects of the combination use against carbapenem-resistant Enterobacteriaceae (CRE). Using killing assays in *P. aeruginosa*, the survival rates in the presence of antibiotics and AIA-1 significantly decreased in comparison with those with antibiotics alone. Furthermore, dual treatment of biapenem and AIA-1 was more effective than biapenem alone in a mouse infection model. AIA-1 did not change the MICs in *P. aeruginosa*, suggesting that AIA-1 acts on the mechanism of antibiotic tolerance. Conversely, the MICs of antibiotics decreased in the presence of AIA-1 in some CRE strains, indicating that AIA-1 may require additional mechanism to act on CRE. In conclusion, AIA-1 may be a potent drug for clinical treatment of infections caused by antibiotic-resistant bacteria. *J. Med. Invest.* 64 : 101-109, February, 2017

Keywords : *Pseudomonas aeruginosa*, CRE, antibiotic resistance

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacteria and a major nosocomial pathogen that also causes opportunistic infections in immunocompromised hosts, especially patients with cystic fibrosis. This pathogen possesses high intrinsic antibiotic resistance and the ability to develop resistance to multiple antimicrobial agents (1), therefore the spread of multidrug-resistant *P. aeruginosa* (MDRPA) represents a major concern in the world (2). Some of the known mechanisms of antibiotic resistance are linked to : 1) the impermeability across membranes and the efflux of the drug from the cytoplasm, 2) drug inactivation and modification by enzymes (e.g., β-lactamases, aminoglycoside-modifying enzymes), and 3) changes in the targets of antibiotics (3). Treatments of infections provoked by antibiotic-resistant bacteria often include cocktails of multiple antimicrobial agents such as β-lactams and fluoroquinolones or aminoglycosides (4-6). Alternatively, combining antibiotics with efflux pump inhibitors (EPIs) is considered (7-9).

Carbapenem-resistant Enterobacteriaceae (CRE) are defined as Enterobacteriaceae that are resistant to carbapenem-class of antibiotics (imipenem, meropenem, doripenem, and ertapenem) or possess a carbapenemase (10). Among the Enterobacteriaceae genera, *Enterobacter* spp. (e.g., *Enterobacter aerogenes*, *Enterobacter*

cloacae), *Escherichia coli*, *Klebsiella pneumoniae* and *Citrobacter* spp. are shown to be resistant to carbapenems (11-13). CRE infection causes high mortality in infected patients, placing these organisms as a major clinical problem worldwide (12-14). Currently available treatment options for CRE are few, therefore further studies are needed to determine the appropriate treatments (14, 15).

Quorum-sensing (QS) system is a bacterial cell-to-cell communication process that relies on the bacterial population density (16). It involves small diffusible signaling molecules called autoinducers, and *P. aeruginosa* produces two main acyl-homoserine lactone (AHL) autoinducers ; OdDHL (3-oxo-C₁₂-HSL) and BHL (C₄-HSL) (16). In previous studies, Smith *et al.* synthesized a library of *P. aeruginosa* autoinducer analogs (AIAs) of the AHL (17, 18).

AIA-1, an analog of OdDHL, was synthesized as QS autoinducer analog, but it did not inhibit the production of QS-related virulence factors. The aim of this study was to investigate the combination effects of AIA-1 and antibiotics against *P. aeruginosa* resistant strains and CRE strains.

MATERIALS AND METHODS

Reagents

The structure of AIA-1 is shown in Figure 1A, and Figure 1B shows the structure of OdDHL. AIA-1 was dissolved in water to reach a stock concentration of 32 μg mL⁻¹ or 64 μg mL⁻¹. Biapenem was purchased from Meiji Seika Pharma Co., Ltd. (Tokyo, Japan). Levofloxacin and tobramycin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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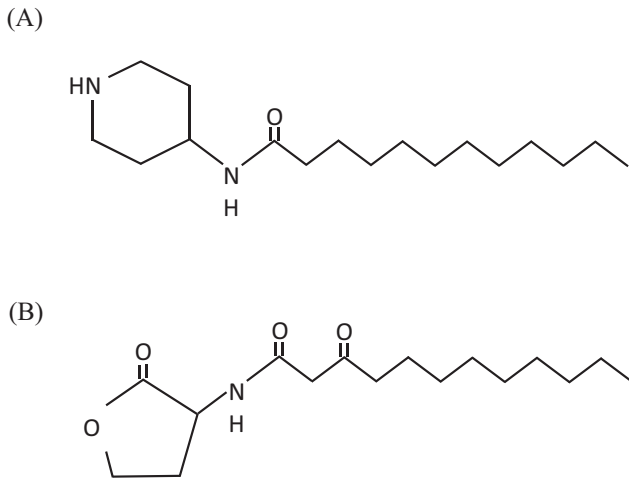


Figure 1. (A) The structure of AIA-1, the analog of OddHL. (B) The structure of OddHL, a typical autoinducer of *P. aeruginosa*.

Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The strains were incubated at 37°C in lysogeny broth (LB) or on LB agar plates. Antibiotics were used in selective media at the

following concentrations for *E. coli*: tetracycline 20 mg L⁻¹, gentamycin 20 mg L⁻¹, and ampicillin 100 mg L⁻¹, and for *P. aeruginosa*: tetracycline 200 mg L⁻¹, carbenicillin 500 mg L⁻¹, gentamycin 200 mg L⁻¹, and when necessary, supplemented with 10% sucrose for conjugation experiments.

Plasmid constructions and generation of mutant strains

In order to construct an *oprD* in-frame deletion mutant, approximately 500 bp of the upstream and downstream regions of *oprD* gene were amplified by primers (*oprDup-F*: 5'-CGACTCTAGAGGATCACGCATTTCGCCACAGACA-3', *oprDup-R*: 5'-GTCCGGT-CGATTACAGTTTCATTGTGATTGCTCCTTT-3', *oprDdown-F*: 5'-CTGTAATCGACCGACAGG-3', *oprDdown-R*: 5'-CCATGAT-TACGAATTTGAGGAGTCAGCAGGCAA-3') from PAO1 chromosomal DNA, and cloned into pEX18Gm (19) using *Bam*HI and *Eco*RI restriction sites to generate pEX18Gm-*oprD*. This plasmid DNA was mobilized from *E. coli* S17-1 (20) into *P. aeruginosa* by mating. Colonies were screened for gentamycin sensitivity and the loss of sucrose sensitivity. For *in vivo* bioluminescence imaging, miniCTX-*lux* plasmid was constructed by ligating *Sac*I/*Bam*HI-digested miniCTX-2 (21) with *Sac*I/*Bam*HI fragment digested from pXen13 (Xenogen, CA, USA), encompassing the *lux* operon. The *lac* promoter region was amplified by PCR using pUCP18 plasmid (22) as a template. The amplified *lac* promoter region and miniCTX-*lux* were digested with *Xho*I/*Bam*HI and ligated, resulting in miniCTX-*P_{lac}*::*lux*. PAO1 Δ *oprD*-*P_{lac}*::*lux* was created by integration of miniCTX-*P_{lac}*::*lux* at the *attB* site of PAO1 Δ *oprD*, followed by FLP recombinase mediated excision of the plasmid

Table 1 Strains and plasmids

Strains	Relevant characteristics	Reference
<i>P. aeruginosa</i>		
PAO1	Wild type	
PAO1 Δ <i>oprD</i>	PAO1 in-frame deletion of <i>oprD</i>	This study
PAO1 Δ <i>oprD</i> <i>P_{lac}</i> :: <i>lux</i>	One copy of the <i>lac</i> promoter linked to the <i>luxCDABE</i> gene in the <i>attB</i> site of the PAO1 Δ <i>oprD</i> chromosome	This study
TUH44	<i>oprD</i> ::IS <i>Ppu12</i> , <i>gyrA</i> (83Thr-Ile)	Clinical isolate
TUH81	<i>nfxC</i> -type mutant	Clinical isolate
TH5	<i>gyrA</i> (83Thr-Ile)	Clinical isolate
Carbapenem-resistant Enterobacteriaceae		
T-CRE1	<i>Citrobacter freundii</i>	Clinical isolate
T-CRE2	<i>Enterobacter aerogenes</i>	Clinical isolate
T-CRE3	<i>Enterobacter aerogenes</i>	Clinical isolate
T-CRE4	<i>Enterobacter cloacae</i>	Clinical isolate
T-CRE5	<i>Enterobacter aerogenes</i>	Clinical isolate
<i>E. coli</i>		
S17-1	<i>thi endA recA hsdR</i> Chromosome::RP4-2Tc::Mu-Km::Tn7	20
DH5 α	F ⁻ , Φ 80d <i>lacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (τ_K , m κ^+), <i>phoA</i> , <i>supE44</i> , λ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	TaKaRa
Plasmids		
miniCTX-2	Plasmid for the integration of genes into the <i>att</i> site of the <i>P. aeruginosa</i> chromosome, Tc ^R	21
pXen13	Cloning vector carrying the original <i>Photobacterium luminescens luxCDABE</i> operon	Xenogen
pUCP18	<i>P. aeruginosa</i> - <i>E. coli</i> shuttle vector	22
miniCTX- <i>P_{lac}</i> :: <i>lux</i>	One copy of the <i>lac</i> promoter linked to the <i>luxCDABE</i> gene inserted into miniCTX-2	This study
pFLP2	FLP recombinase expressing plasmid	19
pEX18Gm	Broad-host-range suicide vector; <i>sacB</i> , Gm ^R	19
pEX18Gm- <i>oprD</i>	<i>oprD</i> deletion suicide vector	This study

backbone (19). Colonies were screened for tetracycline and carbencillin sensitivity and the loss of sucrose sensitivity.

Susceptibility assay

The minimum inhibitory concentration (MIC) was assessed by the standard microbroth dilution method (23). In susceptibility assays including both antibiotics and AIA-1 for *P. aeruginosa*, AIA-1 was used at concentrations of 32 $\mu\text{g mL}^{-1}$. For CRE, AIA-1 concentrations ranging from 8 to 16 $\mu\text{g mL}^{-1}$ were used.

Time kill assay

PAO1 ΔoprD , TUH44, and TH5 were grown overnight in LB medium, then the cultures were diluted 100-fold and grown to an OD_{600} of 0.2 to 0.25, or approximately 2×10^8 CFU mL^{-1} . For TUH81, the culture was grown to stationary phase, and then, the culture was adjusted 2×10^8 CFU mL^{-1} . At this point, the cells were incubated in the presence of antibiotics and/or AIA-1 for various periods of time. Biapenem was used at concentrations of 64 $\mu\text{g mL}^{-1}$ for PAO1 ΔoprD , or 128 $\mu\text{g mL}^{-1}$ for TUH44 and TUH81. Levofloxacin was used at a concentration of 64 $\mu\text{g mL}^{-1}$ for TUH44 and TUH81. Tobramycin was used at a concentration of 64 $\mu\text{g mL}^{-1}$ for TH5. AIA-1 was used at concentrations of 32 $\mu\text{g mL}^{-1}$ for PAO1 ΔoprD , TUH44 and TH5, and at a concentration of 64 $\mu\text{g mL}^{-1}$ for TUH81. The number of viable cells was measured by culturing the cells on LB agar plates. By assuming that survival at time 0 was 100%, CFU values were converted to percent survival relative to the number of untreated cells at time zero.

Mouse infection model by biapenem-resistant *P. aeruginosa* and bioluminescence imaging

In a mouse infection model experiment, neutropenic mice were prepared by two intraperitoneally administrations of cyclophosphamide monohydrate to ICR mice aged 5 to 7 weeks, at 150 mg kg^{-1} at day -4 and 100 mg kg^{-1} at day -1 from infection. Then, mice were infected with a luminescent carbapenem-resistant strain PAO1 ΔoprD - $P_{lac}::lux$. An inoculum containing approximately 10^6 CFU was injected per thigh. AIA-1 (50 mg kg^{-1} body weight) was intraperitoneally administered twice (at the point of 2 and 6 hours) in 8 hours. Two hours after the inoculum injection, mice were subcutaneously administered the selected concentrations of biapenem (12.5 mg kg^{-1} body weight) 4 times over a period of 8 hours. At 0, 2, 4, 6, 8 and 10 h after the inoculum injection, mice were anesthetized using a constant flow of 2.5% isoflurane mixed with oxygen, and bioluminescence images were acquired using an IVIS[®] Lumina system (Caliper, Alameda, CA, USA). At 10 h, photons emitted per second by mice were quantified, and thighs from infected mice were homogenized in saline. A portion of the homogenate was serially diluted and then spread on a nutrient agar plate to count CFUs. Mouse infection studies were approved by the Animal Care and Use Committee, Okayama University, Process number

OKU-2013055. All procedures were performed according to the Policy on the Care and Use of the Laboratory Animals, Okayama University.

RESULTS

Susceptibility assay of *P. aeruginosa* to antibiotics and AIA-1.

The MICs of antibiotics and AIA-1 are shown in Table 2. The MICs of AIA-1 ranged from 128 to 512 $\mu\text{g mL}^{-1}$, demonstrating very weak antibacterial activity against *P. aeruginosa* strains. The MICs of biapenem for PAO1 ΔoprD , TUH44 and TUH81 were 8 to 16 $\mu\text{g mL}^{-1}$, implying intermediate-level of resistance to biapenem. Furthermore, TUH44 and TUH81 also demonstrated intermediate levofloxacin resistance, and TH5 displayed intermediate levofloxacin and tobramycin resistance. In the presence of AIA-1 at 32 $\mu\text{g mL}^{-1}$, the MICs of antibiotics did not change in the tested strains, indicating that AIA-1 did not affect the susceptibility to antibiotics in *P. aeruginosa*.

Characteristics of *P. aeruginosa* clinical isolates about antibiotic resistance.

Since TUH44 and TUH81 demonstrated both biapenem and levofloxacin resistance (Table 2), we investigated the potential factors underlying resistance observed in these strains. Carbapenem resistance in *P. aeruginosa* is often caused by the loss or alteration of the outer membrane porin protein OprD (24), therefore we sought to investigate the genetic structure of the *oprD* gene. In TUH44, the genome sequence analyses revealed an insertion of an approximately 3 kbp IS element (ISPpu12, GenBank Accession Number : AY128707) within the *oprD* gene, resulting in the inactivation of OprD. The major molecular mechanism of fluoroquinolone resistance is mediated by mutations in the *gyrA* gene, which encodes DNA gyrase enzyme (3, 24-26). Using the method of Weile *et al.* (27), in TUH44 strain, we demonstrated the presence of a mutation in the *gyrA* gene at position 83 (Thr-Ile, 242C→T). Next, we focused on the efflux pump MexEF-OprN, which is involved in imipenem resistance (28, 29). We used immunoblotting to detect specific proteins such as MexE and OprD from the clinical strains. In TUH44, OprD and MexE were not detected, however, in TUH81 the overexpression of MexE and low expression of OprD was observed (data not shown), indicating that TUH81 was *nfxC*-type mutant (30). Furthermore TH5 demonstrated levofloxacin resistance, and had a mutation in the *gyrA* gene (83Thr-Ile), as observed in TUH44.

Efficacy of combined use of AIA-1 and biapenem against carbapenem-resistant *P. aeruginosa*.

We initially performed the killing assay on PAO1 ΔoprD mutant in the presence of AIA-1 at a concentration of 1/4 MIC (32 $\mu\text{g mL}^{-1}$)

Table 2 Susceptibility to antibiotics and AIA-1 for *P. aeruginosa*

Strain	MIC ($\mu\text{g mL}^{-1}$)						
	BIPM	LVFX	TOB	AIA-1	BIPM +AIA-1	LVFX +AIA-1	TOB +AIA-1
PAO1	0.5	0.5	2	128	0.5	0.5	2
PAO1 ΔoprD	8	1	2	128	8	0.5	2
TUH44 ^a	16	16	2	256	16	16	2
TUH81 ^b	8	8	4	128	8	8	4
TH5 ^c	2	32	16	512	4	32	16

^a OprD inactivation by an IS element insertion and a mutation of *gyrA* gene (83Thr-Ile).

^b *nfxC*-type mutant (Overexpression of efflux pump MexE).

^c A mutation of *gyrA* gene (83Thr-Ile)

and biapenem at a concentration of $64 \mu\text{g mL}^{-1}$. The survival rate after 10-h of exposure to biapenem and AIA-1 was approximately 1000-fold lower relative to the survival in the presence of biapenem alone (Figure 2A). In clinical isolates, we performed the killing assays in the presence of AIA-1 at a concentration of $32 \mu\text{g mL}^{-1}$ and $64 \mu\text{g mL}^{-1}$, for TUH44 and TUH81, respectively, and biapenem at a concentration of $128 \mu\text{g mL}^{-1}$. The survival rates after 24-h were approximately 1000-fold lower with the combined use of biapenem and AIA-1 than those with biapenem alone (Figure 2B, C). When AIA-1 was used alone, no killing effects were observed in the tested strains (data not shown).

Efficacy of combined use of AIA-1 and biapenem against mouse infection model by carbapenem-resistant strain, PAO1 $\Delta oprD$.

Next, we focused on investigating the effects of combination use

of biapenem and AIA-1 against biapenem-resistant strain in an *in vivo* mouse infection model. The bioluminescence images obtained in mice infected with PAO1 $\Delta oprD$ - $P_{lac}::lux$ showed no apparent therapeutic effects of the treatment with AIA-1 alone. However, markedly stronger therapeutic effects were observed in mice infected with the PAO1 $\Delta oprD$ - $P_{lac}::lux$ when AIA-1 was used in combination with biapenem than when biapenem alone was administered (Figure 3A, B). These therapeutic effects were confirmed by counting the number of surviving cells in the thigh after 10 h. In the group of combined treatment of AIA-1 and biapenem, the numbers of cells demonstrated the tendency to decrease relative to the ones observed in the group of biapenem alone (Figure 3C). These results suggested that the combination use of biapenem and AIA-1 was efficacious in treating carbapenem-resistant *P. aeruginosa* infections.

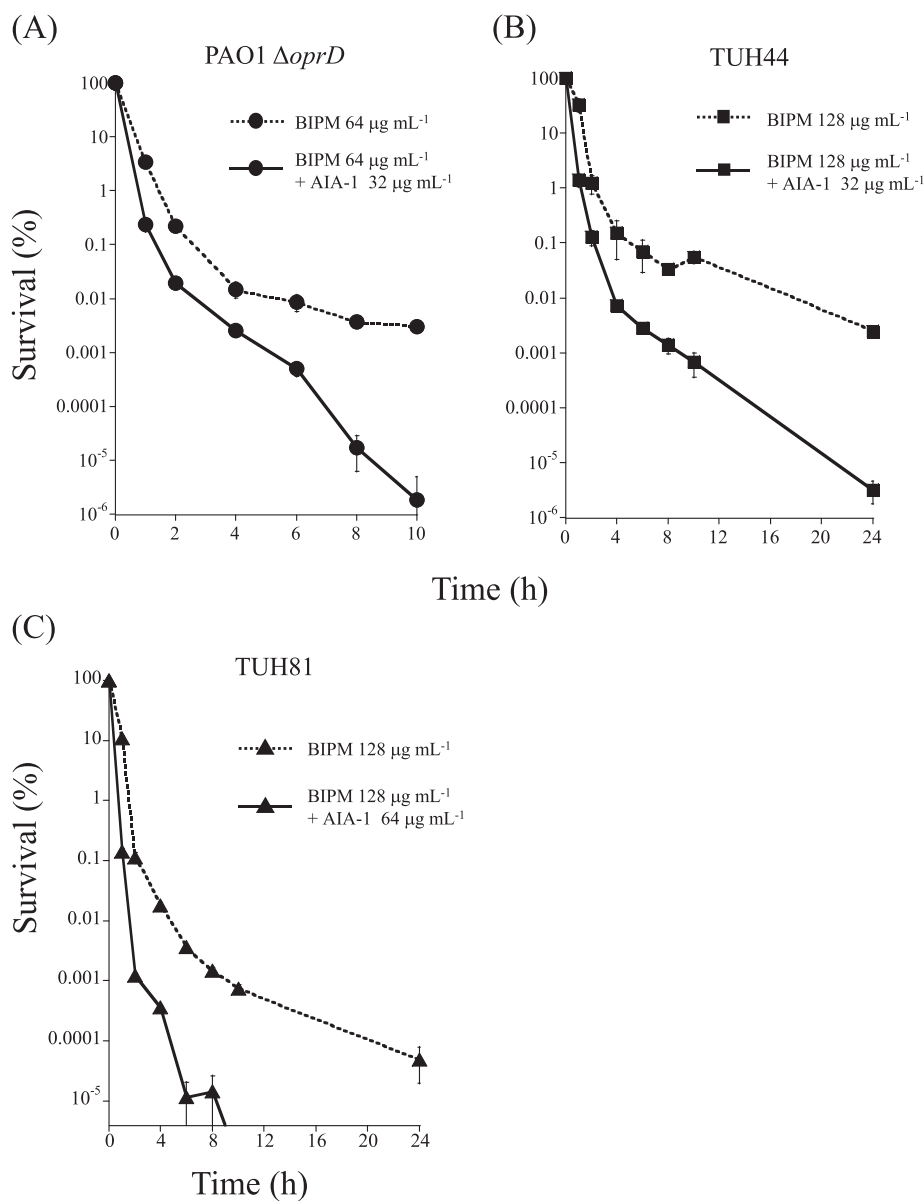


Figure 2. Time kill assays for biapenem-resistant *P. aeruginosa*. (A) Time kill assay for PAO1 $\Delta oprD$ in the presence of biapenem at $64 \mu\text{g mL}^{-1}$ and AIA-1 at $32 \mu\text{g mL}^{-1}$. Biapenem alone; filled circle on the dotted line, biapenem with AIA-1; filled circle on the solid line. (B) Time kill assay for TUH44 in the presence of biapenem at $128 \mu\text{g mL}^{-1}$ and AIA-1 at $32 \mu\text{g mL}^{-1}$. Biapenem alone; filled square on the dotted line, biapenem with AIA-1; filled square on the solid line. (C) Time kill assay for TUH81 in the presence of biapenem at $128 \mu\text{g mL}^{-1}$ and AIA-1 at $64 \mu\text{g mL}^{-1}$. Biapenem alone; filled triangle on dotted line, biapenem with AIA-1; filled triangle on solid line. Under the assumption that the survival at time 0 was 100%, the numbers of CFU were converted to percentages. Experiments were performed in triplicate. Error bars, SDs for three experiments.

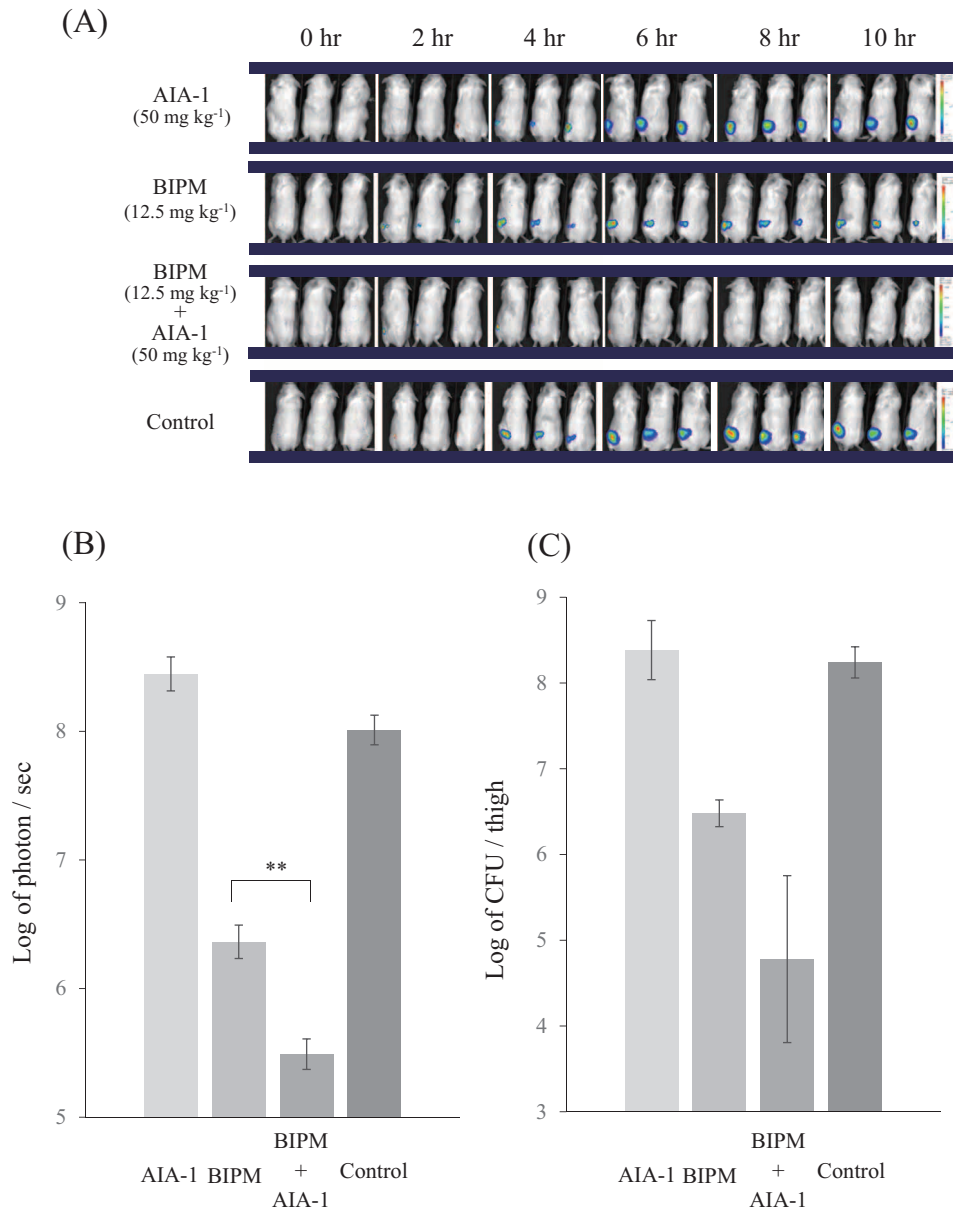


Figure 3. Effects of biapenem and AIA-1 on *in vivo* PAO1 $\Delta oprD-P_{lac}::lux$ bioluminescence signals. PAO1 $\Delta oprD-P_{lac}::lux$ (10^6 CFU) was incubated per thigh. Therapy was performed with biapenem alone or combined with AIA-1, or AIA-1 alone. (A) Representative *in vivo* bioluminescence on color scale images of three mice thighs. (B) Bacterial counts as measured by *in vivo* bioluminescence (photons/second) at 10 hours. (C) Bacterial counts as measured by CFU at 10 hours. **; $P < 0.01$ (Student's *t*-test)

Efficacy of combined use of AIA-1 and levofloxacin or tobramycin against resistant *P. aeruginosa*.

We further investigated whether the observed effects of AIA-1 are also linked to additional mechanisms of antibiotic resistance. Two carbapenem-resistant clinical isolates, TUH44 and TUH81, additionally showed fluoroquinolone resistance (Table 2), therefore we performed the time kill assays for these clinical isolates in the presence of AIA-1 at concentrations of $32 \mu\text{g mL}^{-1}$ and $64 \mu\text{g mL}^{-1}$, for TUH44 and TUH81, respectively, and levofloxacin at a concentration of $64 \mu\text{g mL}^{-1}$. Both TUH44 and TUH81, when treated with levofloxacin and AIA-1, demonstrated the survival rates at the 4-h time point approximately 10-fold lower than those observed for levofloxacin alone (Figure 4A, B). TH5 strain was resistant to tobramycin, therefore we investigated the effects of combination use of tobramycin and AIA-1 by killing assay. The survival rate at

the 6-h time point was approximately 10-fold lower with the combined use of AIA-1 and tobramycin than that with tobramycin alone (Figure 4C).

Susceptibility assay of CRE to antibiotics and AIA-1.

Since AIA-1 enhanced the effects of antibiotics against *P. aeruginosa* resistant strains, we further addressed whether the same effect may be observed in other bacterial species, e.g., CRE. In CRE, the MICs of AIA-1 were 16 or $32 \mu\text{g mL}^{-1}$, demonstrating intermediate antibacterial activity in comparison to that of *P. aeruginosa* (Table 3). In addition, the MICs of carbapenems decreased by 2- to 16-fold in the presence of 1/2 MIC of AIA-1 except for T-CRE1 (Table 3). The 1/2 MIC of AIA-1 did not affect the growth (data not shown), suggesting that AIA-1 may affect the susceptibility to antibiotics in CRE without affecting the growth rate.

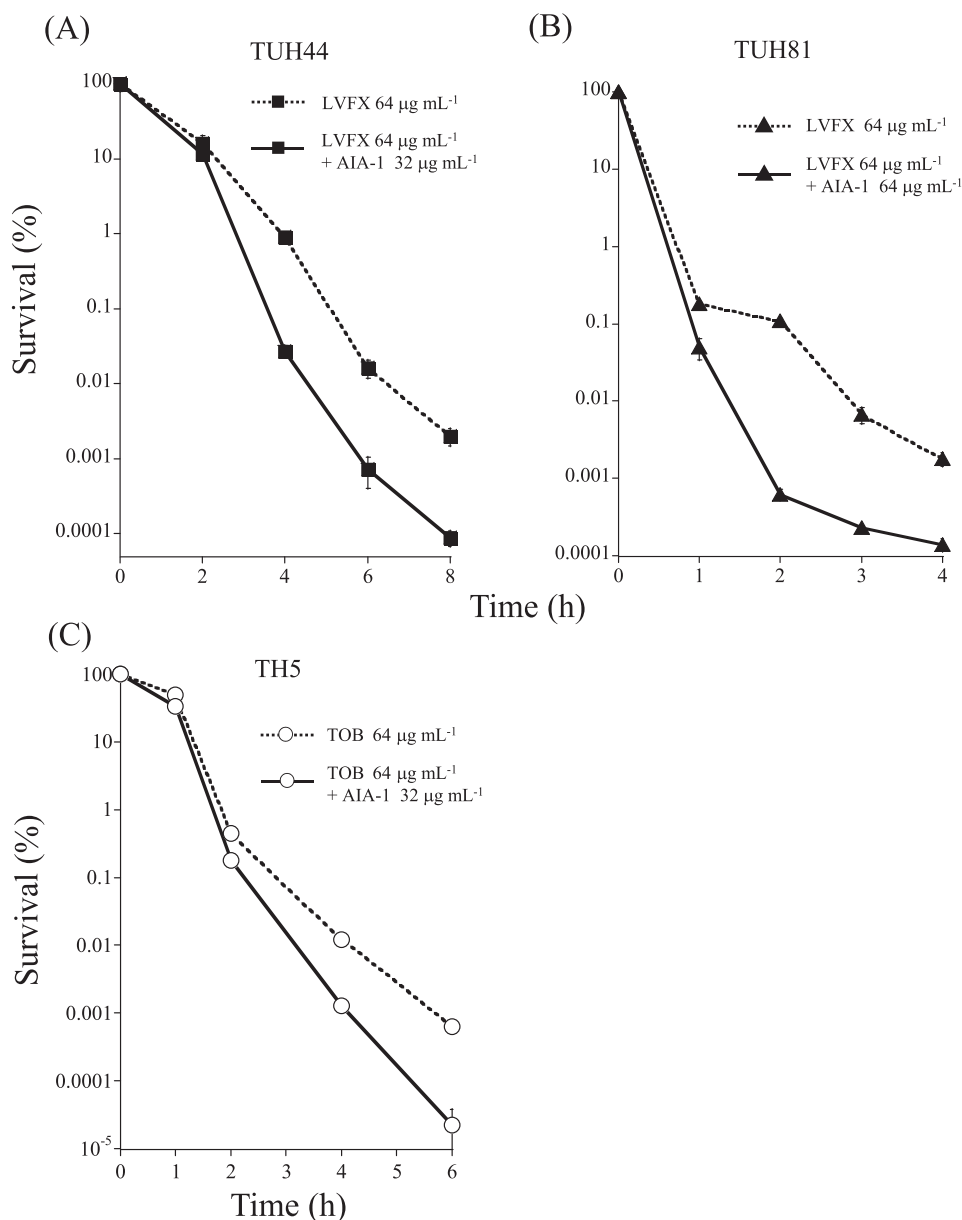


Figure 4. Time kill assays for levofloxacin- and tobramycin-resistant *P. aeruginosa*. (A) Time kill assay for TUH44 in the presence of levofloxacin at 64 µg mL⁻¹ and AIA-1 at 32 µg mL⁻¹. Levofloxacin alone; filled square on the dotted line, levofloxacin with AIA-1; filled square on the solid line. (B) Time kill assay for TUH81 in the presence of levofloxacin at 64 µg mL⁻¹ and AIA-1 at 64 µg mL⁻¹. Levofloxacin alone; filled triangle on the dotted line, levofloxacin with AIA-1; filled triangle on the solid line. (C) Time kill assay for TH5 on the presence of tobramycin at 64 µg mL⁻¹ and AIA-1 at 32 µg mL⁻¹. Tobramycin alone; open circle on dotted line, tobramycin with AIA-1; open circle on solid line. Under the assumption that the survival at time 0 was 100%, the numbers of CFU were converted to percentages. The experiments were performed in triplicate. Error bars, SDs for three experiments.

DISCUSSION

β-lactams (e.g., carbapenems), fluoroquinolones and aminoglycosides are commonly used in treatment of *P. aeruginosa* infections (24, 25). However, these infections are often difficult to cure because *P. aeruginosa* displays multiple intrinsic and acquired mechanisms of antibiotic resistance (31). β-lactam resistance commonly occurs as a result of drug inactivation by β-lactamases, target site alterations, diminished permeability and efflux (30). The production of β-lactamases, such as extended-spectrum β-lactamases (ESBLs) or metallo-β-lactamases, is a major mechanism of β-lactam resistance in Gram-negative bacteria (30). However in *P. aeruginosa*, carbapenem resistance is mostly related to the loss of OprD

and more rarely to carbapenemases (32). An outer membrane protein OprD is a substrate-specific outer membrane porin that facilitates the uptake of carbapenem antibiotics (33), and the loss of OprD significantly reduces the susceptibility to carbapenems (34). Our observations suggest that a mutation in the *oprD* gene in PAO1 Δ*oprD* mutant and *oprD*-inactivated clinical isolate TUH44, resulted in increased biapenem resistance (Table 2). Combination of AIA-1 and biapenem was more efficacious against these *oprD* mutants than treatment with biapenem alone, not only *in vitro* (Figure 2A, B) but also *in vivo* mouse infection model (Figure 3).

P. aeruginosa has 12 resistance-nodulation-division (RND) systems (35, 36), whereof a set of four RND pumps contributes most significantly to antibiotic resistance: MexAB-OprM, MexCD-OprJ,

Table 3 Susceptibility to antibiotics and AIA-1 for Carbapenem-resistant Enterobacteriaceae

	AIA-1 conc ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)				
		BIPM	IPM	LVFX	TOB	AIA-1
T-CRE1	0	0.13	1	0.25	8	16
	8	0.06	1	0.25	8	-
T-CRE2	0	1	2	1	64	32
	16	0.06	0.5	0.25	64	-
T-CRE3	0	1	1	0.06	16	32
	16	0.25	0.5	<0.03	4	-
T-CRE4	0	0.06	0.5	0.06	8	32
	16	<0.03	<0.03	<0.03	<0.03	-
T-CRE5	0	1	2	0.13	16	32
	16	0.06	0.25	<0.03	4	-

MexEF-OprN and MexXY-OprM (36). Strains that overexpress MexEF-OprN, known as *nfxC*-type mutants, exhibit resistance to fluoroquinolones and chloramphenicol (28, 29, 35), and *nfxC*-type mutant additionally displays imipenem resistance because of a transcriptional activator MexT, which positively regulates the expression of MexEF-OprN, and represses the expression of *oprD* gene (1, 24, 37, 38). To establish the effect of AIA-1 on the strain overexpressing MexEF-OprN, we performed the killing assay using a clinical isolate TUH81, an *nfxC*-type mutant. In the killing assays, for all strains except TUH81, the logarithmic phase cultures were used, however MexE is not expressed during the logarithmic phase (38). Therefore, for TUH81, the stationary phase cultures adjusted to approximately 2×10^8 CFU mL^{-1} were used. The combination use of AIA-1 and biapenem also showed stronger efficacy against TUH81 than the use of biapenem alone (Figure 2C). These results suggested that AIA-1 enhanced biapenem activity and the combination use was more efficacious against carbapenem-resistant *P. aeruginosa* than biapenem alone.

Fluoroquinolone resistance is predominantly mediated by mutations in the DNA gyrase and topoisomerase IV enzymes that are the targets for fluoroquinolones (24, 39). Previous studies reported that the main mechanism of fluoroquinolone resistance is mediated through mutations in *GyrA* (25, 26). Alternatively, the above-mentioned four efflux systems which are the members of the RND family are known to adjust their function to fluoroquinolones, and these efflux systems have been involved in fluoroquinolone resistance in clinical isolates (40). TUH44 and TUH81 showed the resistance to levofloxacin because of a mutation in the *gyrA* gene and the overexpression of the efflux pump MexEF-OprN, respectively. The combination use of AIA-1 and levofloxacin demonstrated more pronounced decrease in the survival rates for both TUH44 and TUH81 than those of levofloxacin alone (Figure 4A, B). This observation indicated that AIA-1 in combination with levofloxacin also affects the clinical isolates which express fluoroquinolone resistance.

The mechanisms of aminoglycoside resistance include outer membrane impermeability, active efflux pump and enzymatic modification of the drug (24, 32, 41). In *P. aeruginosa*, the most common aminoglycoside-resistance mechanism is enzymatic modification of the drug by aminoglycoside-modifying enzymes (AMEs) and 16s rRNA methylases, leading to high level of resistance (32). In addition to the mechanism described above, the overexpression of efflux pump MexXY often causes aminoglycoside-resistance in clinical isolate (42), and this efflux pump also accommodates fluoroquinolones (28, 42). A clinical isolate used in this study, TH5, showed intermediate tobramycin and fluoroquinolone resistance (Table 2), suggesting that aminoglycoside-resistance of this

strain was not caused by AMEs or 16s rRNA methylases but by overexpression of MexXY. In the killing assay for TH5, combination of AIA-1 and tobramycin produced a decrease in the survival in comparison to tobramycin alone (Figure 4C).

In this study we have presented a new compound, AIA-1, an analog of *P. aeruginosa* QS autoinducer, which enhanced antibacterial effect of biapenem in *in vitro* time kill assay and *in vivo* mouse infection model against carbapenem-resistant strains (Figure 2, 3). In addition, this compound enhanced antibacterial effect of levofloxacin and tobramycin against fluoroquinolone- and aminoglycoside-resistant clinical isolates (Figure 4). Although AIA-1 is the analog of QS autoinducer, this compound does not inhibit the production of QS-related virulence factors such as elastase and pyocyanin (data not shown), suggesting that the effects of AIA-1 are not mediated through inhibition of QS system. It was reported previously that the efflux pump inhibitors (EPIs) increased bacterial susceptibility to antibiotics when used in combination with antibiotics (7-9). We hypothesized that if AIA-1 acts on EPIs, the MICs of antibiotics would decrease. However, our results show that AIA-1 did not change the MICs (Table 2), implying that AIA-1 probably exerts its effect by affecting the mechanism of antibiotic tolerance in *P. aeruginosa*.

Antibiotic tolerance is the ability of bacteria to survive but not grow under the stress of antibiotics (43). It is not caused by a gene mutation but rather by the presence of a dormant phenotypic variation of gene expression, referred to as persisters (44). Few studies have examined the compounds which decrease antibiotic tolerance, and herein we propose that AIA-1 can serve as a new type of drug that inhibits antibiotic-resistant *P. aeruginosa*.

In carbapenem-resistant Enterobacteriaceae, the addition of AIA-1 produced an increase in the susceptibility to antibiotics (Table 3). Few treatment options are currently available for CRE infections (14, 15), and this observation additionally suggests that AIA-1 may be a new type of drug for treatment of infections caused by CRE.

CONCLUSION

The results presented in this study indicate that a new compound AIA-1, which is an analog of *P. aeruginosa* QS autoinducer, enhances the antibacterial activity of antibiotics against antibiotic-resistant strains by promoting a decrease in antibiotic tolerance in *P. aeruginosa*. On the other hand, AIA-1 also increased the susceptibility of CRE to antibiotics.

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AUTHOR CONTRIBUTIONS

Conceived and designed experiments : TA, KM, RK, HS, HK, YM. Performed the experiments : TA, KM, KeH. Analyzed data : RK, KaH. Wrote the paper : TA, KM, YI, DV. Synthesis of AIA-1 : JI, HS.

DISCLOSURE

The authors report no conflict of interest in this work.

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