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-$C_{12}$-HSL) and BHL ($C_4$-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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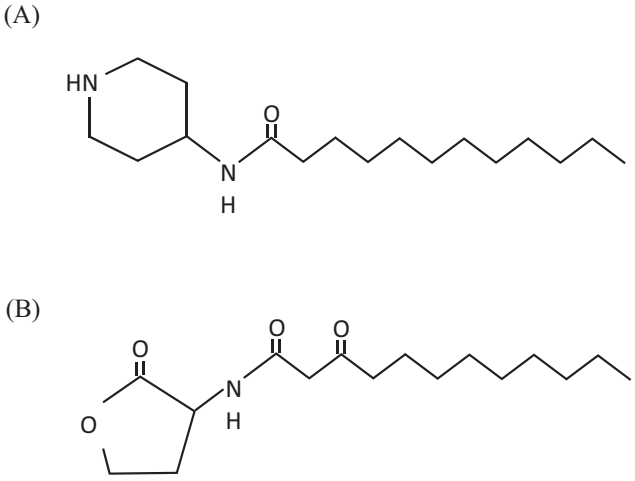


Figure 1. (A) The structure of AIA-1, the analog of OdDHL. (B) The structure of OdDHL, a typical autoinducer of *P. aeruginosa*.

*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 (*oprD*up-F : 5'-CGACTCTAGAGGATCACGCATTCGCCACAGACA-3', *oprD*up-R : 5'-GTCGGTCGATTACAGTTTCATTGTGATTGCTCCTTT-3', *oprD*down-F : 5'-CTGTAATCGACCGACAGG-3', *oprD*down-R : 5'-CCATGATTACGAATTTGAGGAGTCAGCAGGCAA-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 |
|---|---|---|
| *P. aeruginosa* | | |
| PAO1 | Wild type | |
| PAO1 Δ*oprD* | PAO1 in-frame deletion of *oprD* | This study |
| PAO1 Δ*oprD* $P_{lac}$ : : *lux* | One copy of the *lac* promoter linked to the *luxCDABE* gene in the *attB* site of the PAO1 Δ*oprD* chromosome | This study |
| TUH44 | *oprD* : : IS*Ppu12*, *gyrA* (83Thr-Ile) | Clinical isolate |
| TUH81 | *nfxC*-type mutant | Clinical isolate |
| TH5 | *gyrA* (83Thr-Ile) | Clinical isolate |
| Carbapenem-resistant Enterobacteriaceae | | |
| T-CRE1 | *Citrobacter freundii* | Clinical isolate |
| T-CRE2 | *Enterobacter aerogenes* | Clinical isolate |
| T-CRE3 | *Enterobacter aerogenes* | Clinical isolate |
| T-CRE4 | *Enterobacter cloacae* | Clinical isolate |
| T-CRE5 | *Enterobacter aerogenes* | Clinical isolate |
| *E. coli* | | |
| S17-1 | *thi endA recA hsdR* | 20 |
| | Chromosome : : RP4-2Tc : : Mu-Km : : Tn7 | |
| DH5α | $F^-$, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1*, *endA1*, *hsdR*17($r_K^-$, $m_K^+$), *phoA*, *supE44*, $\lambda^-$, *thi*-1, *gyrA*96, *relA*1 | TaKaRa |
| Plasmids | | |
| miniCTX-2 | Plasmid for the integration of genes into the *att* site of the *P. aeruginosa* chromosome, $Tc^R$ | 21 |
| pXen13 | Cloning vector carrying the original *Photorhabdus luminescens luxCDABE* operon | Xenogen |
| pUCP18 | *P. aeruginosa-E. coli* shuttle vector | 22 |
| miniCTX-$P_{lac}$ : : *lux* | One copy of the *lac* promoter linked to the *luxCDABE* gene inserted into miniCTX-2 | This study |
| pFLP2 | FLP recombinase expressing plasmid | 19 |
| pEX18Gm | Broad-host-range suicide vector ; *sacB*, $Gm^R$ | 19 |
| pEX18Gm-*oprD* | *oprD* deletion suicide vector | This study |

backbone (19). Colonies were screened for tetracycline and carbenicillin 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 μg $mL^{-1}$. For CRE, AIA-1 concentrations ranging from 8 to 16 μg $mL^{-1}$ were used.

*Time kill assay*

PAO1 Δ*oprD*, 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 x $10^8$ CFU $mL^{-1}$. For TUH81, the culture was grown to stationary phase, and then, the culture was adjusted 2 x $10^8$ 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 μg $mL^{-1}$ for PAO1 Δ*oprD*, or 128 μg $mL^{-1}$ for TUH44 and TUH81. Levofloxacin was used at a concentration of 64 μg $mL^{-1}$ for TUH44 and TUH81. Tobramycin was used at a concentration of 64 μg $mL^{-1}$ for TH5. AIA-1 was used at concentrations of 32 μg $mL^{-1}$ for PAO1 Δ*oprD*, TUH44 and TH5, and at a concentration of 64 μg $mL^{-1}$ 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 Δ*oprD*-$P_{lac}$ : : *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 μg $mL^{-1}$, demonstrating very weak antibacterial activity against *P. aeruginosa* strains. The MICs of biapenem for PAO1 Δ*oprD*, TUH44 and TUH81 were 8 to 16 μg $mL^{-1}$, 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 μg $mL^{-1}$, 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 (IS*Ppu12*, 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 Δ*oprD* mutant in the presence of AIA-1 at a concentration of 1/4 MIC (32 μg $mL^{-1}$)

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

| Strain | MIC (μg $mL^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 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 |
| PAO1Δ*oprD* | 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 μg $mL^{-1}$. 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 μg $mL^{-1}$ and 64 μg $mL^{-1}$, for TUH44 and TUH81, respectively, and biapenem at a concentration of 128 μg $mL^{-1}$. 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 ΔoprD.*

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 Δ*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 Δ*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.

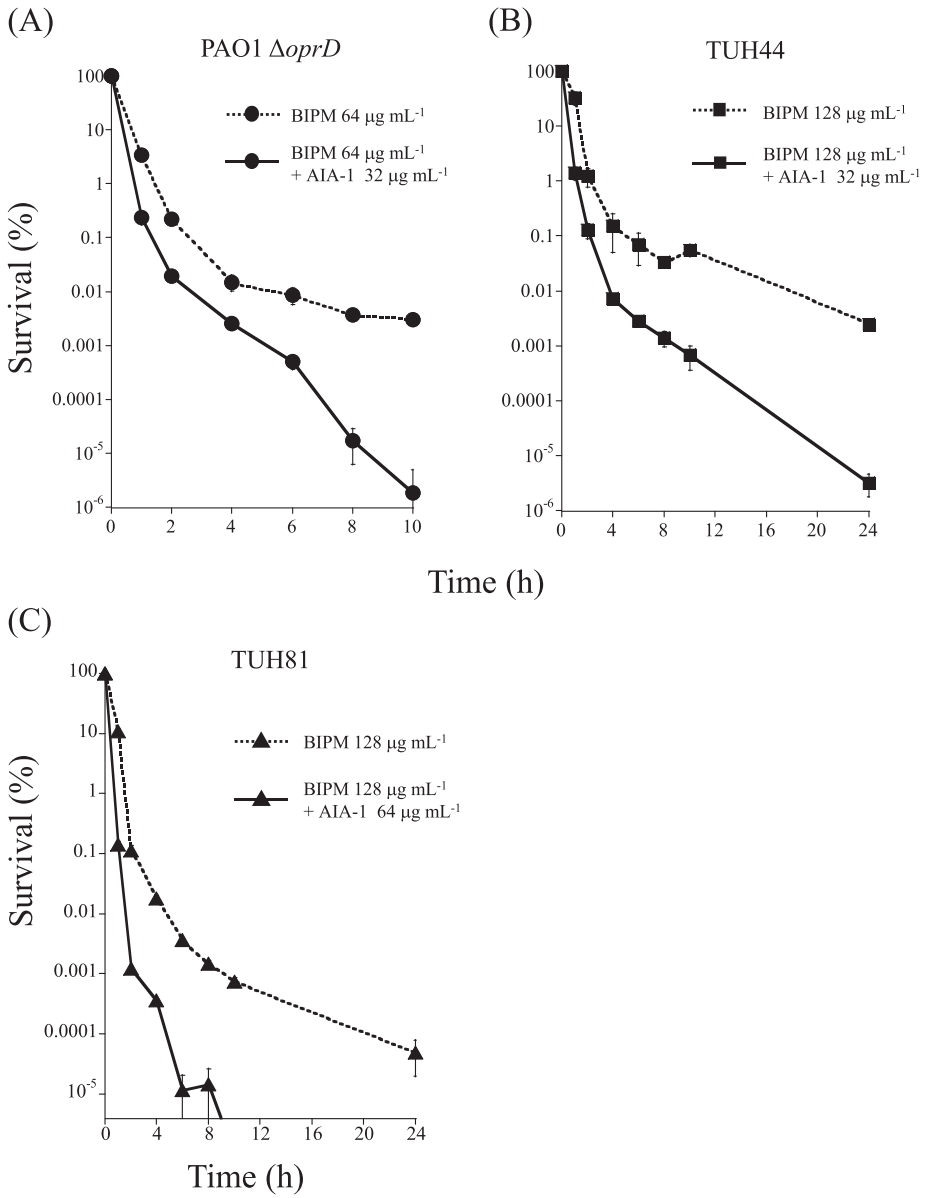


Figure 2. Time kill assays for biapenem-resistant *P. aeruginosa*. (A) Time kill assay for PAO1 Δ*oprD* in the presence of biapenem at 64 μg $mL^{-1}$ and AIA-1 at 32 μg $mL^{-1}$. Biapenem alone ; filled circle on the dotted line, biapenem with AIA-1 ; filled circle on the solid line. (B) Time kill assay for TUH44 in the presence of biapenem at 128 μg $mL^{-1}$ and AIA-1 at 32 μg $mL^{-1}$. 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 μg $mL^{-1}$ and AIA-1 at 64 μg $mL^{-1}$. 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.

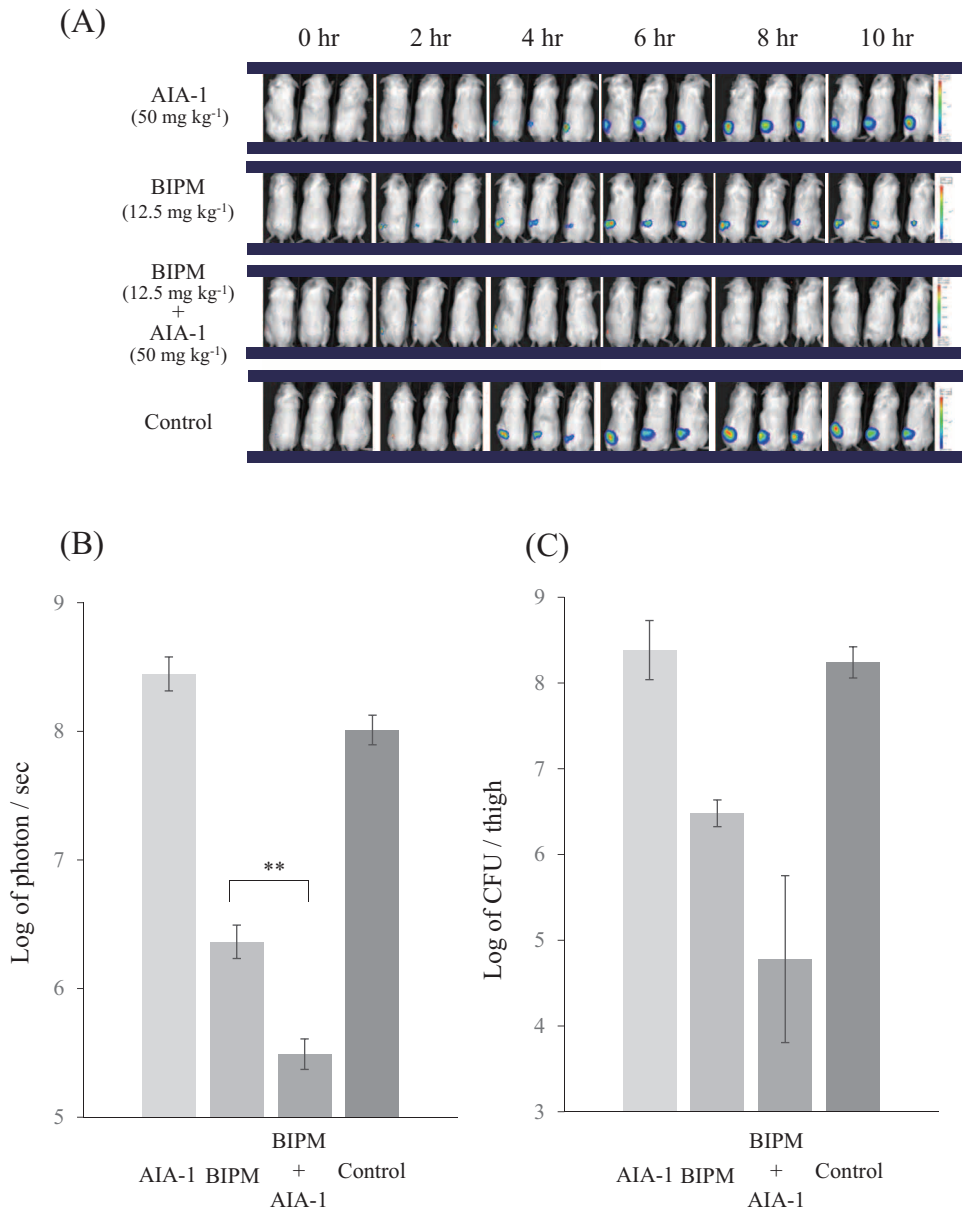


Figure 3. Effects of biapenem and AIA-1 on *in vivo* PAO1 Δ*oprD*-$P_{lac}$ : : *lux* bioluminescence signals. PAO1 Δ*oprD*-$P_{lac}$ : : *lux* ($10^6$ CFU) was incubated per thigh. Therapy was performed with biapenem alone or combined with AIA-1, or AIA-1 alone. (A) Representative *in vivo* bioluminescence 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 μg $mL^{-1}$ and 64 μg $mL^{-1}$, for TUH44 and TUH81, respectively, and levofloxacin at a concentration of 64 μg $mL^{-1}$. 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 μg $mL^{-1}$, 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.

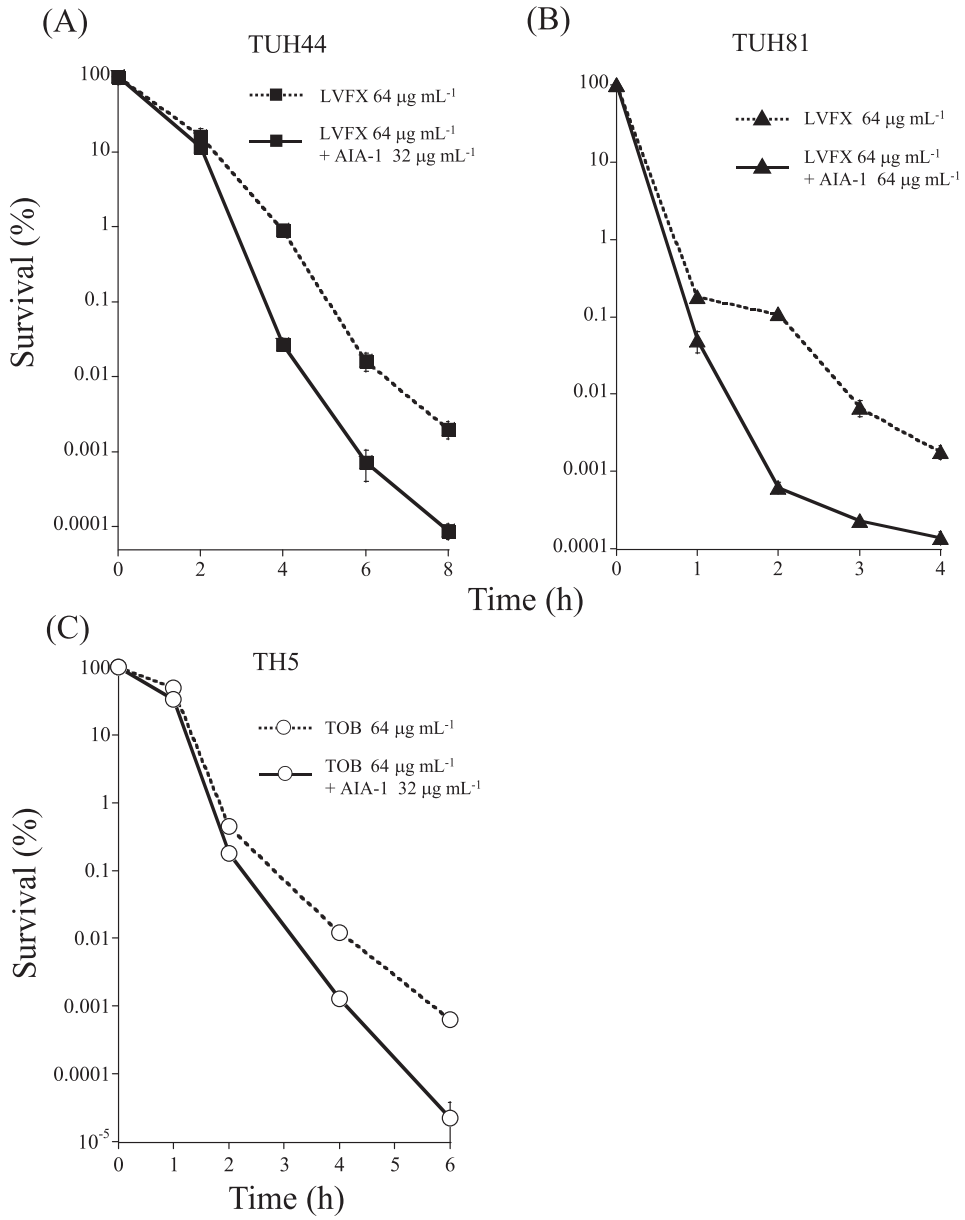


Figure 4. Time kill assays for levofloxacin- and tobramycin-resistant *P. aeruginosa*. (A) Time kill assay for TUH44 in the presence of levofloxacin at 64 μg mL$^{-1}$ and AIA-1 at 32 μg mL$^{-1}$. Levofloxacin alone ; filled square on the dotted line, levofloxacin with AIA-1 ; filled square on the solid 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 (μg mL$^{-1}$) | MIC (μg 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 x $10^8$ 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 abovementioned 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 Enterobacteriacae, 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.

## REFERENCES

1. Köhler T, Epp SF, Curty LK, Pechère JC : Characterization of MexT, the regulator of the MexE-MexF-OprN Multidrug Efflux System of *Pseudomonas aeruginosa*. J Bacteriol 181 : 6300-6305, 1999
2. Fusté E, López-Jiménez L, Segura C, Gainza E, Vinuesa T, Viñas M : Carbapenem-resistance mechanisms of multidrug-resistant *Pseudomonas aeruginosa*. J Med Microbiol 62 : 1317-1325, 2013
3. Lambert PA : Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. J R Soc Med 95(Supple. 41) : 22-26, 2002
4. Tamma PD, Cosgrove SE, Maragakis LL : Combination Therapy for Treatment of Infections with Gram-Negative Bacteria. Clin Microbiol Rev 25 : 450-470, 2012
5. Siqueira VL, Cardoso RF, Caleffi-Ferracioli KR, Scodro RBL, Fernandez MA, Fiorini A, Ueda-Nakamura T, Dias-Filho BP, Nakamura CV : Structural Changes and Differentially Expressed Genes in *Pseudomonas aeruginosa* Exposed to Meropenem-Ciprofloxacin Combination. Antimicrob Agents Chemother 58 : 3957-3967, 2014
6. Krezdorn J, Adams S, Coote PJ : A *Galleria mellonella* infection model reveals double and triple antibiotic combination therapies with enhanced efficacy versus a multidrug-resistant strain of *Pseudomonas aeruginosa*. J Med Microbiol 63 : 945-955, 2014
7. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshino K, Ishida H, Lee VJ : Identification and Characterization of Inhibitors of Multidrug Resistance Efflux Pumps in *Pseudomonas aeruginosa* : Novel Agents for Combination Therapy. Antimicrob Agents Chemother 45 : 105-116, 2001
8. Morita Y, Nakashima K, Nishino K, Kotani K, Tomida J, Inoue M, Kawamura Y : Berberine Is a Novel Type Efflux Inhibitor Which Attenuates the MexXY-Mediated Aminoglycoside Resistance in *Pseudomonas aeruginosa*. Front Microbiol 7 : 1233, 2016
9. Telebi-Taher M, Majidpour A, Gholami A, Rasouli-kouhi S, Adabi M : Role of efflux pump inhibitor in decreasing antibiotic cross-resistance of *Pseudomonas aeruginosa* in a burn hospital in Iran. J Infect Dev Ctries 10 : 600-604, 2016
10. Cheung CY, Chan SY, Yeung CS, Chak WL, Wu TC, Chau KF : Carbapenem Resistant Enterobacteriaceae as a Cause of Peritonitis in a Peritoneal Dialysis Patient. Nephrology 21 : 906-907, 2016
11. Lee HJ, Choi JK, Cho SY, Kim SH, Park SH, Choi SM, Lee DG, Choi JH, Yoo JH : Carbapenem-resistant Enterobacteriaceae : Prevalence and Risk Factors in a Single Community-Based Hospital in Korea. Infect Chemother 48 : 166-173, 2016
12. Xu Y, Gu B, Huang M, Liu H, Xu T, Xia W, Wang T : Epidemiology of carbapenem resistant *Enterobacteriaceae* (CRE) during 2000-2012 in Asia. J Thorac Dis 7 : 376-385, 2015
13. Netikul T, Kiratisin P : Genetic Characterization of Carbapenem-Resistant *Enterobacteriaceae* and Spread of Carbapenem-Resistant *Klebsiella pneumonia* ST340 at a University Hospital in Thailand. PLoS ONE 10 : e0139116, 2015
14. Morrill HJ, Pogue JM, Kaye KS, LaPlante KL : Treatment Options for Carbapenem-Resistant Enterobacteriaceae Infections. Open Forum Infect Dis 2 : ofv050, 2015
15. van Duin D, Kaye KS, Neuner EA, Bonomo RA : Carbapenem-resistant Enterobacteriaceae : a review of treatment and outcomes. Diagn Microbiol Infect Dis 75 : 115-120, 2013
16. Rutherford ST, Bassler BL : Bacterial Quorum Sensing : Its Role in Virulence and Possibilities for Its Control. Cold Spring Harb Perspect Med 2 : a012427, 2012
17. Smith KM, Bu Y, Suga H : Induction and Inhibition of *Pseudomonas aeruginosa* Quorum Sensing by Synthetic Autoinducer Analogs. Chem Biol 10 : 81-89, 2003
18. Smith KM, Bu Y, Suga H : Library Screening for Synthetic Agonists and Antagonists of a *Pseudomonas aeruginosa* Autoinducer. Chem Biol 10 : 563-571, 2003
19. Hoang TT, Karkhoff-Schweizer RAR, Kutchma AJ, Schweizer HP : A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences : application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212 : 77-86, 1998
20. Simon R, Priefer U, Puhler A : A broad host range mobilization system for in vivo genetic engineering transposon mutagenesis in gram negative bacteria. Nat Biotechnol 1 : 784-791, 1983
21. Hoang TT, Kutchma AJ, Becher A, Schweizer HP : Integration-Proficient Plasmids for *Pseudomonas aeruginosa* : Site-Specific Integration and Use for Engineering of Reporter and Expression Strains. Plasmid 43 : 59-72, 2000
22. Schweizer HP : *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene 97 : 109-112, 1991
23. Taniguchi K, Ono T, Murakami K, Viducic D, Kayama S, Hirota K, Nemoto K, Miyake Y : Novel *Pseudomonas aeruginosa* Gene That Suppresses Tolerance to Carbapenems. Antimicrob Agents Chemother 47 : 2997-3001, 2003
24. Poole K : *Pseudomonas aeruginosa* : resistance to max. Front Microbiol 2 : 65, 2011
25. Cambau E, Perani E, Dib C, Petinon C, Trias J, Jarlier V : Role of Mutation in DNA Gyrase Gene in Ciprofloxacin Resistance of *Pseudomonas aeruginosa* Susceptible or Resistant to Imipenem. Antimicrobiol Agents Chemother 39 : 2248-2252, 1995
26. Higgins PG, Fluit AC, Milatovic D, Verhoef J, Schmitz FJ : Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. Int J Antimicrob Agents 21 : 409-413, 2003
27. Weile J, Schmid RD, Bachmann TT, Susa M, Knabbe C : DNA microarray for genotyping multidrug-resistant *Pseudomonas aeruginosa* clinical isolates. Diagn Microbiol Infect Dis 59 : 325-338, 2007
28. Aeschlimann JR : The Role of Multidrug Efflux Pumps in the Antibiotic Resistance of *Pseudomonas aeruginosa* and Other Gram-Negative Bacteria. Pharmacotherapy 23 : 916-924, 2003
29. Maseda H, Yoneyama H, Nakae T : Assignment of the Substrate-Selective Subunits of the MexEF-OprN Multidrug

Efflux Pump of *Pseudomonas aeruginosa*. Antimicrobial Agents Chemother 44 : 658-664, 2000
30. Poole K. Resistance to β-lactam antibiotics : Cell Mol Life Sci 61 : 2200-2223, 2004
31. Tenover FC : Mechanisms of antimicrobial resistance in bacteria. Am J Med 119(6 Supple 1) : S3-10 ; discussion S62-70, 2006
32. Potron A, Poirel L, Nordmann P : Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumanii* : Mechanisms and epidemiology. Int J Antimicrob Agents 45 : 568-585, 2015
33. Sanbongi Y, Shimizu A, Suzuki T, Nagaso H, Ida T, Maebashi K and Gotoh N : Classification of OprD sequence and correlation with antimicrobial activity of carbapenem agents in *Pseudomonas aeruginosa* clinical isolates collected in Japan. Microbiol Immunol 53 : 361-367, 2009
34. Fang ZL, Zhang LY, Huang YM, Qing Y, Cao KY, Tian GB, Huang X : OprD mutations and inactivation in imipenem-resistant *Pseudomonas aeruginosa* isolates from China. Infect Genet Evol 21 : 124-128, 2014
35. Lister PD, Wolter DJ, Hanson ND : Antibacterial-Resistant *Pseudomonas aeruginosa* : Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. Clin Microbiol Rev 22 : 582-610, 2009
36. Dreier J, Ruggerone P : Interaction of antibacterial compounds with RND efflux pumps in *Pseudomonas aeruginosa*. Front Microbiol 6 : 660, 2015
37. Ochs MM, McCusker MP, Bains M, Hancock REW : Negative Regulation of the *Pseudomonas aeruginosa* Outer Membrane Porin OprD Selective for Imipenem and Basic Amino Acis. Antimicrob Agents Chemother 43 : 1085-1090, 1999
38. Maseda H, Uwate M, Nakae T : Transcriptional regulation of the *mexEF-oprN multidrug* efflux pump operon by MexT and an unidentified repressor in *nfxC*-type mutant op *Pseudomonas aeruginosa*. FEMS Microbiol Lett 311 : 36-43, 2010
39. Hooper DC, Jacoby GA : Mechanisms of drug resistance : quinolone resistance. Ann N Y Acad Sci 1354 : 12-31, 2015
40. Poole K : Efflux-Mediated Resistance to Fluoroquinolones in Gram-Negative Bacteria. Antimicrob Agents Chemother 44 : 2233-2241, 2000
41. Bonomo RA, Szabo D : Mechanisms of Multidrug Resistance in *Acinetobacter* Species and *Pseudomonas aeruginosa*. Clin Infect Dis 43 (Supple 2) : S49-S56, 2006
42. Guénard S, Muller C, Monlezun L, Benas P, Broutin I, Jeannot K, Plésiat P : Multiple Mutations Lead to MexXY-OprM-Dependent Aminoglycoside Resistance in Clinical Strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 58 : 221-228, 2014
43. Handwerger S, Tomasz A : Antibiotic tolerance among clinical isolates of bacteria. Ann Rev Pharmacol Toxicol 25 : 349-80, 1985
44. Lewis K : Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5 : 48-56, 2007