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수의학석사학위논문

Effect of a synthetic antimicrobial peptide RP557 on carbapenem-resistant *Pseudomonas aeruginosa* biofilm

합성 항균펩타이드 RP557 이 카바페넴 내성 *Pseudomonas aeruginosa* 바이오필름에 미치는 효과

2023 年 2 月

서울대학교 대학원

수의학과

수의병인생물학 및 예방수의학 전공

송운기

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By

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February, 2023

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(Major: Veterinary Pathobiology and Preventive medicine)
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A Dissertation
Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master

To the Faculty of College of Veterinary Medicine
Department of Veterinary Medicine
The Graduate School
Seoul National University

By

Song Yunqi

2022

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Abstract

Effect of a synthetic antimicrobial peptide RP557 on carbapenem-resistant *Pseudomonas aeruginosa* biofilm

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Pseudomonas aeruginosa infection has become a global problem because of its increasing antibiotic resistance. As a typical biofilm-forming bacteria, *P. aeruginosa* biofilm infection is often found in nosocomial infections in immunodeficient patients and chronic lung infections in cystic fibrosis infections. Both of these characteristics make *P. aeruginosa* biofilm infection refractory. Therefore, many alternative compounds have been developed. Among them, antimicrobial peptides have attracted much attention. A newly synthesized peptide, RP557, derived from human cathelicidin LL- 37 was

evaluated for its antimicrobial and antibiofilm effect toward carbapenem-resistant *P. aeruginosa.* The results showed that regardless of the resistance to carbapenems, the minimal inhibition concentrations of RP557 and LL-37 against *P. aeruginosa* were 32 μg/ml and 256 μg/ml, respectively. Both RP557 and LL-37 were able to inhibit *P. aeruginosa* biofilm formation at sub-MICs (p <0.05), while sub-MICs of carbapenems induced biofilm formation (p <0.05). RP557 could also remove approximately 50% of the mature biofilm at a concentration of 64 μg/ml (p <0.05), while 256 μg/ml LL-37 was needed to remove it (p <0.05). A quarter MIC of RP557 and LL-37 were used together with carbapenems (ertapenem, imipenem, and meropenem). The results show that both RP-557 and LL-37 might increase the susceptibility of CRPA at 4~16 times. Significant gene expression level changes were observed in RP557- or LL-37-treated CRPA (p <0.05). Confocal images showed that biofilm structures were significantly reduced in the LL-37- or RP557-treated groups (p <0.05), and biofilm cell viability was significantly repressed. Therefore, RP557 and its structural origin, LL-37, could be potential treatments for carbapenem-resistant *P. aeruginosa* infection, especially for chronic biofilm infection.

Keywords: *Pseudomonas aeruginosa*, carbapenem resistance, antimicrobial peptide, biofilm

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Contents

List of figures

List of tables

List of abbreviations

AMP	Antimicrobial peptides
CAMHB	Cation-adjusted Muller Hinton broth
cDNA	Complementary DNA
CF	Cystic fibrosis
CLSI	Clinical laboratory standards institute
CPRA	Carbapenem-resistant *Pseudomonas aeruginosa*
eDNA	Exocellular DNA
ICU	Intensive Care Unit
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
OD	Optical density
PBS	phosphate buffered saline
PI	propidium iodide
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing

1. Introduction

Bacteria on the solid surface often grow as biofilm forms, with multiple compositions in the biofilm matrix including exocellular DNA, exopolysaccharide, and proteins [1]. Biofilm is considered to be the survival strategy of bacteria to resist tough environmental conditions such as pH, temperature and also, to resist the immune system during infection. Bacteria growing in biofilm form often have higher resistance than planktonic forms cells [2, 3]. The antibiotic resistance of biofilm is a comprehensive system including the substance of the matrix, the gene expression network, and the different metabolism inside biofilm [4]. Biofilm matrix delays the penetration of antibiotics because the anionic substance within the biofilm can bind positively charged antibiotics such as aminoglycosides, making them less likely to penetrate bacterial cells [2]. Another important mechanism of drug resistance in biofilms is the cellular heterogeneity within the biofilm. The mature biofilm of *P. aeruginosa* biofilm has a typical mushroom-like structure, and persister cells exist at its stalk portion that expression levels of virulence production upregulate and is less susceptible to antibiotics [5]. It has been presumed that persister cells are the main cause of chronic infections because their inactive metabolism makes them less sensitive to antibiotics aimed at bacteria cells in the log phase, while they can switch to metabolically active cells when the antibiotics in the environment are cleared [6].

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen that often causes nosocomial infection, with approximately 16.2% of ICU infections

being caused by *P. aeruginosa* [7]. As typical biofilm-forming bacteria, *P. aeruginosa* biofilms often causes wounds infection, medical device-related infections, and chronic lung infections in cystic fibrosis patients [8]. CF patients lung infection is usually caused by *P. aeruginosa* mucoid strains whose biofilm contains a large amount of alginate, while other infections are more likely caused by non-mucoid strains [9]. In addition to its intrinsic resistance to β-lactam antibiotics, development of resistance to carbapenems makes *P. aeruginosa* infection harder to cure. The carbapenem resistant mechanism of *P. aeruginosa* are similar to other carbapenem-resistant gram-negative bacteria. It can be divided into 4 types: overexpression of efflux pumps, porin mutation, derepressing the intrinsic β-lactamase and production of carbapenemase. Among them, mutations in porins are the main mechanism of carbapenem resistance in *P. aeruginosa*, especially to imipenem [6, 10, 11]. Combined with its strong biofilm forming ability, CRPA results in higher mortality than carbapenem resistant Enterobacteriaceae [12]. Thus, new therapeutic compounds should be developed for treating CRPA infection.

Antimicrobial peptides (AMPs) are peptides displaying promising antimicrobial effects. They can be naturally produced by humans, animals, plants and fungi. Most AMPs are positively charged and achieve their antimicrobial effect by disrupting microorganism cell membranes through electrostatic interactions with anionic phospholipids, which further leads to cell death [13, 14]. The antimicrobial effect of AMP can be enhanced by adjusting its structure, which provides a basis for the synthesis of new AMPs. LL-37 is the only cathelicidin secreted in humans and is often found in

mucosal surfaces. LL-37 has been shown to have antimicrobial effects by disrupting the lipid bilayer. However, compared to its moderate antimicrobial effect on planktonic cells, LL-37 has an outstanding effect on biofilms that reduces biofilm formation at sub- MICs [15, 16]. Thus, many antimicrobial peptides have been designed based on LL-37. RP557 is a novel synthetic peptide derived from LL-37 and Tachyplesin 1. It has a broad-spectrum antibacterial and anti-biofilm effect against common gram-negative and gram-positive bacteria and even fungi. RP557 is reported to have low cytotoxicity and to be less likely to develop resistance in *P. aeruginosa* and *S. aureus* [17]. In another study, RP557 was found to be effective in the biofilm of multidrug-resistant *Mycobacterium abscessus* and can increase susceptibility to other antibiotics when coadministrated [18].

However, the effect of RP557 and LL-37 on CRPA have not been studied yet. Therefore, RP557 and LL-37 were assessed for their antimicrobial effect, biofilm inhibition effect and biofilm removing effect in the current study. Consequently, to provide new therapeutic options for treating CRPA, the synergetic effect of RP557 or LL-37 with carbapenems was assessed. To further reveal the mechanism of RP557 and LL-37 on CRPA biofilm, the gene expression level of biofilm formation-related genes was measured and analyzed by qPCR. And to assess the effect of RP557 and LL-37 on biofilm structure, confocal laser scanning microscopy images were taken.

2. Materials and Methods

2.1. Bacterial strains, growth media, and culture conditions

Pseudomonas aeruginosa reference strain ATCC 27853 was obtained from the American Type Culture Collection (Manassas, VA, USA). Carbapenem-resistant *P. aeruginosa* strain 16079 was obtained from Korean National Culture Collection for Pathogens (NCCP, Cheongju, South Korea). Other Carbapenem-resistant *P. aeruginosa* strains were sampled from companion animals by veterinarians and preserved in the infectious disease laboratory (Seoul National University, Seoul, South Korea). Bacteria were maintained on tryptic soy agar during the experimental process (BD, Franklin Lakes, USA) and enriched in tryptic soy broth (BD, Franklin Lakes, USA).

2.2. Antibiotics and antimicrobial peptide

Meropenem and imipenem were purchased from Sigma Aldrich (Saint Louis, MO, USA). Ertapenem was purchased from European Pharmacopoeia Reference Standard (Strasbourg Cedex, France). Antimicrobial peptides LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and RP557 (RFCWKVCYKGICFKKCK) were synthesized by GL Biochem (Shanghai,

China) and purified to 95% by high-performance liquid chromatography. Lyophilized peptide was stored at -20 °C until use.

2.3. Minimal inhibitory concentration assays

The MIC assay was performed following the guidelines from the Clinical and Laboratory Standards Institute with slight modifications [4]. *P. aeruginosa* strains were cultured on tryptic soy agar before the experiment. A single colony was picked and inoculated to 10 ml tryptic soy broth and incubated at 37 °C overnight. After enrichment, bacteria suspension was diluted to approximately 1×10^6 CFU/ml. Carbapenem and peptides were dissolved in cation-adjusted muller hinton broth (BD, Franklin Lakes, USA) with a starting concentration of 2048 μg/ml and 512 μg/ml, respectively. An aliquot of 100 μl solution was added to a 96-well U-bottom microplates (Greiner bio-one, Frickenhausen, Germany) and make a 2-fold serial dilution to 0.25 μg/ml. Then 50 μl bacteria suspension was inoculated into each well. Growth control was made of 50 μl bacteria suspension and 50 μl sterile CAMHB, and a column containing 100 μl sterile CAMHB was used as sterility control. After inoculation, the initial OD_{600} was measured using VersaMax absorbance microplate reader (Molecular Devices, California, USA). The plates were incubated at 37 °C, overnight. After incubation, the final OD_{600} was measured and the minimal inhibition concentration was determined by OD_{600} value increase less than 0.005. The assay was performed in triplicate with each sample performed in triplicate wells.

2.4. Biofilm inhibition assay

To assess the effect of RP557, LL-37, and carbapenems on *P. aeruginosa* biofilm, biofilm inhibition assays were performed using the microtiter plate method and crystal staining method previously described with slight modifications [19]. Briefly, *P. aeruginosa* overnight culture was prepared using TSB and diluted using CAMHB to a concentration of approximately 1×10^6 CFU/ml. Twofold diluted solutions of LL-37 and RP557 with concentrations from 512 μg/ml to 0.25 μg/ml were prepared. Ertapenem, imipenem ranging from 512 μg/ml to 1 μg/ml, and meropenem from 256 μg/ml to 0.5 μg/ml were prepared on 96-well U-bottom microtiter plates. Aliquot of 50 μl bacterial suspension was inoculated into each well. Wells without peptides were used as growth control and wells with 100 μl sterile CAMHB were used as sterility control. Plates were covered and sealed and incubated at 37 ℃ for 24 hours without agitation to form mature biofilm. After biofilm formed, the bacterial suspension was discarded and wells were washed with 150 μl sterile phosphate buffered saline (PBS) 3 times to wash out unattached bacteria cells. Each well was stained with 120 μl 0.1% crystal violet dye for 30 min. After staining, the dye was discarded, and wells were washed with distilled water 3 times to wash out excess dye. 120 μl 100% ethanol was added to each well for dissolving the dye. After incubation for 20 min, optical absorbance at 570 nm was measured. Biofilm mass was normalized by comparing it to growth control group and represented as a percentage. All assays were performed in triplicate with each sample

performed in triplicate wells.

2.5. Biofilm removal assay

Overnight cultures of *P. aeruginosa* were prepared and diluted to approximately 1×10^6 CFU/ml. An aliquot of 100 μl of bacterial suspension was inoculated into each well and incubated at 37 °C for 24 h to establish a mature biofilm. Then the suspension was discarded and each well was washed three times using PBS to wash out unattached cells. LL-37 and RP557 were dissolved in CAMHB at concentrations ranging from 0.25 μg/ml to 256 μg/ml. Each well was loaded with peptide solution and further incubated at 37 °C for 24 h without agitation. The remaining biofilm mass was evaluated by 0.1% crystal violet staining followed by measurement of optical absorbance at 570 nm. Biofilm mass was normalized by comparison to the growth control group and represented as a percentage. The experiments were performed in triplicate, and each sample performed in triplicate wells.

2.6. Evaluation of the synergetic effect between antimicrobial peptides and carbapenems

To assess the combined effect of antimicrobial peptides and carbapenems, 25 μl peptide solution at the MIC and 25 μl of carbapenem solution with concentrations ranging from 1 μg/ml to 2048 μg/ml were loaded onto 96-well plates. Overnight cultures of *P. aeruginosa* were diluted to 1×10^6 CFU/ml

with CAMHB, and 50 μl of bacterial suspension was loaded into 96-well plates. The final concentration of peptide was 1/4 MIC. Plates were covered and sealed following incubation at 37 ℃ for 24 h without agitation. After incubation, the final OD_{600} was measured and minimal inhibition concentration was decided by wells with OD_{600} value increase of less than 0.005. The assay was performed in triplicate with each sample performed in triplicate wells.

2.7. Total RNA extraction of *P. aeruginosa*

Overnight cultures= of *P. aeruginosa* was diluted to 1×10^6 CFU/ml with CAMHB and 50 μl of bacteria suspension was added to 96-well plates. Fifty microliters of LL-37 and RP557 solution at half MIC was added to each well to reach a final concentration of a quarter MIC. Wells added with 50 μl sterile CAMHB instead of peptide solution were used for growth control. The plates were covered and incubated at 37 ℃ for 24 h without agitation. After incubation, total RNA was extracted using RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany). RNA purity was assessed using absorbance ratios at 260/280 nm and 260/230 nm. Samples with ratio 1.9 ~ 2.1 at 260/280 nm and 2.0~2.2 at 260/230 nm were qualified to proceed cDNA synthesis. The complementary DNA of the extracted RNA was synthesized using High-Capacity cDNA Reverse Transcript Kit (Applied Biosystems, Lithuania, UAB). The experiments were performed in triplicate. All samples were stored at -80 ℃ until further analysis was taken.

2.8. Quantitative real-time PCR

The expression level of genes related to biofilm formation was assessed by qPCR using Rotor-Gene Q (Qiagen, Hilden, Germany). The 20 µl reaction system consisted of 10 µl EzAmp qPCR 2X master mix (SYBR Green, Low Rox, Daejeon, South Korea), 2 µg of cDNA, 10 pmol of forward primer, 10 pmol of reverse primer and ultrapure water (MilliQ, Darmstadt, Germany) to a total volume of 20 µl. The cycling conditions of qPCR was as followed: initial denaturation at 94 ℃ for 3 min, followed by 40 cycles of 15 s at 94 ℃, 25 s at 56 ℃ and 30 s at 72 ℃. The melting curve was obtained from 60 ℃ to 95 ℃. Housekeeping gene *rpoD* was used for normalization. The relative changes in expression level were calculated using the $2^{-\Delta\Delta ct}$ method. The primers used in this study were listed in Table.1

Table 1. Primers used in this study

Gene	Gene description		Forward (5'-3')	Reverse (5'-3')	Reference
lasI	Acyl-homoserine-lactone synthase	Quorum sensing system *las*	CTACAGCCTGCAGAACGACA	ATCTGGGTCTTGGCATTGAG	[20]
lasR	Transcriptional regulator LasR		ACGCTCAAGTGGAAAATTGG	GTAGATGGACGGTTCCCAGA	[20]
lasA	Protease LasA		CTGCTGGCTTTCAAGGTTTC	CCAGCAAGACGAAGAGGAAC	[21]
rhlA	Rhamnosyltransferase subunit A	Quorum sensing system *rhl*	AGCTGGGACGAATACACCAC	GACTCCAGGTCGAGGAAATG	[21]
rhlB	Rhamnosyltransferase subunit B		GAGCGACGAACTGACCTACC	CGTACTTCTCGTGAGCGATG	[21]
rhlI	Acyl-homoserine-lactone synthase		AAACCCGCTACATCGTCGC	TCTCGCCCTTGACCTTCTGC	[22]
pslB	Sugar-nucleotide production protein PslB	Exopolysaccharide *psl* synthesis	CAACGAATCCACCTTCATCC	ACTCGCCGCTCTGTACCTC	[22]
pslD	Outer membrane protein/secretion protein PslD		CGCTATCACGCCTACTTCCT	GACTTGGGCACGAAGACGAT	This study
pelB	Outer membrane protein/secretion protein PelB	Exopolysaccharide *pel* synthesis	AGCGCTTGCAACAGATTCTC	AACAGGTTCCAGTGGGTTTC	This study
algA	Sugar-nucleotide production AlgA	Alginate synthesis	CCATGATGATCGCCCACAAG	ACCTCGCAGTGGTTCTGGGT	[23]
alg44	c-di-GMP binding Alg44		CCACCAGATGAAAGGGACC	AAGATCACCTGGCCCTTGTT	This study
algD	GDP-mannose 6-dehydrogenase AlgD		CGCCGAGATGATCAAGTACA	AGGTTGAGCTTGTGGTCCTG	[24]
algE	Alginate production protein AlgE		GTGGCAGGACACCAACATC	GTGCGGTATTCGCTGAAACG	This study
algK	Outer membrane protein/secretion protein AlgK		CTCAAGCGCGAACAACAGAG	AACGGGAGCTGTTCATAGGC	This study
rpoD	RNA polymerase sigma factor RpoD (housekeeping gene)	Housekeeping gene	GCGACGGTATTCGAACTTGT	CGAAGAAGGAAATGGTCGAG	[24]

2.9. Confocal laser scanning microscopy analysis

P. aeruginosa overnight cultures were prepared and diluted to 1×10^6 CFU/ml. An aliquot of 125 μl bacterial suspension was cultured with 125 μl LL-37 and RP557 solution at concentration of a quarter MIC in 8-well chambered glass (LAB TEK, USA), respectively. The chambers were cultured at 37 ℃ for 48 h to form mature biofilms. After incubation, the bacterial suspension was discarded and the biofilm was washed using 0.85% saline 3 times. Biofilm was stained with FilmTracer LIVE/DEAD Biofilm Viability Kit (Life Technologies Corporation, Eugene, USA) for 20 min away from light. Then the dye was discarded and each well was rinsed to remove the excess dye. Biofilms were examined by confocal laser scanning microscopy on ZEISS LSM 800 (ZEISS, Germany) using 20 × objective. Live cells were stained green by SYTO 9 and dead cells were stained red by propidium iodide. Cell viability was determined by the intensity of red fluorescence and green fluorescence using ImageJ (NIH, Maryland, USA).

2.10. Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's T3 method by GraphPad Prism 9.4 (GraphPad Software, San Diego, USA). Data are presented as the mean ± SD, and significance was established at $p < 0.05$.

3. Results

3.1. MICs of carbapenems, LL-37 and RP557 against carbapenem-resistant *P. aeruginosa*

The minimal inhibitory concentrations of carbapenems and antimicrobial peptides were determined by the microtiter dilution method according to CLSI guideline, 30th edition. Except for reference strain ATCC 27853, all strains were resistant to carbapenems. Among the carbapenem-resistant *P. aeruginosa* strains, NCCP 16079 isolated from human patients was extremely resistant to carbapenems, with MICs against ertapenem and meropenem greater than 2048 μg/ml. For other strains isolated from companion dogs, the MIC against carbapenems were 16 times larger than the resistance criteria (Table 2). Peptide LL-37 was able to inhibit ATCC 27853 and other CRPA growth at 256 μg/ml, and RP557 was able to inhibit the reference strain and most CRPA at 32 μg/ml.

Table 2. Minimal Inhibitory Concentrations (MICs, μg/ml) of Carbapenem-Resistant *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa Strain	Minimal Inhibitory Concentrations (μg/ml)				
	Ertapenem	Imipenem	Meropenem	LL-37	RP557
ATCC 27853	4	4	0.5	256	32
NCCP 16079	>2048	256	>2048	256	32
D7	128	64	32	256	32
D16	128	64	32	256	32
D25	128	256	32	256	32
D26	128	64	64	256	32
B4	128	128	32	256	32

3.2. Biofilm inhibition effect of LL-37 and RP557

Concentrations ranging from 512 μg/ml to 1 μg/ml of LL-37 and from 128 μg/ml to 0.25 μg/ml were used to assess the biofilm inhibition effect. LL-37 was able to inhibit biofilm formation of all 7 strains at a concentration lower than the MIC (Figure 1). For RP557, biofilm formation of 6 strains was inhibited at sub-MIC, except for D16, in which biofilm formation was inhibited at the MIC of 32 μg/ml (Figure 2). However, in the groups treated with carbapenems, low concentrations of carbapenems caused significant increases in biofilm mass (Figure 3, 4, and 5).

3.3. Biofilm removal effect of LL-37 and RP557

Concentrations ranging from 512 μg/ml to 1 μg/ml and from 128 μg/ml to 0.5 μg/ml were used to assess its effect on mature *P. aeruginosa* biofilm of LL-37 and RP557 respectively. LL-37 removed 25~70% of the mature biofilm of the *P. aeruginosa* strains used in this study at 256 μg/ml. For strains ATCC 27853, NCCP 16079, D16, and D26, LL-37 was even able to remove part of the mature biofilm at a concentration lower than its MIC of 256 μg/ml. (Figure 6). For all 7 strains, RP557 was effective to remove the mature biofilm at concentrations of 2~4 times the MIC (Figure 7).

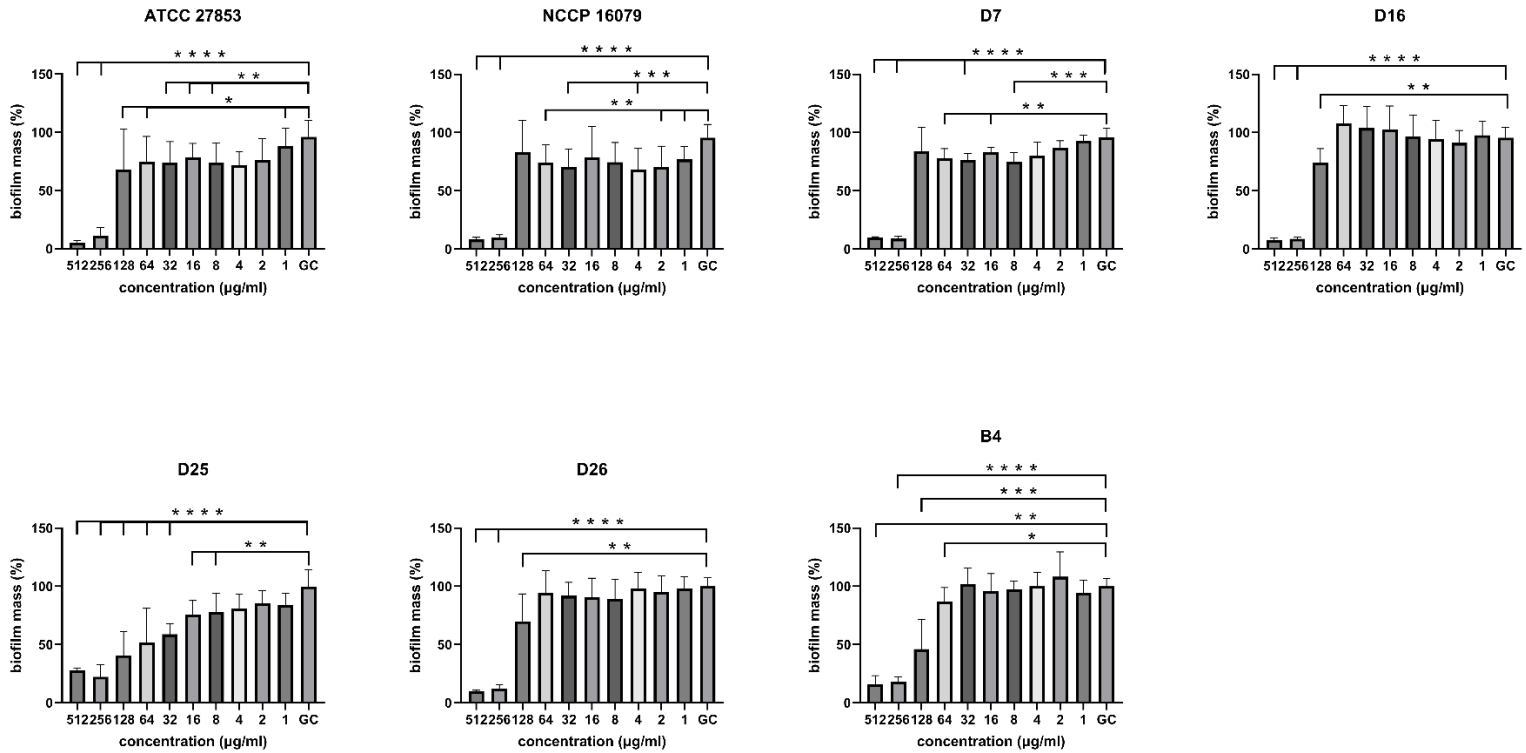

Figure 1. Inhibitory effect of LL-37 to carbapenem-resistant *Pseudomonas aeruginosa* biofilm formation. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

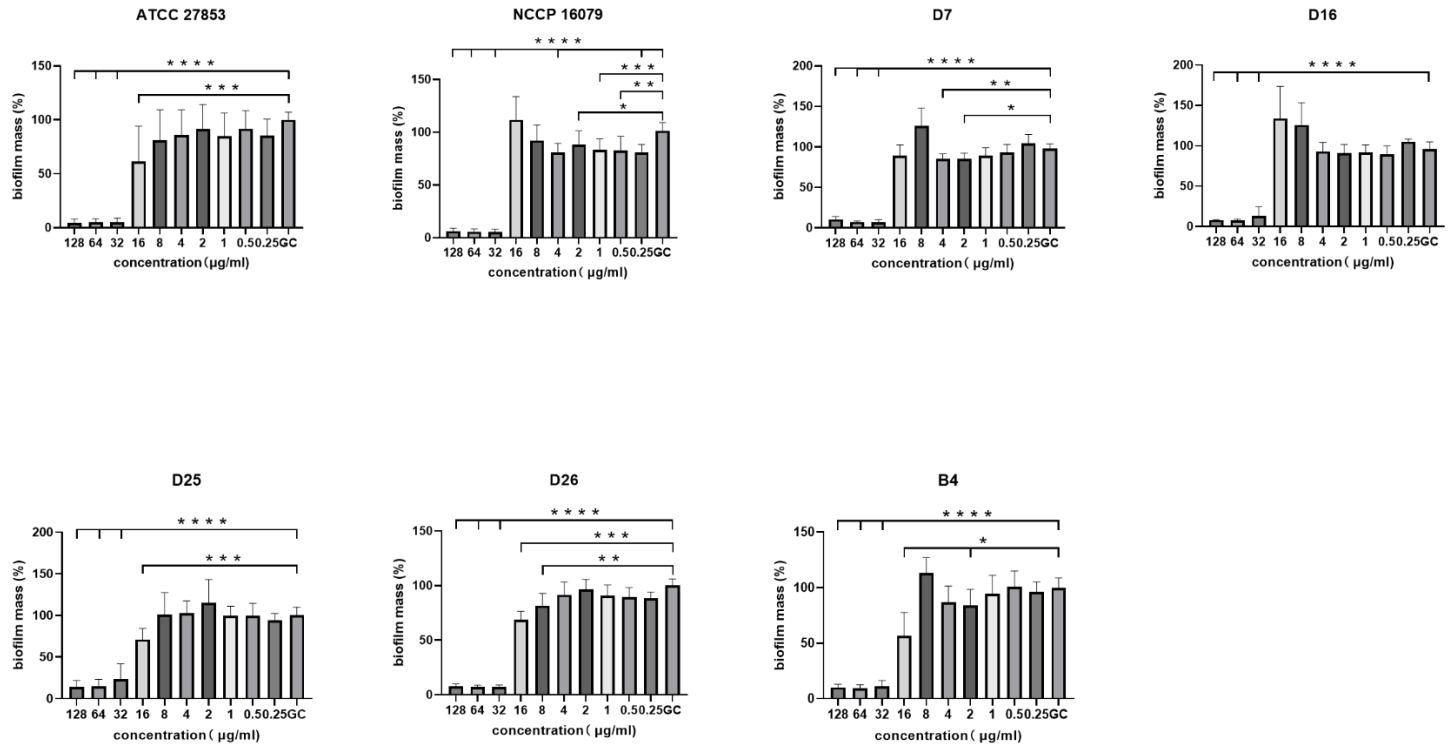

Figure 2. Inhibitory effect of RP557 to carbapenem-resistant *Pseudomonas aeruginosa* biofilm formation. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

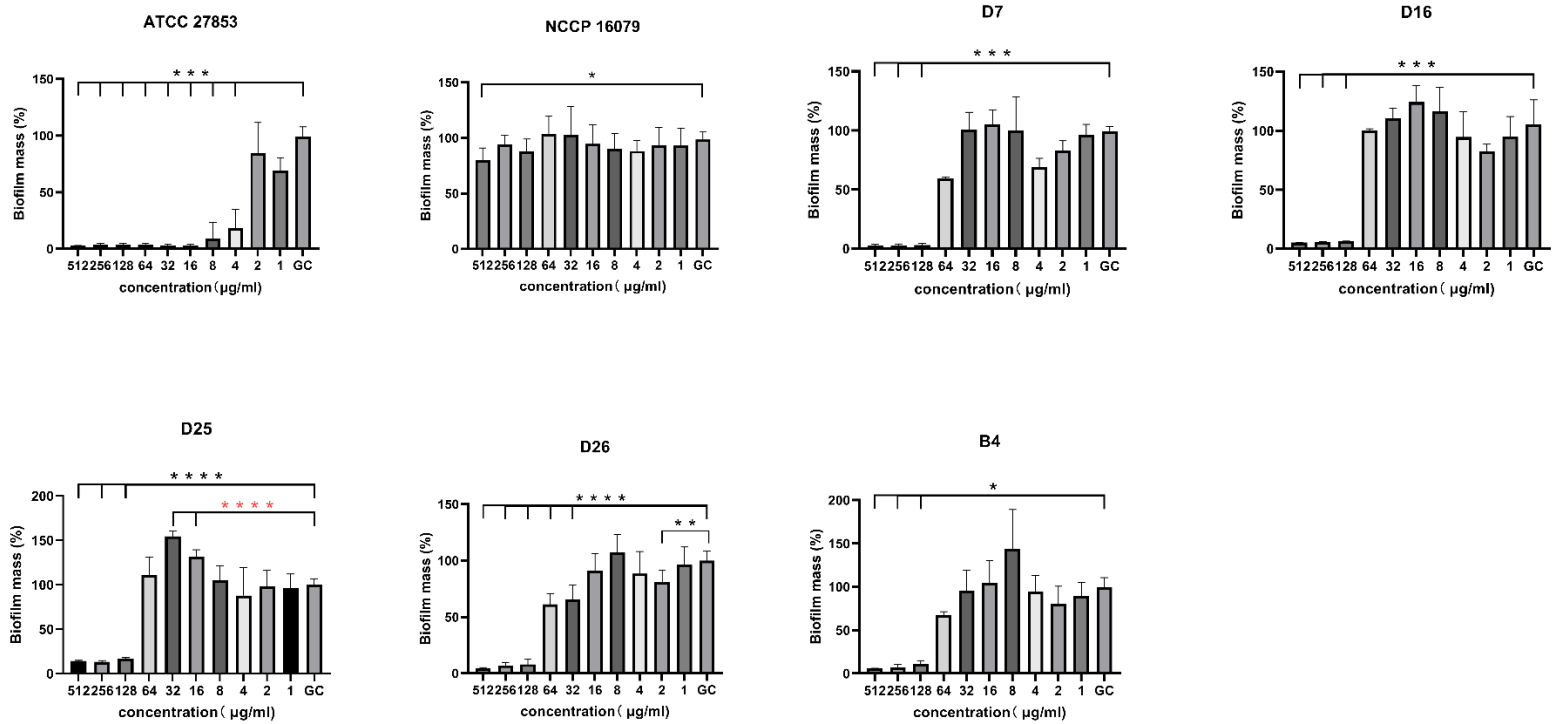

Figure 3. Effect of ertapenem on carbapenem-resistant *Pseudomonas aeruginosa* biofilm. Asterisk in black represents significantly decrease, asterisks in red represents significantly increase. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

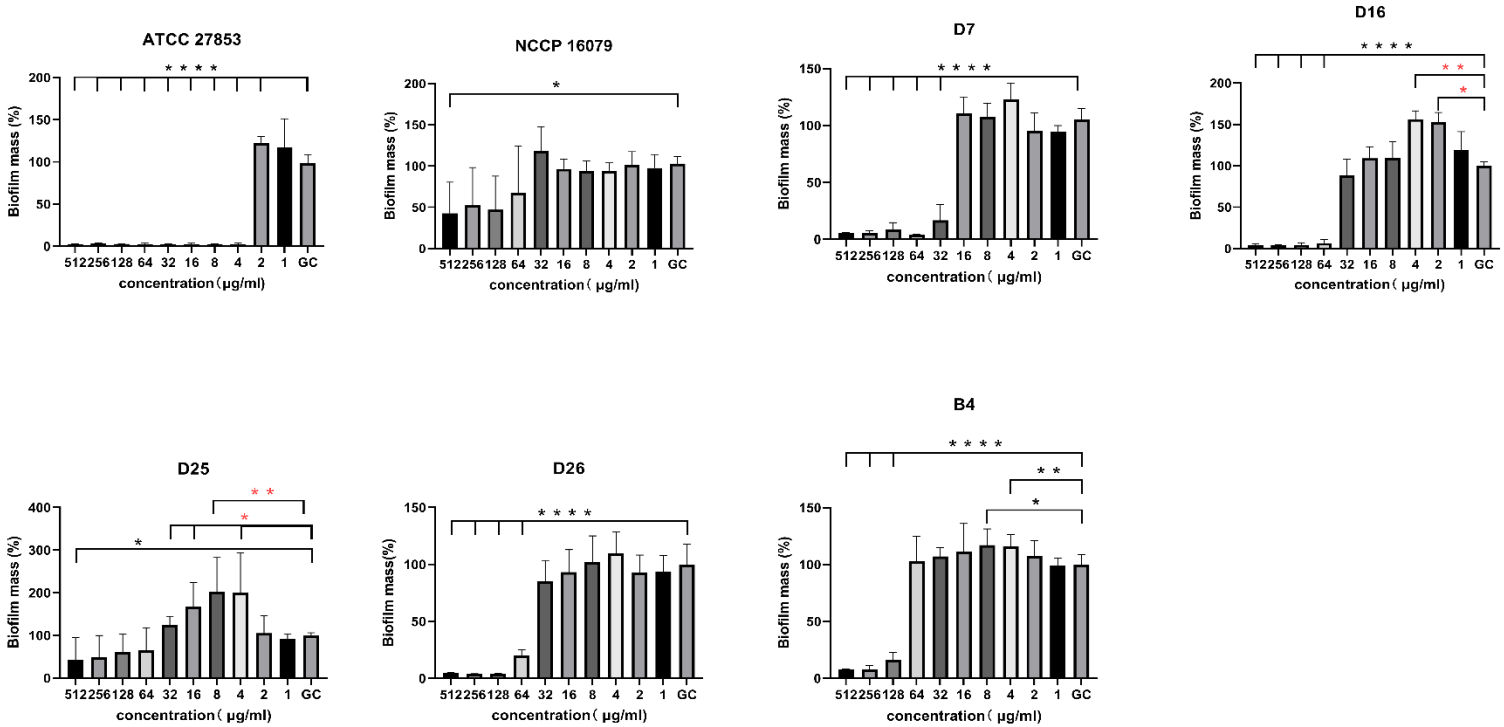

Figure 4. Effect of imipenem on carbapenem-resistant *Pseudomonas aeruginosa* biofilm. Asterisk in black represents significantly decrease, asterisks in red represents significantly increase. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

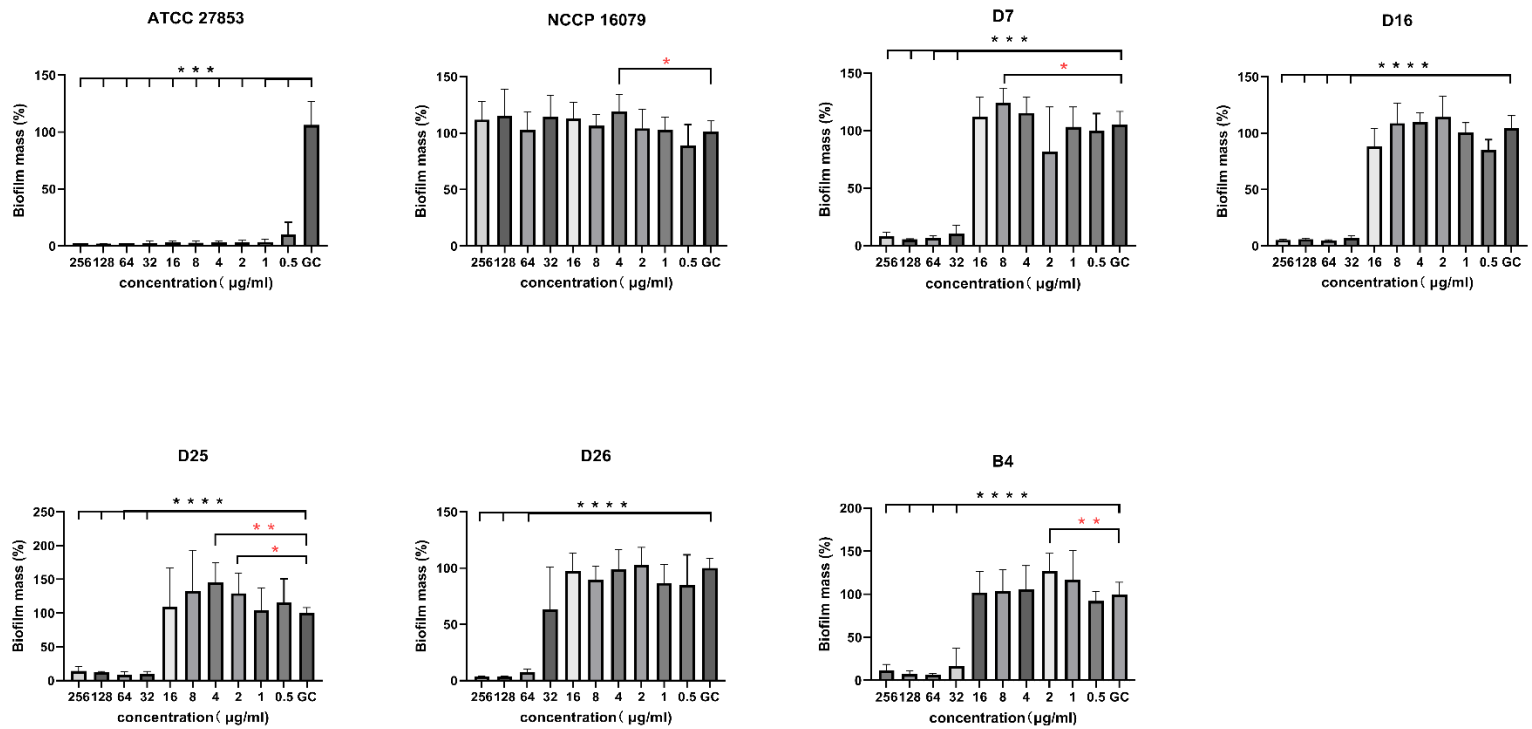

Figure 5. Effect of meropenem on carbapenem-resistant *Pseudomonas aeruginosa* biofilm. Asterisk in black represents significantly decrease, asterisks in red represents significantly increase. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

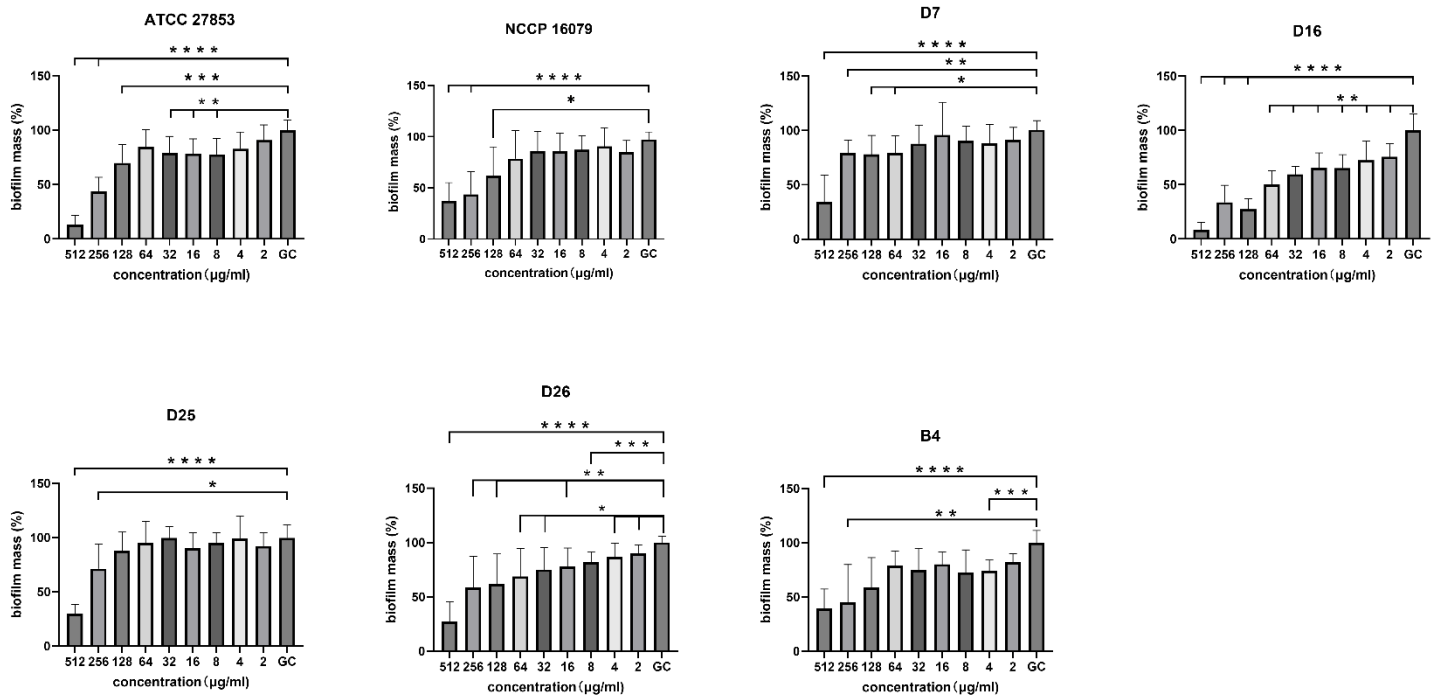

Figure 6. Biofilm removing effect of LL-37. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

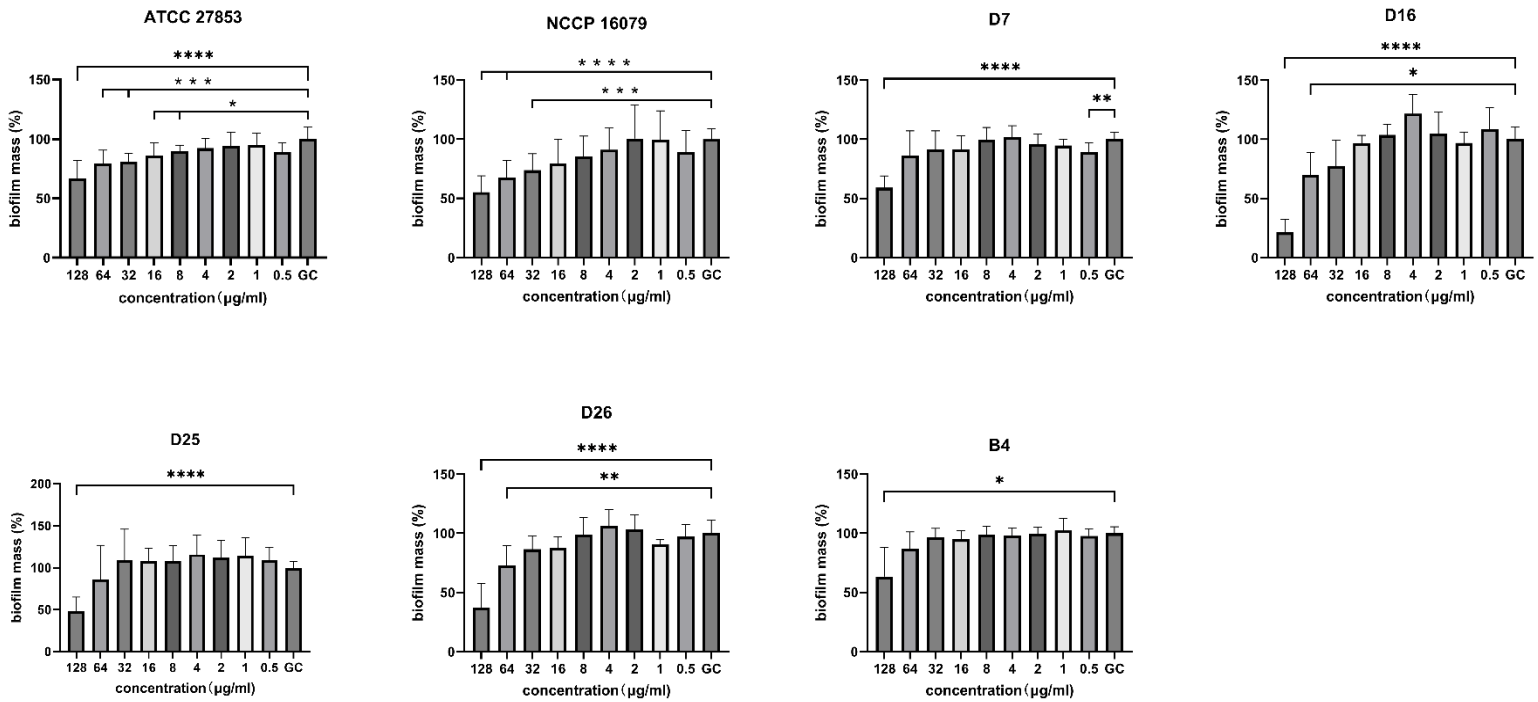

Figure 7. Biofilm removing effect of RP557. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

3.4. Synergetic effects of RP557 and LL-37 with carbapenems on carbapenem-resistant *P. aeruginosa*

A quarter MIC of LL-37 (64 μg/ml) and RP557 (8 μg/ml) were used together with carbapenems to assess the combined effect of the two antimicrobial peptides with carbapenems. Coadministration of LL-37 at a quarter MIC and carbapenems increased the susceptibility of carbapenem-resistant *Pseudomonas aeruginosa* to carbapenems by 4 to 32-fold (Table 3). Among them, the susceptibility of 4 CRPA strains to imipenem were increased from high resistance (MIC 64~256 μg/ml) to the resistance border. The susceptibility of 2 CRPA strains to meropenem was increased to resistance border and the susceptibility of 3 strains was increased to intermediate zone. The combination of sub-MIC RP557 and carbapenems had a synergetic effect on inhibiting CRPA growth (Table 4).When 8 μg/ml RP557 was used together, the susceptibility of CRPA to carbapenems increased by 4 ~ 16-fold.

Table 3. Synergetic effect of LL-37 and carbapenems against carbapenem-resistant *Pseudomonas aeruginosa.*

Pseudomonas aeruginosa Strain	Minimal inhibitory concentrations (μg/ml) combined use with LL-37 (initial MIC)		
	Ertapenem	Imipenem	Meropenem
ATCC 27853	4 (4)	1 (4)	0.5 (0.5)
NCCP 16079	>256 (>2048)	64 (256)	>256 (>2048)
D7	16 (128)	8 (64)	8 (32)
D16	16 (128)	8 (64)	8 (32)
D25	16 (128)	8 (256)	4 (32)
D26	16 (128)	16 (64)	4 (64)
B4	16 (128)	8 (128)	4 (32)

Table 4. Synergetic effect of RP557 and carbapenems against carbapenem-resistant *Pseudomonas aeruginosa.*

Pseudomonas aeruginosa Strain	Minimal Inhibitory Concentration (μg/ml) Combined Use With RP557 (initial MIC)		
	Ertapenem	Imipenem	Meropenem
ATCC 27853	2(4)	1 (4)	0.5 (0.5)
NCCP 16079	>256 (>2048)	32 (256)	>256 (>2048)
D7	16 (128)	16 (64)	8 (32)
D16	16 (128)	16 (64)	8 (32)
D25	16 (256)	16 (256)	4 (32)
D26	16 (256)	16 (64)	4 (64)
B4	16 (128)	16 (128)	8 (32)

3.5 Biofilm related gene expression levels changed in *P. aeruginosa* treated with LL-37 and RP557

Fourteen genes related to the quorum sensing system and biofilm information were selected for gene expression level analysis by qPCR. Among the 14 genes selected, 5 genes had significant expression differences in the LL-37- or RP557-treated groups (Figure 8A, 8B and 8C). Within the total of 7 strains, 5 strains treated with antimicrobial peptides showed significant downregulation of *rhlA* and *rhlB* expression, and 2 strains showed upregulation of *rhlA* or *rhlB* in the treated groups. *rhlI* expression was significantly suppressed in the treatment groups except for strains NCCP 16079, D7, and D16. For *pslD*, its expression was significantly upregulated in RP557-treated ATCC 27853 and LL-37 treated D25. However, there was a significant decrease in *pslD* expression in D25, and B4 treated with RP557 and D26 treated with LL-37. For *algK*, its expression in ATCC 27853, NCCP 16079, and D16 treated by antimicrobial peptides was induced, while in treated D7, D25, and D26, its expression was repressed.

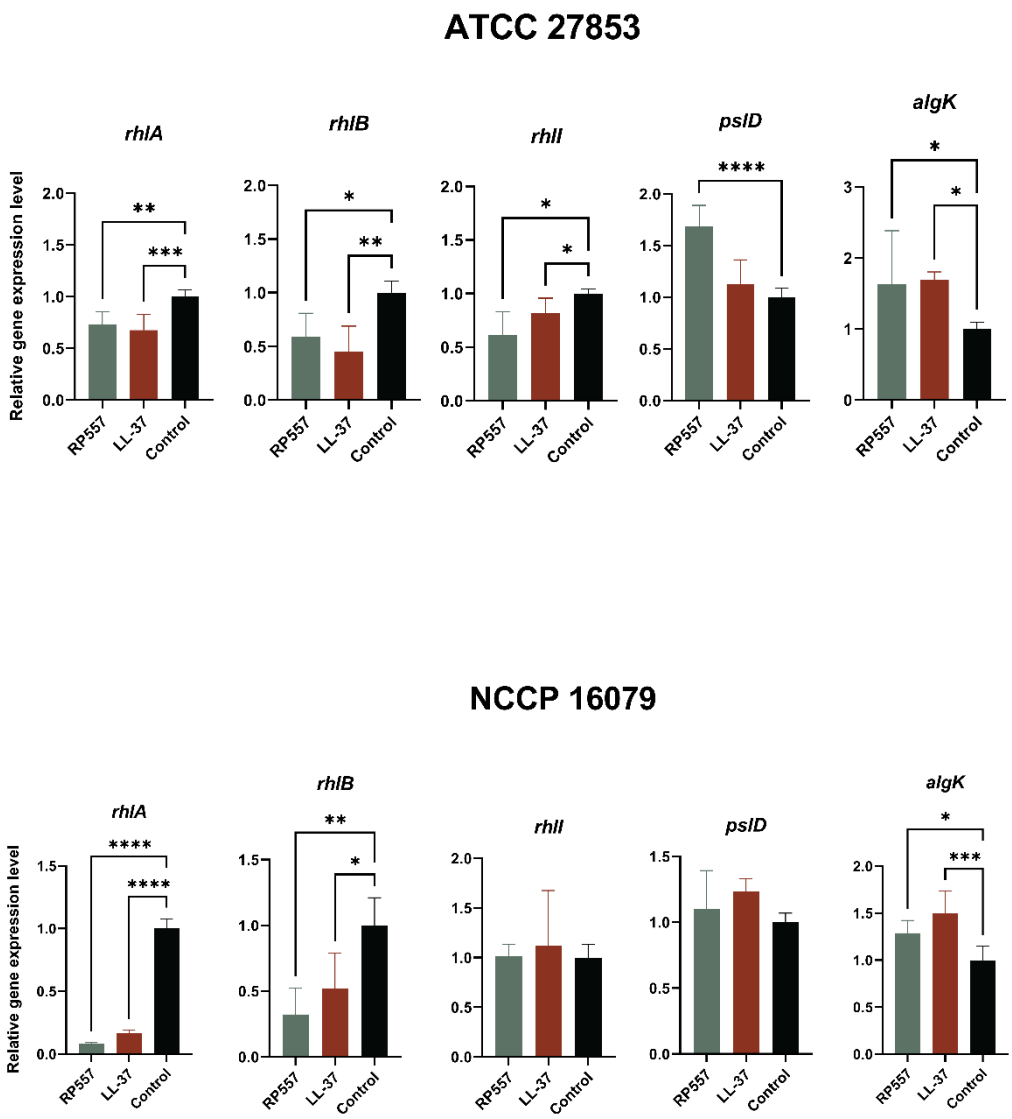

Figure 8A. The effects of LL-37 and RP557 on biofilm formation related genes. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

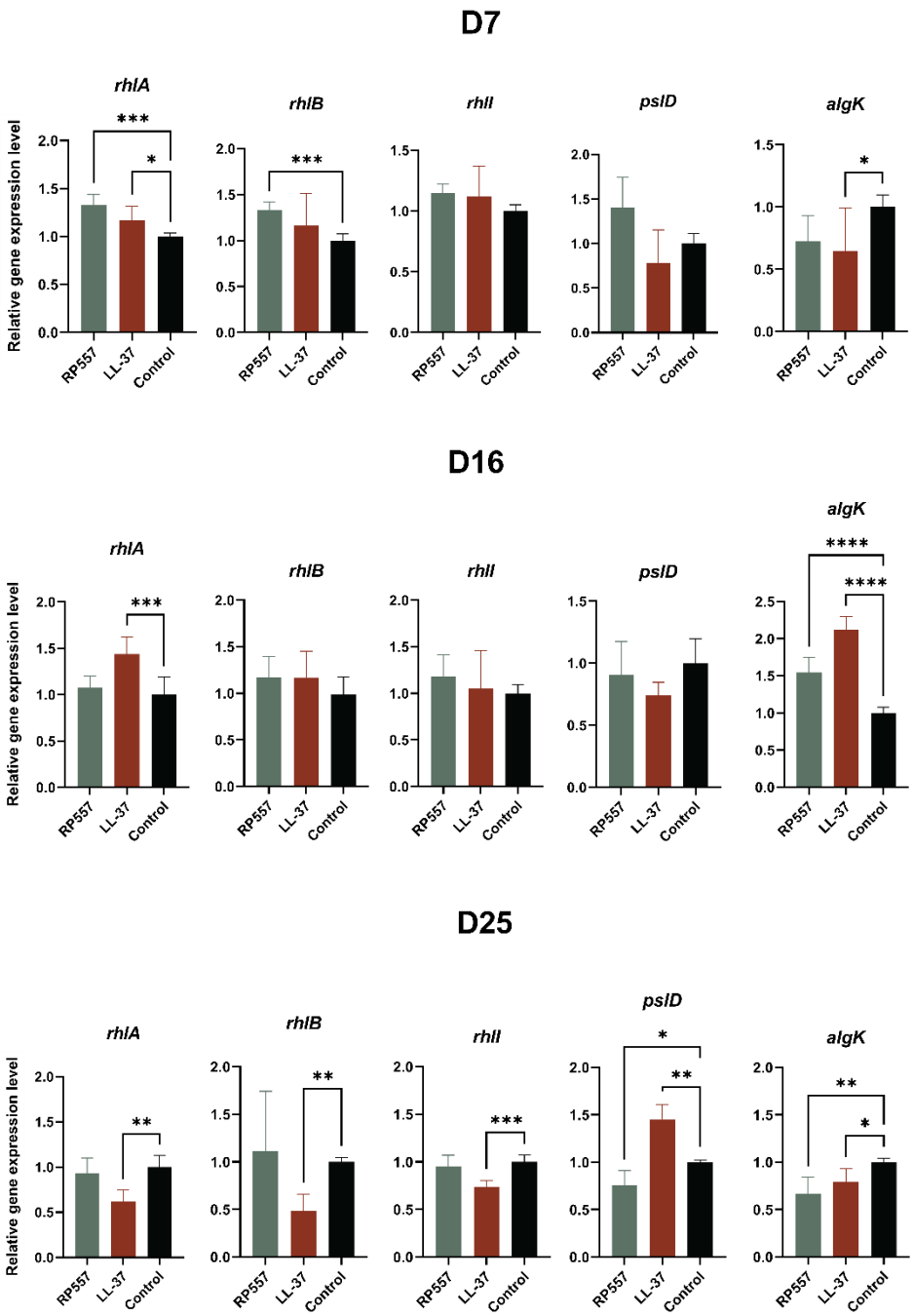

Figure 8B. The effects of LL-37 and RP557 on biofilm formation related genes. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

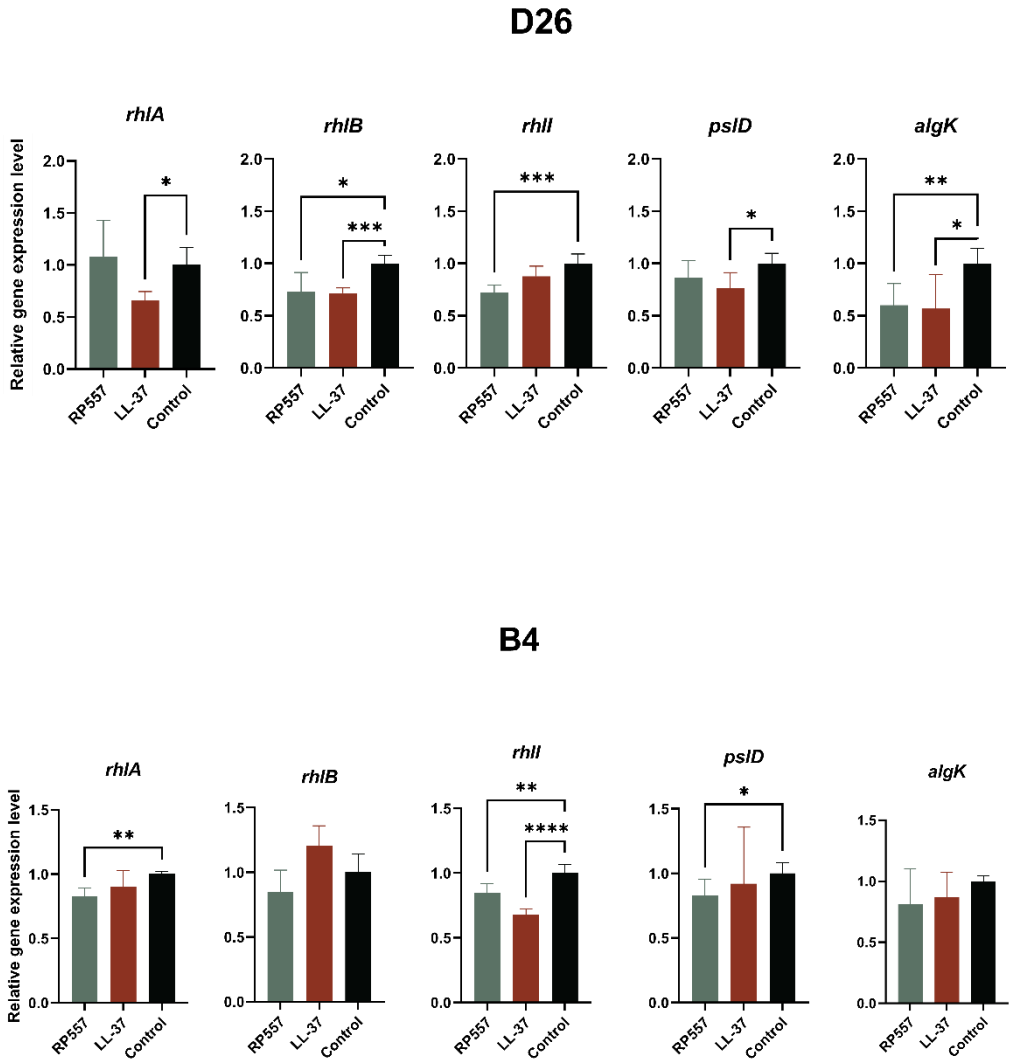

Figure 8C. The effects of LL-37 and RP557 on biofilm formation related genes. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

3.6 The biofilm structure and cell viability changed after treatment with LL-37 or RP557

The biofilm structure was visualized by confocal laser scanning microscopy and the biofilm cell viability was assessed by comparing fluorescence intensity. The live cells were stained green by SYTO 9 and dead cells were stained red by PI. The three-dimensional structures were significantly reduced in the LL-37- and RP557- treated groups (Figure 9). In addition, the cell viability of the RP557- treated group was significantly decreased in all 7 strains, and 4 strains treated with LL-37 had lower viability than the control group (Figure 10).

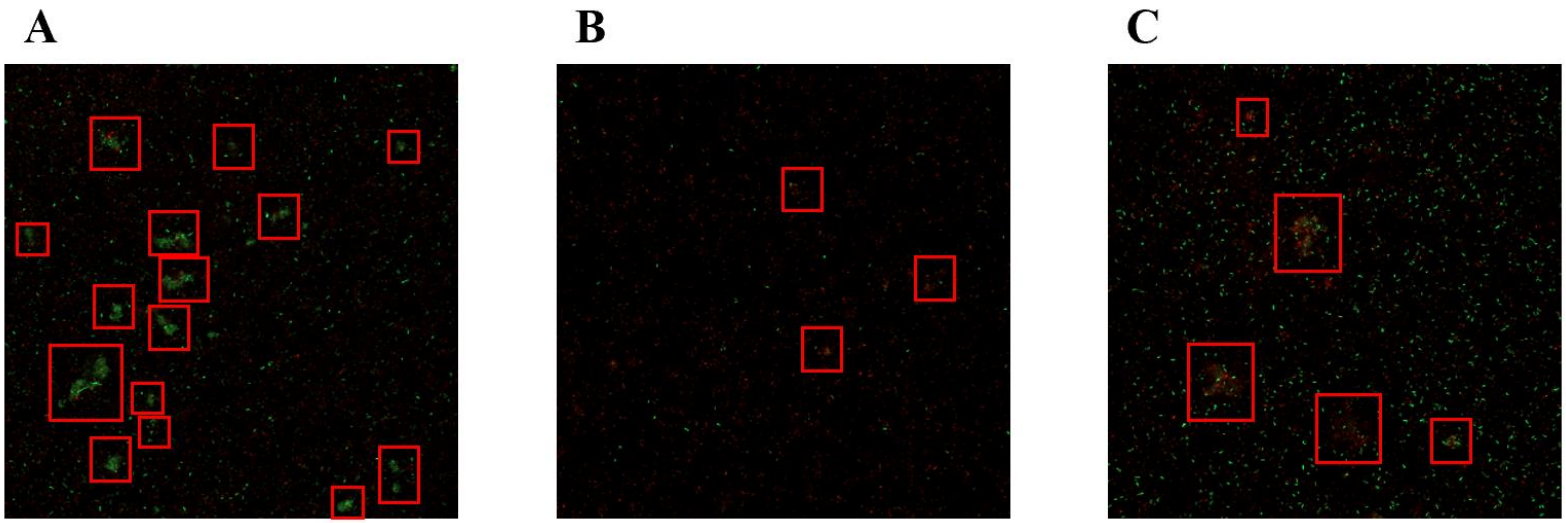

Figure 9. Confocal laser scanning microscopy images after staining showing the biofilm structure of *P. aeruginosa*. Mature biofilm structure was tagged with red box. (A) Confocal images of ATCC 27853 without LL-37 or RP557 treatment. (B) Confocal images of ATCC 27853 taken at 48 h with LL-37 treatment. (C) Confocal images of ATCC 27853 taken at 48 h with RP557 treatment.

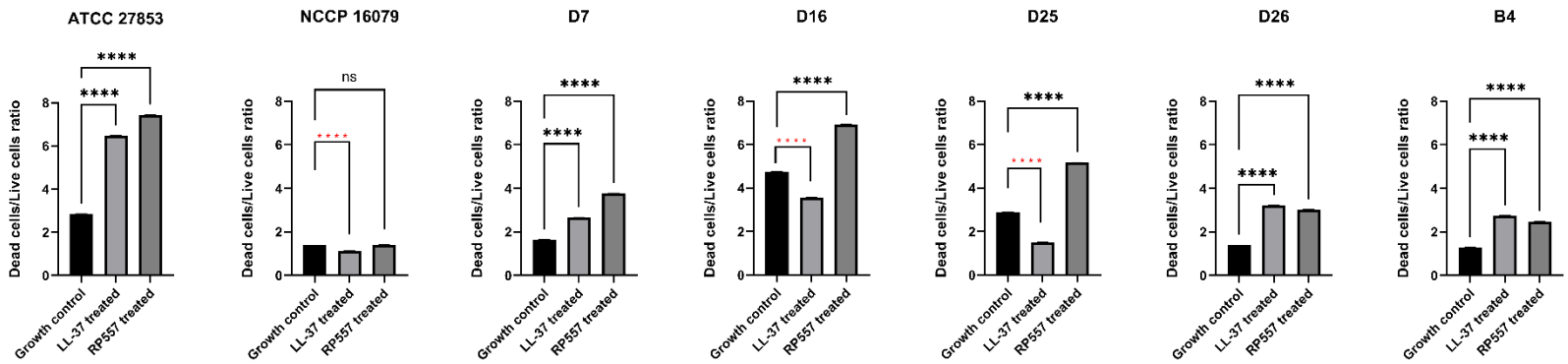

Figure 10. Biofilm cell viability of *P. aeruginosa* in different-treated groups. The cell viability was represented by the ratio of red and green fluorescence intensity. Asterisks in black represent cell viability significantly decrease, asterisks in red represent cell viability significantly increase. * : $p < 0.05$; * * : $p < 0.01$; * * *: $p < 0.001$; * * * *: $p < 0.0001$.

4. Discussion

Pseudomonas aeruginosa has received a lot of concerns due to its biofilm-forming ability and increasing antimicrobial resistance[8]. Both biofilm-forming ability and antimicrobial resistance make *P. aeruginosa* infection hard to cure. As the most effective β-lactam antibiotic, carbapenems are used to treat severe ESBL-producing bacteria infection, but since carbapenem resistance was first found in the 1980s, carbapenem-resistant *P. aeruginosa* infections have been reported widely, and now it has been listed as critical pathogens together with carbapenem-resistant Enterobacteriaceae and carbapenem-resistant *Acinetobacter baumannii* [25]. It has been reported that almost 300 cases of CRPA infection in 274 patients and most of the infected patients were long-term hospitalized patients [26]. Therefore, new antimicrobial compounds need to be developed to combat carbapenem-resistant *P. aeruginosa* infections. In this study, a new synthetic peptide RP557 was selected for assessing its antimicrobial and antibiofilm effect. Derived from human cathelicidin LL-37, RP557 had a similar antimicrobial effect and antibiofilm effect against the *P. aeruginosa* reference strain [27]. Our results were also consistent with the previous study of the effect of RP557 on several biofilm-forming bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, Methicillin- resistant *Staphylococcus aureus* and *Mycobacterium abscessus* [17, 18]. Furthermore, to our knowledge, there are no previous studies that have revealed the effect of LL-37 and RP557 on carbapenem-resistant *P. aeruginosa*. Therefore our study provides a new way

of dealing with carbapenem-resistant *P. aeruginosa*.

The antimicrobial effect of LL-37 has been reported previously. However, compared to its antimicrobial effect, its antibiofilm effect is more important since it can inhibit biofilm formation at very low concentrations [28]. Our results show that LL-37 at 256 μg/ml inhibits the growth of both *P. aeruginosa* reference and carbapenem-resistant strains. In addition to inhibiting planktonic cell growth, it inhibited the biofilm information at concentrations lower than MIC. As for RP557, it showed a better bacterial inhibition effect because its MIC for both *P. aeruginosa* reference and CPRA strains was 32 μg/ml, which is one-eighth of the LL-37 MIC. Our results are consistent with those previously reported in which both LL-37 and RP557 can inhibit *P. aeruginosa* growth regardless of the resistance to other antibiotics, although the MIC of LL-37 and RP557 against reference strains are different from those previously reported [17]. Thus, it is speculated that the difference during the synthesis process is responsible for the difference in MIC. RP557 also has an antibiofilm effect at concentrations lower than MIC. The effective concentration of anti-biofilm varies among strains, but most of them are effective at 1/8 to half MIC, which is from 4 μg/ml to 16 μg/ml. The effective concentration of RP557 in the current study is also safe to use, as it didn't represent cytotoxity to mamalian cells even at a high concentration of 256 μg/ml [29]. In contrast, biofilm mass in CPRA treated with low a concentration of carbapenems was significantly increased, indicating that it may induce biofilm formation, and increasing its resistance against carbapenems.

Mature biofilm is usually more resistant to drugs because there are many components in the biofilm matrix including eDNA, exopolysaccharides, and proteins. The penetration of some antimicrobial compounds can be delayed by biofilm matrices, especially in *P. aeruginosa* mucoid strain in which the biofilm contains a large amount of alginate [5]. In addition, the existence of anionic eDNA will reduce the antimicrobial activity of cationic antimicrobial peptides such as polymyxin B. Additionally, the pesister cells in the mushroom-like structure of biofilm are not susceptible to antibiotics that act in the growth phase, such as β-lactam antibiotics, making the biofilm more resistant and hard to eliminate [4]. Thus, the effects of LL-37 and RP557 on mature biofilm have been assessed. We found that 256 μg/ml of LL-37 removed approximately 30-70% of the mature biofilm and at sub-MIC from 2 ~ 128 μg/ml, LL-37 was also able to remove mature biofilm of some strains. RP557 reduced approximately 40~80% of mature biofilm at 128μg/mL which is 4 times the MIC. In our study, LL-37 showed better biofilm removal ability than RP557 compared to their MIC. LL-37 has been reported to be effective in eradicating *P. aeruginosa* biofilm at sub-MIC, which is consistent with our results [15, 30]. In the previous study, RP557 was able to kill 50% of *P. aeruginosa* biofilm cells at a concentration of 32 μg/ml, and killed 90% of biofilm cells at a concentration approximately 100 μg/ml [17].

Since biofilms contribute a lot to antibiotic resistance, LL-37 and RP557 in subinhibitory concentration were coadministrated with carbapenems to investigate their combined effect [31]. The results showed that coadministration of LL-37 or RP557 with carbapenems resulted in a

significant increase in the susceptibility of CRPA to carbapenems, with MIC reduced by 4 to 8 times compared to its initial MIC. The synergetic effect of the antimicrobial peptide with other antibiotics has been verified in other studies[30, 32]. It suggests that the carbapenem resistance of CRPA may be associated with its biofilm. However, strain NCCP 16079 maintained a high level of carbapenem resistance when coadministered with LL-37 or RP557, suggesting that carbapenem resistance of NCCP 16079 is more likely associated with other resistance mechanisms such as the carbapenemase it produced.

To further investigate the effect of LL-37 and RP557 on CRPA biofilms, 14 genes related to biofilm formation were selected for qPCR analysis. Among the 14 genes tested, 5 genes showed significant expression level changes when treated with LL-37 or RP557 at a quarter MIC. *rhlA* and *rhlB* were repressed in ATCC 27853, D25, D26 and B4. *rhlA* and *rhlB* are the genes essential for rhamnolipid synthesis in *P. aeruginosa*. Rhamnolipid, a kind of biosurfactant produced by *P. aeruginosa*, is not only one of the main virulence factors of *P. aeruginosa*, but it also have an important role in maintaining the typical mushroom-like structure in *P. aeruginosa* and inducing the dispersion of biofilm [33]. Downregulations of *rhlA* and *rhlB* result in decrease in the swarming ability of *P. aeruginosa*, which is essential for the initial attachment of *P. aeruginosa* to the surface thus influencing biofilm formation [34]. This result was consistent with the results of the biofilm inhibition assay which showed that at a quarter MIC, the biofilm mass of these downregulated strains was significantly less than the untreated group. Additionally, the repressed

production of rhamnolipid will lead to forming a flat biofilm rather than a mushroom-shaped structure. This could be the reason for the synergistic effect of LL-37 and RP557 with carbapenem since the persister cells inside of the mushroom structure contribute a lot to antimicrobial resistance, especially in chronic infection [6].

rhlI expression level was reduced in ATCC 27853, D25, D26 and B4. RHLI is responsible for synthesizing N-butyryl-L-homoserine lactone, the cognate AHL of RhlR, together with RhlR forming the RHL QS system [35]. The RHL system regulates biofilm formation through regulating exopolysaccharide production by regulating the *pel* genes [36]. Reduction in pel polysaccharides affects pellicle formation and biofilm structure [37]. The fold changes in expression of *pslD* and *algK* differed in different strains. PslD is the secretion protein in exopolysaccharide synthesis thus involving biofilm formation, and AlgK is a secretion protein related to alginate synthesis [38]. The changes in *pslD* and *algK* expression were also according to the results from the biofilm inhibition assay, as the biofilm mass of groups with upregulation of *pslD* and *algK* treated by a quarter MIC of LL-37 or RP557 was neither increased nor reduced. These results suggest that biofilm formation is a comprehensive process regulated by many genes, and changes in some genes may not affect overall biofilm matrix production.

To study the effect of LL-37 and RP557 on *P. aeruginosa* biofilm structure, the confocal laser scanning microscopy images were taken. The confocal laser scaning microscopy images showed that the mushroom-like structure reduced in LL-37 or RP557 treated groups. This result was consistent with

the decreased expression level of *rhlA* and *rhlB as* rhamnolipid reduction could induce the biofilm to form a flat form rather than the typical mushroom structure. The monolayer was considered more sensitive to antibiotics [39], which could be the reason for the synergetic effect between RP557 and antibiotics observed in the present study. Compared to the nontreated group, the biofilm cell viability of *P. aerugionsa* in LL-37 or RP557-treated groups decreased, as well. Peptides were reported to have potential ability to kill biofilm cells [40-42]. RP557 was proved effective in killing MRSA, *P. aeruginosa*, MDR *S. epidermidis*, and *M. abscessus* biofilm cells in the previous study [17, 18]. Most peptides play their antimicrobial effect by disrupting bacteria cell membranes. Along with biofilm inhibition ability, peptides are easier to penetrate into bacteria membrane, thus increasing the killing effciency.

5. Conclusion

The synthetic peptide RP557 and its structural origin LL-37 have antimicrobial effect on carbapenem-resistant *Pseudomonas aeruginosa*, and both of the two peptides have an antibiofilm effect that can inhibit biofilm formation at sub-MICs. The effect of both RP557 and LL-37 on the mature biofilm was manifested at 2X MIC which can remove approximately 50% of the mature biofilm. Both LL-37 and RP557 could affect biofilm structure at sub- MIC. Furthermore, two peptides have a synergetic effect on CRPA that increased the susceptibility of CRPA to carbapenems. Therefore, RP557 and LL-37 could be the potential treatment for carbapenem-resistant *P. aeruginosa* infections especially for chronic biofilm infections.

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국문초록

합성 항균펩타이드 RP557 이 카바페넴 내성 *Pseudomonas aeruginosa* 바이오필름에 미치는 효과

서울대학교 대학원

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송운기

(지도교수: 유 한 상)

Pseudomonas aeruginosa 항생제 내성의 증가는 세계적인 문제가 되고있다. 대표적인 바이오필름 형성 세균으로서 *P. aeruginosa* 바이오필름 감염은 면역결핍 환자의 병원성 감염과 낭포성 섬유증 감염의 만성 폐렴에서 종종 발견된다. 이 두 가지 특성에 의해 *P. aeruginosa* 바이오필름의 감염 치유는 현재까지도 어려운 실정이다. Human cathelicidin LL-37 에서 유래한 인공합성 펩타이드 RP557 은 카바페넴 내성 *P. aeruginosa* 에 대한 항균 효과와 바이오필름 억제효과를 통해 평가되었다. 카바페넴 내성과 상관없이 *P. aeruginosa* 에 대한 RP557 과 LL-37 의 최소 억제 농도는 각각 32

μg/ml 와 256 μg/ml 인 것으로 나타났다. RP557 과 LL-37 모두 Sub-MIC 에서 *P. aeruginosa* 바이오필름 형성을 억제할 수 있었고, Carbapenems 의 Sub-MIC 는 바이오필름 형성을 유도했다. 또한 RP557 은 64 μg/ml 의 농도로 약 50% 형성된 바이오 필름을 제거할 수 있었으며, 256 μg/ml 의 LL-37 은 약 50% 형성된 바이오 필름을 제거했다. RP557 과 LL-37 의 1/4 MIC 는 서로다른 카바페넴(에타페넴, 이미페넴, 메로페넴)과 함께 사용하였다. RP-557 과 LL-37 모두 4~8 배에서 CRPA 의 민감도를 증가시킬 수 있음을 보여주었다. RP557 또는 LL-37 처리된 CRPA 에서 바이오필름 관련 유전자 발현 수준의 변화가 관찰되었다. 공초점 현미경 영상으로 관찰했을 때 LL-37 또는 RP557 처리된 그룹에서 바이오필름 구조가 감소되었음을 보여주었다. LL-37 또는 RP557 처리된 그룹에서 바이오필름 세포활성이 억제되었다. 따라서, RP557 과 LL-37 은 카바페넴 내성 *P. aeruginosa* 감염에 대한 잠재적인 치료물이 될 수 있다.

주요어: *Pseudomonas aeruginosa,* 카바페넴 내성, 항균펩타이드, 바이오필름

학번: 2021-28252

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