



Peptide dendrimers G3KL and TNS18 inhibit *Pseudomonas aeruginosa* biofilms

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Abstract

Herein we report that peptide dendrimers G3KL and TNS18, which were recently reported to control multidrug-resistant bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, strongly inhibit biofilm formation by *P. aeruginosa* PA14 below their minimum inhibitory concentration (MIC) value, under which conditions they also strongly affect swarming motility. Eradication of preformed biofilms, however, required concentrations above the MIC values. Scanning electron microscopy observation and confocal laser scanning micrographs showed that peptide dendrimers can destroy the biofilm morphological structure and thickness in a dose-dependent manner, even make the biofilm dispersed completely. Membrane potential analysis indicated that planktonic cells treated with peptide dendrimers presented an increase in fluorescence intensity, suggesting that cytoplasmic membrane could be the target of G3KL and TNS18 similarly to polymyxin B. RNA-seq analysis showed that the expressions of genes in the *arnBCADTEF* operon-regulating lipid A modification resulting in resistance to AMPs are differentially affected between these three compounds, suggesting that each compound targets the cell membrane but in different manner. Potent activity on planktonic cells and biofilms of *P. aeruginosa* suggests that peptide dendrimers G3KL and TNS18 are promising candidates of clinical development for treating infections.

Keywords *Pseudomonas aeruginosa* · Biofilm · Peptide dendrimers · Membrane · Polymyxin B · Mingqiang Qiao and Jean-Louis Reymond contributed equally to this work.

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Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen with the ability to cause infections in pulmonary and urinary tracts, burns, and wounds, which can occasionally be fatal to immunocompromised patients (Balasoïu et al. 2014). This pathogen can form biofilm by colonizing on various surfaces or at air-liquid interfaces (Kong et al. 2015). The bacterial cells adhere to each other and are wrapped in a protective extracellular matrix that consists of self-produced extracellular polymeric substances to form mature biofilm structures (Abdel et al. 2016; Fazli et al. 2014; Pires et al. 2015; Stach et al. 2012, 2014; Thien and George 2001). Biofilm formation leads to resistance of bacteria to many harsh environments, including starvation and conventional antimicrobial drugs, and there are no treatments available to address biofilm infections (Fazli et al. 2014; Hoffman et al. 2005; Taylor et al. 2014; Thien and George 2001). As a result, there is an urgent need for novel antibacterial agents to inhibit and kill these multidrug-resistant bacteria.

Peptide dendrimers were recently reported as a new possible class of antimicrobial agents to treat bacteria such as *Staphylococcus aureus*, *P. aeruginosa*, and *Acinetobacter baumannii*, including multidrug-resistant clinical isolates (Abdel et al. 2016; Pires et al. 2015; Siriwardena et al. 2018; Stach et al. 2014). Remarkably, these peptide dendrimers as well as some dendrimers with cell penetrating, some glycopeptide dendrimers with drug delivery of cytotoxic drugs and esterase peptide dendrimers as enzyme models with catalytic activities (Eggimann et al. 2014; Reymond and Darbre 2012) showed excellent stability in serum, owing to the branching points in the peptide chain hindering the action of proteases (Sommer et al. 2009). Here, we focused on G3KL and TNS18, which are two of the most potent peptide dendrimers reported to date (Pires et al. 2015; Siriwardena et al. 2018). An antimicrobial peptide Polymyxin B (PMB) used in clinic, a cyclic peptide last-resort antibiotic with membrane-disruptive properties, is known to interact primarily with lipid A of lipopolysaccharide (LPS) (Bilecen et al. 2015). We asked the question whether G3KL and TNS18 might act similarly to PMB against bacterial biofilms, which play an important role on bacterial infections.

Although biofilm inhibition by peptide dendrimers was previously reported only for the case of glycosylated dendrimers acting via lectin binding (Johansson et al. 2008; Kadam et al. 2011; Michaud et al. 2016), we found that both G3KL and TNS18, which are simply polycationic hydrophobic dendrimers without carbohydrate appendages, also strongly inhibited biofilm formation below the minimum inhibitory concentration (MIC) value, and discovered that they affect swarming motility at these low concentrations. On the other hand, dispersal of established biofilms required much higher concentrations; nevertheless, peptide dendrimers were significantly more active against biofilms compared to the reference antibiotic PMB. Imaging by scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM), membrane potential, RNA-seq, and qPCR studies suggest that the effects of peptide dendrimers on bacteria are mediated by membrane interactions.

Methods

Bacterial strains

Pseudomonas aeruginosa PA14 strain (BEI Resources No. NR-50573) (Liberati et al. 2006) was collected and stored in our laboratory.

Synthesis of G3KL and TNS18

G3KL and TNS18 were received from Prof. Dr. Jean-Louis Reymond. Peptide dendrimers were synthesized using standard solid-phase peptide synthesis. Lipidated peptide

dendrimers synthesis was carried out as above with some modifications. Dendrimer TNS18 (70 mg of HPLC-purified trifluoroacetate salt) was exchanged from trifluoroacetate salt to acetate salt, and the dendrimer was obtained as a white foamy solid after exchange (54 mg, 93%). The dendrimer G3KL (94 mg of HPLC purified trifluoroacetate salt) was obtained as a white foamy solid after exchange (80 mg, 99%) (Siriwardena et al. 2018).

Minimum inhibitory concentration assay

MIC values of the peptide dendrimers were determined by twofold serial dilution as suggested by 96-well microtiter plate as previously described (Rahman et al. 2004). Before the twofold serial dilution test, bacteria were firstly cultured in Luria-Bertani (LB) medium at 37 °C, with shaking for 12 h at a speed of 200 rpm, and then inoculated into 5 mL of LB with 50 µL of the bacterial culture, shaking bacterial liquid to an OD₆₀₀ value of 0.6. One hundred microliters were added to each well in the prepared microplate and double-diluted concentrations of peptides were added to the wells, followed by incubation for 16 h at 37 °C. The MICs were read out by 2,3,5-triphenyltetrazolium chloride (TTC) staining testing. Five microliters of 5 g/L indicator (TTC) was added to each well of the microplates for 1 h at 37 °C. The control was obtained by incubating the cells with PMB. The MICs were examined by using at least two independent measurements.

Inhibition of biofilm formation

Pseudomonas aeruginosa static biofilm was grown in a 96-well microtiter plate to determine the inhibitory effects of peptide dendrimers on biofilm formation. The overnight culture of *P. aeruginosa* was diluted to OD₆₀₀ = 0.01 with fresh LB medium. The suspension was transferred to each well of a 96-well plate and incubated with peptide dendrimers for 24 h at 37 °C without agitation to allow biofilm formation. The negative control was LB medium. The assay was performed using the concentrations of 0, 1/2, 1/4, and 1/8× MIC of TNS18, G3KL, and PMB, respectively.

Crystal violet staining

The supernatant was removed gently and the biofilm cells attached to the sample well surface were washed three times with physiological-buffered saline (PBS) and air-dried. Absolute methanol was added per well for 15 min for fixation and removed, and plates were allowed to dry (Zhang et al. 2016). Wells were stained using 0.1% crystal violet for 10 min then washed with deionized water to remove unbound crystal violet, and the bounded crystal violet was eluted in 70% ethanol for 10 min. The OD values of the eluted ethanol samples were measured at 595 nm. Experiments were done in triplicate.

TTC staining

After washing, 100 μL of 0.1% TTC (Sigma, USA) was added to a final concentration of 0.02%. The remaining LB medium/TTC solution was then removed and the plates were air-dried. Bound TTC dye was dissolved using 20% acetone/80% ethanol and the A_{540} of the solution was measured. Experiments were done in triplicate.

Biofilm eradication assay

The overnight culture of *P. aeruginosa* was diluted to $\text{OD}_{600} = 0.01$ with fresh LB medium and incubated for 24 h at 37 °C without agitation to allow biofilm formation. The medium of 24-h biofilm was replaced with fresh medium containing different concentrations of peptide dendrimers or PMB and incubated for 5 h at 37 °C under static condition. The positive controls were *P. aeruginosa* strains in LB without peptide. After incubation, the wells were washed with PBS. Subsequently, 0.1% crystal violet staining was performed as described earlier to measure the biomass and measured at 595 nm. Experiments were performed in triplicate.

Swarming motility assay

Motility assays were undertaken in petri dishes (diameter of 82 mm). Swarming motility test was conducted on a specific medium (0.5% agar, 8 g/L nutrient broth, and 5 g/L glucose) (Rashid and Kornberg 2000). A small quantity of the *P. aeruginosa* PA14 glycerol stock (stored at -80 °C) was streaked onto LB agar plate and incubated at 37 °C overnight. A single colony of *P. aeruginosa* from overnight LB agar plates (1.5%, w/v) was inoculated onto the agar surface of the swarming plates. Plates were incubated at 37 °C for 18 h and photographs were taken. The motility assay was performed in triplicate.

SEM

SEM was used to observe the effects of peptide dendrimers on biofilm formation. To observe the morphological changes of *P. aeruginosa* PA14 biofilms following peptide dendrimers treatment, the biofilms were allowed to grow on sterile cover slip, which was inserted into a flat-bottom 24-well polystyrene tissue culture plate. The overnight culture of *P. aeruginosa* was diluted to $\text{OD}_{600} = 0.5$ with fresh LB medium and incubated for 24 h at 37 °C without agitation to allow biofilm formation. Then, the cover slips were inserted into tissue culture centrifuge tube with or without peptide dendrimers for 5 h.

After incubation, the samples were fixed with 3% glutaraldehyde for 12 h at 4 °C. After washing with PBS, the samples were dehydrated in a series of ethanol solutions of increasing concentration (30–100%). Then, the cells were

washed with different concentrations of tert-butyl alcohol (50% and 100%) and dried with a critical point dryer (Wang et al. 2016). The dried samples were gently cut into 1.0-cm small pieces and were coated with 15-nm gold using an argon automatic sputter coater. After processing, the samples were viewed under a QUANTA 200 scanning electron microscope (Quanta200, FEI, USA).

CLSM

Overnight *P. aeruginosa* PA14 biofilms formed on slides were rinsed with PBS (pH 7.0) and transferred to 10-mL tubes containing fresh LB medium supplemented with three peptides at concentrations of 0, 1, 4, and 16 \times MIC. The control slides were transferred to 10-mL tubes containing only fresh medium. After incubation, the medium was removed and washed three times with PBS. The bacterial viability in biofilm cultures was assessed using the Live/Dead BacLight bacterial viability assay, with 1.5 $\mu\text{L}/\text{mL}$ 1 mM SYTO9 and 1.5 $\mu\text{L}/\text{mL}$ 1 mM propidium iodide (PI) dyes and incubated for 30 min at room temperature, protected from light. The samples were submerged into the 3.7% (w/v) paraformaldehyde for 30 min, removed, and air-dried. The biofilms were observed under confocal laser scanning microscopy (CLSM; Leica TCS SP8, Solms, Germany) using a 60 \times oil immersion objective. The biofilm images were acquired in 0.4- μm optical sections for the entire thickness of the biofilm. SYTO9 was excited with the 480-nm argon laser and the emission was collected with a 500-nm filter. PI was excited with the 543-nm HeNe laser and the emission was collected with the 635-nm filter. To quantify biofilm formation, COMSTAT biofilm software (Heydorn et al. 2000) was used to measure mean thicknesses (μm).

Gram-negative membrane potential measurements

Overnight cultures of *P. aeruginosa* were diluted to $\text{OD}_{600} = 0.05$ in fresh medium and grown to $\text{OD}_{600} = 0.5$. Cells were collected by centrifugation, washed in HEPES buffer (5 mM HEPES at pH 7.2 and 5 mM glucose), and resuspended in the same buffer containing 0.4 μM diSC 3 (5) and 0.2 mM EDTA (to $\text{OD}_{600} = 0.05$). The mixture was incubated in the dark for 1 h to allow maximal uptake of the diSC 3 (5) dye. The osmotic gradient was equilibrated to a final concentration of 100 mM KCl. Subsequently, the mixture was treated with increasing amounts of peptides in HEPES buffer. The diSC 3 (5)-derived fluorescence was monitored with a Spectramax M5 spectrophotometer at excitation of 622 nm and emission of 670 nm. Relative fluorescence intensities were normalized to PBS (set to a value of 0%) and 99% (v/v) isopropanol (set to a value of 100%) standards (McGrath et al. 2013).

Transcriptome analysis

The overnight culture was diluted to a ratio of 1:100 and 1/8 MIC G3KL or TNS18 was added to the cell suspension to a final concentration of 2 or 1 µg/mL, respectively, and the cells were grown till the stationary phase (OD_{600} of about 2) (Chin et al. 2015). Then, appropriate volume of the cell suspension was mixed with two volumes of RNA protect reagent (Tiangen, China), and the total RNA was extracted using RNeasy Mini Kit (Tiangen). The quality of the total RNA obtained was determined, and the integrity of the RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent, USA).

The RNA samples with 1.8–2.2 ratio of absorbance at 260/280 nm, 1.8–2.2 ratio of absorbance at 260/230 nm, and an RNA integrity number of > 7 were used for the analysis. Transcriptome analysis were performed by BGI (Shenzhen, China). All the samples were purified by removing the ribosomal RNA, fragmented, and primed with random primers, and pair-end index libraries were constructed. All the samples were sequenced on Illumina HiSeqTM2000 platform (Illumina, USA), and sequence reads were mapped to the annotated *P. aeruginosa* UCBPP-PA14 genome (www.pseudomonas.com).

RT-qPCR

RT-qPCR was conducted to confirm and compare fold change for the *arnBCADTEF* operon after the bacterial cells were treated with the three peptides. The primer pairs used for RT-qPCR are listed in Table S1. Total RNA was extracted from the bacterial cells by using RNeasy Pure Cell/Bacteria Kit (Tiangen), and reverse-transcribed into cDNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The standards were generated by PCR and RT-qPCR was performed using FastStart Universal Probe Master (Roche, Germany). The RT-qPCR signals were normalized to the constitutively expressed housekeeping gene *rpsL*. Each experiment was performed in triplicate on two separate extractions.

Statistical analysis

Statistical analysis was carried out using SPSS software. Significance was assessed by calculating student's *t* test and a *P* value below 0.05 was considered significant.

Accession number and data availability

The expression data for samples with G3KL or TNS18 and without peptide dendrimer are summarized in Table 1. The RNA-seq data were deposited to SRA under accession number SRP182880.

Results

Antimicrobial activity

To check whether peptide dendrimers G3KL and TNS18 were indeed as active as antibacterial agents as initially reported, we determined their MICs against *P. aeruginosa* PA14 available in our laboratory in comparison to PMB. Both dendrimers showed significant activities against PA14. The MICs of G3KL, TNS18, and PMB against PA14 were 16, 8, and 1 µg/mL, respectively. Dendrimer TNS18 showed stronger activity than G3KL.

TNS18 and G3KL inhibited PA14 biofilm formation

The anti-biofilm activities of peptides were investigated against *P. aeruginosa* PA14 strains in 96-well plates by crystal violet staining (Fig. 1a) and by TTC staining (Fig. 1b). The addition of peptides at the beginning of bacterial culture dose-dependently inhibited *P. aeruginosa* biofilm formation. As shown in Fig. 1a, at 1/8× MIC, the inhibition of *P. aeruginosa* biofilm formation by G3KL, TNS18, and PMB reached approximately 50%, 60%, and 15%, respectively (Fig. 1a). The results obtained by TTC staining also showed that at 1/8× MIC, the inhibition of *P. aeruginosa* biofilm formation by G3KL, TNS18, and PMB reached approximately 50%, 52%, and 18% (Fig. 1b) compared with the untreated control. With further increase in the peptide concentration, the biofilm inhibitory effect of these peptides increased, with TNS18 and PMB presenting the strongest and weakest biofilm inhibitory effect, respectively.

TNS18 eliminated PA14 mature biofilm

Preformed *P. aeruginosa* biofilms were used to determine the ability of the peptide dendrimers to disperse mature biofilms. At concentrations lower than their respective MIC (1/4× MIC), TNS18 was able to disperse approximately 10% of *P. aeruginosa* biofilm, but G3KL presented a relatively low dispersion rate of 5%. However, at 1× MIC, TNS18, G3KL, and PMB could disperse the preformed biofilm by approximately 15%, 6%, and 12% (Fig. 2). With further increase in the concentrations of the peptides to 16× MIC, their biofilm degradation efficiency improved to 55%, 32%, and 40%, with TNS18 exhibiting the strongest biofilm dispersion effect.

TNS18 has an inhibitory effect on swarming motility

At concentrations lower than their MICs, the three peptides could significantly influence *P. aeruginosa* swarming motility. Compared with the control group (untreated), 1/8 MIC of G3KL, TNS18, and PMB caused change in swarming motility (Fig. 3a, d, g); 1/4 MIC of G3KL, TNS18, and PMB affected

Table 1 Validation of transcriptome data of *arnBCADTEF* operon using quantitative and semi-quantitative real-time PCR. Overnight *P. aeruginosa* PA14 cells were 1% diluted to fresh LB medium. 1/8 MIC G3KL or TNS18 was added to the cell suspension to a final

concentration of 2 or 1 $\mu\text{g}/\text{mL}$, respectively, and the cells were grown till the stationary phase (OD_{600} of about 2). Each experiment was performed in triplicate.

Genes	Description	Fold change				
		Transcriptome	qRT-PCR	Transcriptome	qRT-PCR	qRT-PCR
		G3KL	G3KL	TNS18	TNS18	PMB
<i>arnB</i>	AmB	1.22	1.60	NC	NC	1.81
<i>arnC</i>	AmC	1.34	1.68	NC	NC	1.71
<i>arnA</i>	AmA	2.14	1.85	NC	NC	2.00
<i>arnD</i>	AmD	1.65	1.78	NC	NC	1.60
<i>arnT</i>	inner membrane L-Ara4N transferase AmT	NC	NC	NC	NC	1.72
<i>arnE</i>	AmE	2.25	1.71	NC	NC	1.73
<i>arnF</i>	AmF	2.27	1.88	NC	NC	1.79
<i>PA3559</i>	probable nucleotide sugar dehydrogenase	1.89	1.79	NC	NC	1.71

swarming motility (Fig. 3b, e, h); and 1/2 MIC of G3KL, TNS18, and PMB disrupted swarming motility (Fig. 3c, f, i). Although the effects of the three peptides on *P. aeruginosa* swarming motility were noted to be relatively similar, TNS18 presented the strongest effect.

SEM showed removal of PA14 biofilm by TNS18

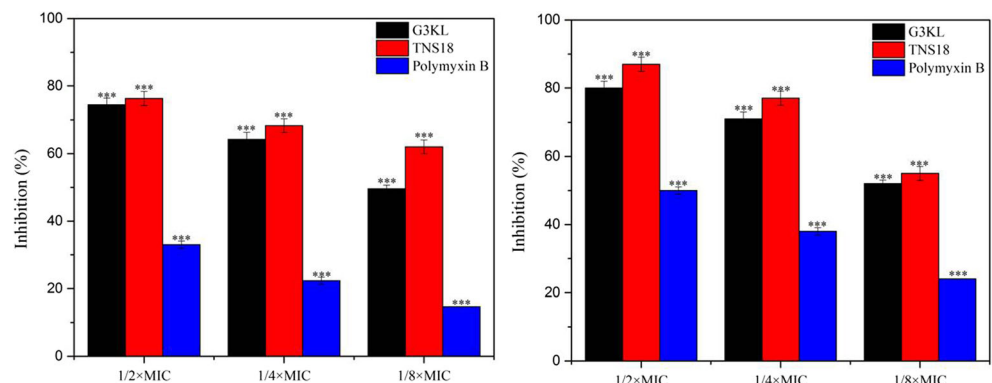
SEM observation showed that after 24-h incubation, *P. aeruginosa* PA14 secreted extracellular polymeric substances and formed compact, thick biofilm in untreated group. However, after 5-h treatment with the three peptides, the biofilm began to disperse and there was a little space between the attached cells (Fig. 4a, d, g) at a concentration equal to 1 \times MIC. With further increase in the concentrations of the peptides to 4 \times MIC, the degradation of the biofilm increased (Fig. 4b, e, h), and finally, the biofilm almost dispersed completely and dispersed bacteria were detected (Fig. 4c, f, i) at 16 \times MIC. At 16 \times MIC, the average number of the bacteria left were 103, 58, and 150 in G3KL, TNS18, and PMB-treated group,

respectively, from different positions. Among the three compounds tested, TNS18 exhibited the strongest *P. aeruginosa* biofilm dispersion effect.

CLSM showed the biofilm thickness decreased by TNS18

CLSM micrographs showed that the *P. aeruginosa* PA14 intact biofilm structure (untreated) was disrupted by TNS18, G3KL, and PMB treatments (0, 1, 4, and 16 \times MIC). The green fluorescence mostly resulting from SYTO9 binding indicated dense nucleic acids and the areas of red fluorescence owing to PI binding signified dead cells. The untreated group had intact, thick, and dense biofilm. The biofilm thickness was reduced obviously with 1 \times MIC of peptide (Fig. 5a, d, g). With the increase in the peptides concentrations equal to 4 \times MIC (Fig. 5b, e, h) and even 16 \times MIC (Fig. 5c, f, i), their effect on the dispersion of the preformed biofilm significantly increased (Fig. 5b, e, h) with lowest number of

Fig. 1 Anti-biofilm activity of peptide dendrimers by crystal violet staining (a) and by TTC staining (b). The peptides inhibited the attachment of *P. aeruginosa* PA14 and inhibited the formation of biofilm. The experiment was performed three times to obtain means and standard errors of the means. *** $P < 0.01$



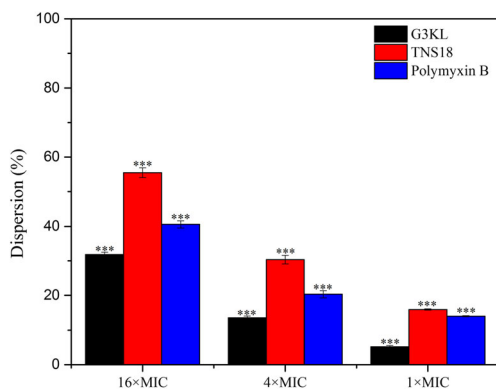


Fig. 2 In vitro activity of peptide dendrimers against preformed biofilm. The peptides dispersed the pre-matured biofilm of *P. aeruginosa*. The concentrations of the peptides were higher than the MICs. The experiment was performed three times to obtain means and standard errors of the means. *** $P < 0.01$

P. aeruginosa cells adhering to the surface (Fig. 5c, f, i). At 16x MIC, the peptides G3KL, TNS18, and PMB showed decreased biofilm thickness from 8.3 to 4.3, 3, and 5.3 μm , respectively. In particular, the biofilm dispersion effects of TNS18 and PMB were the strongest and weakest, respectively.

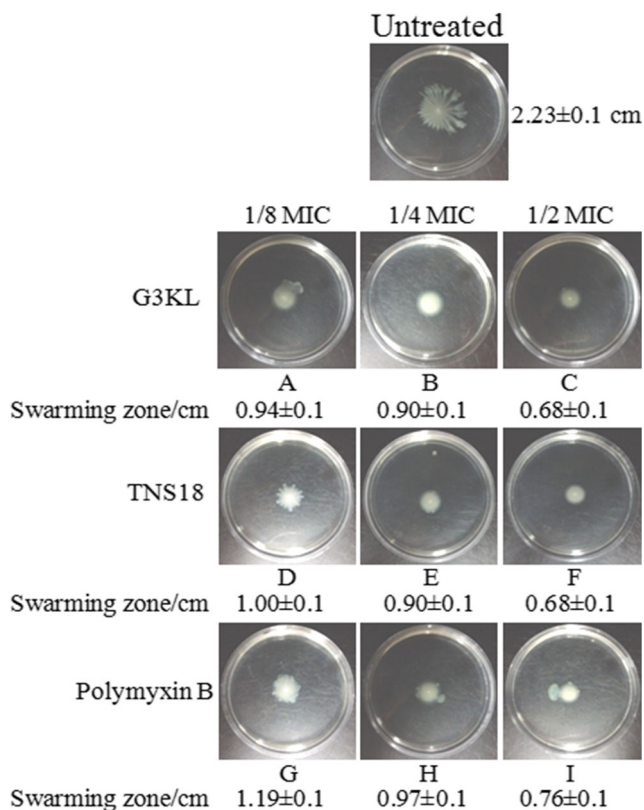


Fig. 3 The effects of the peptides on *P. aeruginosa* PA14 swarming motility. G3KL, TNS18, and PMB (0, 1/8, 1/4, and 1/2 MIC) were added to motility culture (0.5% agar, 8 g/L nutrient broth, and 5 g/L glucose) and motilities were examined. All experiments were repeated three times in two replicates

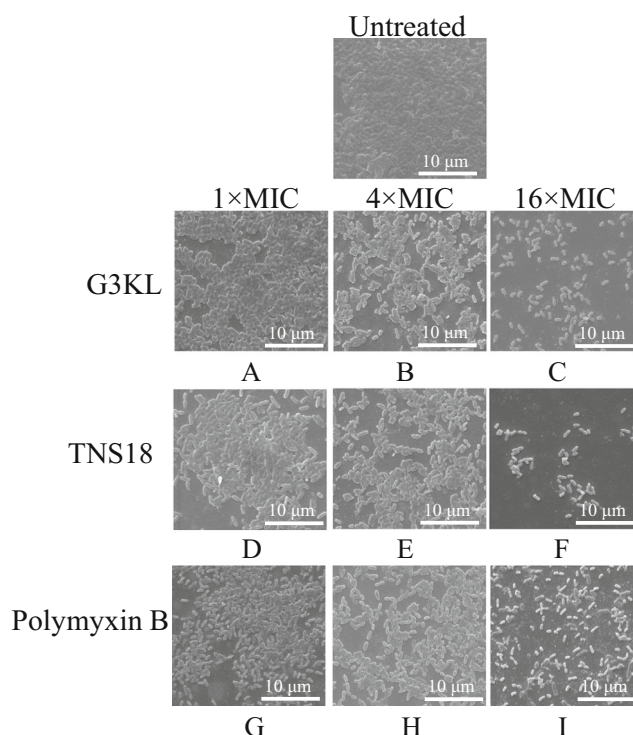


Fig. 4 Scanning electron microscopy images of biofilms of *P. aeruginosa* PA14 strain, treated with different concentrations of G3KL (a–c), TNS18 (d–f), and PMB (g–i), respectively, for 5 h. Representative images (magnification, $\times 10,000$) of *P. aeruginosa* biofilms grown on cover glass in LB medium with the indicated concentrations of peptides at 37 $^{\circ}\text{C}$ for 5 h. The concentrations of three peptides were 0 (untreated); 1x MIC (a, d, g); 4x MIC (b, e, h), and 16x MIC (c, f, i)

Membrane potential analysis indicated that the membrane integrity of *P. aeruginosa* cells was destroyed by treatments with PMB, G3KL and TNS18

Severe membrane injury usually results in membrane potential analysis dissipation and cell death due to loss of lipid bilayer function. Thus, we next assessed whether the peptide can induce loss of membrane integrity by using the lipophilic potentiometric dye disk 3 (5) assay. The addition of peptides triggered an increase in fluorescence intensity, indicating rapid membrane depolarization. Especially when the concentration is 1x MIC, the three peptides (TNS18, G3KL, and PMB)-induced potential loss reached 57%, 32%, and 31%. We observed a dose-dependent loss of membrane potential analysis in *P. aeruginosa* PA14 exposed to peptides (Fig. 6). We concluded that the lipid bilayer damage induced by peptides are followed by membrane potential dissipation and cell death.

Transcriptome analysis and RT-qPCR

To determine the differential gene expression between cells treated with AMPDs and the control, the cDNA libraries of cells treated with the peptides were subjected to high-throughput sequencing. Both RNA sequencing and

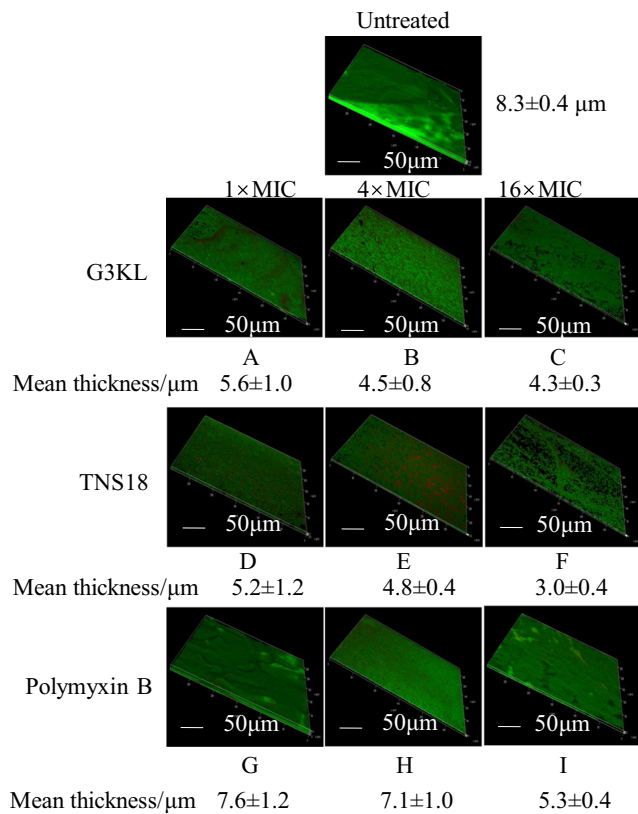


Fig. 5 Confocal laser scanning microscopy images of biofilms of *P. aeruginosa* PA14 strain, treated with different concentrations of G3KL (a–c), TNS18 (d–f), and PMB (g–i), respectively, for 5 h. The concentrations of three peptides were 0 (untreated), 1× MIC (a, d, g); 4× MIC (b, e, h); and 16× MIC (c, f, i)

comparative transcriptome analysis were employed to identify the genes and their respective expression levels in cells subjected to AMPDs treatment. By adopting a q value of ≤ 0.01

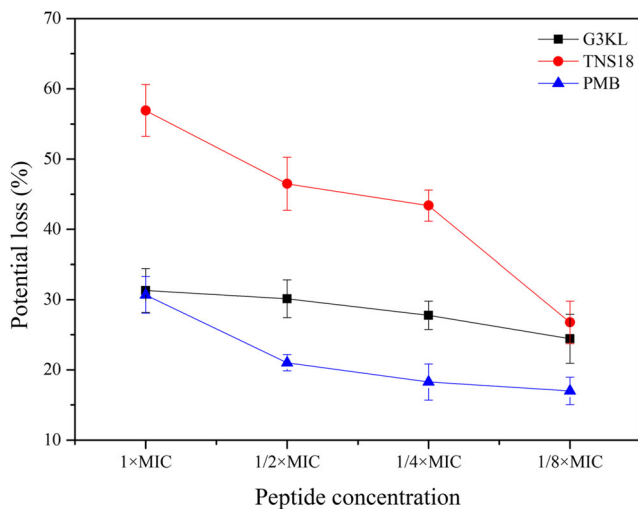


Fig. 6 Membrane potential loss due to G3KL (black squares), TNS18 (red circles), and PMB (blue triangles) exposure (0, 1/8, 1/4, 1/2, and 1 MIC) in *P. aeruginosa* measured with the potentiometric dye disk 3 (5). Data were pooled from samples (two independent experiments with triplicate) (Balasoiu et al. 2014)

and $|\log_2 \text{fold-change}|$ of > 1 to classify a transcript as being differentially expressed, 92 differentially expressed genes (52 upregulated and 40 downregulated genes) in TNS18-treated cells and 60 differentially expressed genes (53 upregulated and 7 downregulated genes) in G3KL-treated cells were identified, relative to distilled water treated cells (Table S2, S3).

It can be observed from Table 1 that the expressions of genes in the *arnBCADTEF* operon in G3KL-treated cells were upregulated, different from those in TNS18-treated group in which no significant changes were observed in the expressions of these genes. NC means that no apparent change in abundance was observed. The differences in some expressions of the transcriptome may lead in part to the distinctions of the mechanisms.

Discussion

Pseudomonas aeruginosa infections and their abilities to acquire resistance to aggressive antibiotic therapy have been the subjects of many investigations all the time. However, there are no suitable drugs against these pathogens because adaptive resistance makes them resistant to these drugs. *P. aeruginosa* usually starts with the adhesion followed by secretion of extracellular polysaccharides, mainly alginate, leading to the biofilm formation which can cause approximately two thirds of all infections (Wolfmeier et al. 2017). Currently, there are no suitable treatments to inhibit and disperse biofilm (Diez et al. 2015; Dosler and Karaaslan 2014).

Peptide dendrimers are emerging drugs that have advantages over standard antibiotics. However, the use of peptide dendrimers is a double-edged sword owing to certain disadvantages, such as toxicity to host cells and enzymatic degradation by proteases (Fuente et al. 2014; Lin et al. 2015; Zhang et al. 2016). In the present study, the activities of peptide dendrimers, which are relatively more resistant to bacterial or host proteases, against *P. aeruginosa* PA14 were investigated. The results revealed that peptide dendrimers have strong anti-biofilm activities, and can inhibit and disperse biofilm formation in a concentration-dependent manner. However, compared to the blank control, the specific growth rates of PA14 in the presence of G3KL, TNS18, and PMB at a concentration of $1/8 \times \text{MIC}$ were reduced by 12.7%, 16.4%, and 11%, respectively (Fig. S1). The biofilm inhibition rates are much higher than the growth inhibition rates. At the same time, AMPDs were found to affect swarming motility which can be described as a bacterial movement on solid medium (Strehmel et al. 2015) by probably having an effect on the flagella and polysaccharides. Therefore, the effect on swimming motility which is mediated mainly by flagella should be examined. All of these three peptides reduce the swimming zone, especially TNS18 (Fig. S2). The other concern is the effect on polysaccharides. In PA14, the main polysaccharide is

Pel and is formed between the air-liquid interface. The effect of peptides on the swarming motility of another type strain PAOI was also checked since that strain has one major polysaccharide Psl which is involved in this process. Consistently, these peptides also significantly decrease the swarming motility of *P. aeruginosa* (Fig. S3). But the effects on the swarming motility are stronger than those on the swimming motility, so the effect on swarming motility is mainly related to polysaccharide. As swarming motility is one of the factors contributing to biofilm formation, the AMPDs may influence the biofilm structure by inhibiting swarming motility.

Furthermore, the effects of these peptides on the thickness and structure of *P. aeruginosa* PA14 biofilm were examined by SEM and CLSM. CLSM revealed that treatment of the three peptides at $1\times$ MIC led to less clearance of biofilm, whereas treatment with higher concentrations of the peptides for 5 h resulted in complete eradication of preformed biofilm. These results indicated that all of the three peptides obviously reduced the biofilm thickness and destroyed the structure, when compared with that observed without peptide treatment. After 5-h incubation with TNS18 and G3KL, the green fluorescence of SYTO9 was less (Fig. 5c, f, i) when compared with that noted in the untreated group. As PI can only enter the cells with damaged membranes, this finding confirmed that biofilms exposed to TNS18 or G3KL rapidly lost membrane integrity (Manner et al. 2015). SEM assay revealed that peptides treatment at $16\times$ MIC significantly reduced the number of bacterial cells that adhered to the surface, with the multi-layer and compact bacterial cluster transformed into dispersed bacteria. In particular, TNS18 exhibited the strongest inhibitory and dispersive effect on the biofilm.

To gain insight into the molecular mechanism of TNS18 action, we performed a series of assays with bacteria. Membrane potential analysis indicated that cells treated with the three peptides presented the loss of membrane integrity which is accompanied by marked membrane depolarization and bacterial cell death.

To determine the differential gene expression between cells treated with AMPDs and the control, the cDNA libraries of cells treated with the peptides were subjected to high-throughput sequencing. The RNA sequencing analysis by RT-qPCR (Table S1) also revealed similar results (Table 1). The transcriptome of PMB-treated group was derived from the indicated reference (Murray et al. 2015). As the genes in *arnBCADTEF* operon in PMB-treated group were expressed differentially, the expression of *arnT* in G3KL-treated cells was slightly different, suggesting that the antimicrobial mechanism of G3KL might be different from that of PMB (Murray et al. 2015). However, the expression of *arnBCADTEF* operon in TNS18-treated group is totally different from those in both PMB- and G3KL-treated groups. Many Gram-negative bacteria can modify lipid A by adding some positive charges (Maria et al. 2015; Raetz and

Whitfield 2002) through regulation of the *arnBCADTEF* operon resulting in resistance to AMPs (McPhee et al. 2006), similar to that noted against PMB (Murray et al. 2015). The two-component regulatory system, PhoP-PhoQ (PhoPQ), usually controls all three modifications of lipid A by activating the expression of *pmrD* gene. This gene can induce the PmrA-PmrB two-component system (PmrAB) by post-translational modifications. These two regulatory systems control many genes related to antimicrobial resistance in bacteria. In *P. aeruginosa*, LPS modification also occurs independent of these two regulatory systems through regulation of the *arnBCADTEF* operon, resulting in resistance to AMPs (Maria et al. 2015). Most importantly, L-Ara4N transferase (ArnT) can transfer L-Ara4N to lipid A. As PMB can combine with lipid A, *P. aeruginosa* resists PMB action by regulating the *arnBCADTEF* operon (Fernandez et al. 2013). Thus, although the mechanisms of the three peptides examined are related to the cell membrane, the transcriptome of *arnBCADTEF* operon are different resulting in different mechanisms.

Peptide dendrimers contain different residues and variable amino acids, and their biological activities depend on the variations in amino acids and fatty acids. G3KL is a third-generation (G3) antimicrobial peptide dendrimer that contains lysine-leucine dipeptides linked by branching lysine residues in three successive generations (Fig. S4) (Stach et al. 2014). TNS18 is a second-generation (G2) peptide dendrimer analog of G3KL with an optimized amino acid sequence and an additional fatty acid chain that increases its activity (Siriwardena et al. 2018). Thus, the difference in their structures results in different biological mechanisms and future studies should focus on designing peptide dendrimers with different structures and compositions to achieve various biological functions.

In summary, G3KL and TNS18 were found to target not only the planktonic bacteria but also show particularly remarkable effects on *P. aeruginosa* biofilms by disrupting the membrane integrity. This study highlights the value of peptide dendrimers G3KL and TNS18 as starting materials for the development of new antimicrobial and anti-biofilm agents useful against *P. aeruginosa* infections. Imaging by SEM, CLSM, and membrane potential studies suggest that the effects of peptide dendrimers on bacteria are mediated by membrane interactions. The expressions of genes in the *arnBCADTEF* operon-regulating lipid A modification resulting in resistance to AMPs differ from each other. These results indicated that the antimicrobial mechanisms of these three peptides are related to cell membrane. In future studies, we will focus on the mechanism research.

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Compliance with ethical standards

This article does not contain any study with human participants or animals performed by any of the authors.

Conflict of interest The authors declare they have no conflict of interest.

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