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Antimicrobial effects and mechanism of action of dendrimer peptides against human pathogens

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항생제 기능성 방사형 구조 펩타이드의 개발 및
항생 활성의 작용 기작 연구

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Antimicrobial effects and mechanism of action of dendrimer peptides against human pathogens

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현재 다양한 항생제가 개발이 되어 많은 질병에 사용되었다. 하지만 항생제의 과다 및 남용으로 인하여 저항성 균이 출현하였고, 새로운 항생제의 대체물질이 요구 되었다. 그 중에 항균 펩타이드(antimicrobial peptide)는 매우 빠른 작용과 세포막을 직접 공격함으로써 내성을 가지기 어렵다는 것으로 알려져 있다. 하지만 항균 펩타이드는 인체내부에 존재 하는 효소들에 의한 안정성이 약한 경우가 많아 펩타이드 결합이 끝어지거나 박테리아에 대한 활성을 잃어버려서 항균 펩타이드가 인체내부에 침입을한 균에 작용을 못 하게 되다. 또한 항균 펩타이드가 박테리아뿐만 아니라 적혈구나 동물세포에 대해서 독성을 가진 경우가 있다. 그 대체방법으로 펩타이드의 구조를 방사형태(Dendrimer)로 만들면 박테리아에 대한 활성이 저하되지 않고, 적혈구와 동물세포에 대한 독성을 줄일 수 있는 것으로 보고됐다. 이에 박테리아에 대한 활성이 좋으나 적혈구와 동물세포에 독성이 있는 펩타이드를 디자인하여 그 구조를 방사형(dendrimer) 형태로 디자인 하여 박테리아에 대한 활성을 유지하며 적혈구 및 동물세포에 대하여 독성을 줄이고자 한다.

이 논문은 KLWK를 기본으로 펩타이드 디자인하여 반복된 구조를 가진 펩타이드를 설계하였다. 즉, 3종류의 일반적인 형태 Linear 펩타이드(LIN1-1: $(KLWK)_2$, LIN1-2: $(KLWK)_4$, LIN1-3: $(KLWK)_8$)와 3종류의 방사형인 Dendrimer(DEN1-1:

$(KLWK)_2[K]$, DEN1-2: $(KLWK)_4[K_2K]$, DEN1-3: $(KLWK)_8[K_2K]_2K$)로 디자인을 하였다. 합성된 linear 펩타이드와 dendrimer 펩타이드를 이용하여 그람 음성 균과 양성균에 대한 항균 활성과 yeast에 대한 항진균 활성을 측정을 하여 linear 펩타이드와 dendrimer 펩타이드가 다양한 균주에 대한 활성이 있음을 확인을 하였고, 항생제에 저항성을 가진 *S. aureus*와 *P. aeruginosa*에 대한 활성을 측정 한 결과 항생제 저항성을 가진 균에서도 항생 활성을 가짐을 확인 하였다. Hemolysis와 cytotoxicy test를 하여 적혈구와 동물세포에 대한 독성이 linear 펩타이드와 비교해서 dendrimer 펩타이드가 독성이 적은 것을 볼 수 있고. 또한 *P. aeruginosa*의 biofilm 형성억제와 제거반응을 확인한 결과 형성에는 좋은 활성을 보였고, biofilm 제거에는 일정농도 이상에서 항생 활성 작용한다는 것을 확인할 수 있었다. 이들 펩타이드의 작용 기작을 확인하기 위하여 time killing실험과 SYTOX green실험을 수행하였다. 그 결과 세포막에 작용 한다는 것을 유추할 수 있었으며, 주사전자현미경(SEM)을 통하여서 세포막을 작용한다는 것을 다시 한 번 확인 할 수 있었다. 결과적으로 dendrimer 펩타이드와 linear 펩타이드의 in vitro 비교 실험을 통해서 dendrimer 펩타이드가 세균에 대해서 강한 항생 활성을 유지하면서 인간 적혈구와 동물세포에대해 세포독성이 줄어든다는 것을 확인 하였다. 기존에 알려진 항균펩타이드 설계 방법 중에서 아미노산 서열을 치환하지 않고 세포독성을 줄일 수 있는 다른 하나의 방법이라 할 수 있다.

Ⅰ. Introduction

Due to the overuse antibiotics, multiple resistant bacteria have developed. Use of methicillin was first reported in 1960, but just year later in 1961, resistant strains started to appear. This has lead to a plethora of antibiotic-resistant strains since then [1-3].

The emergence of microbial pathogens resistant to conventional antibiotics has stimulated the search for new therapeutic drugs. Antimicrobial peptides (AMPs) are of particular interest as their proposed mode of action does not appear to stimulate rapid development of microbial resistance [7-9, 34]. Over the past two decades, numerous AMPs have been identified in both prokaryotes and eukaryotes [10-12]. Common features of these peptides include a positive net charge under physiological conditions, amphipathic secondary structures within membranes, small size and rapid binding to biological membranes. Although their mode of action is not well understood, it is believed that AMPs mediate their effects through disruption of the cytoplasmic membrane as well as cell division and macromolecule synthesis [13, 29]. Most AMPs are known to cause efflux of intercellular materials by destabilizing or disrupting the cytoplasmic membrane either through pore formation via a “barrel-stave” and “toroidal pore [43] ” mechanism or a nonpore “carpet-like” mechanism. Furthermore, to reach their intracellular targets, commonly nucleotides and functional proteins, AMPs must permeate the cell wall and cytoplasmic membrane [14-16].

However, due to their peptide essence, AMPs suffer from poor bioavailability and proteolytic stability, two which have that have severely hampered their clinical progress to date. In my thesis, the new peptides developed [31-33]. The new peptides, which are have repeat amino acids. that repeat amino acids synthesized to

dendrimeric structure. The dendrimeric structure repeated to the existing antimicrobial activities. But decreased the synthesited lost [17-19], as well as greater stability to peptidases and proteases, possibly due to the steric hindrance of the branching core that would limit the cleavage rates of plasma peptidases[4-6].

An amphipathic peptides designed and synthesized based KLWK repeated linear three peptides (LIN1-1: $(KLWK)_2$, LIN1-2: $(KLWK)_4$, LIN1-3: $(KLWK)_8$) and dendrimer three peptides (DEN1-1: $(KLWK)_2$ [K], DEN1-2: $(KLWK)_4$ $[K_2K]$, DEN1-3: $(KLWK)_8$ $[K_2K]_2K$).

The antimicrobial activity was tested against various bacterial strains containing multi-drug resistance strains and yeast strains. Hemolysis and cytotoxicity of the peptides were tested for toxicity in human cells. In additionally I tested inhibit and reduction of biofilm of *P. aeruginosa*. Time killing and SYTOX green experiments using synthetic peptides strains will determine how it works, and scanning electron microscopy (SEM) can determine whether the mechanism of action is mediated through the cell membrane. I found the dendrimeric structure of the amphipathic peptide greatly influences target selection and peameabilzation of the microbial cells.

Ⅱ. Material and Methods

1. Material

1.1. Materials and microorganisms

S. aureus (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 15692) were obtained from the American Type Culture Collection. *C. albicans* (KCTC 7965), *C. catenulate* (KCTC 7642), *C. glabrate* (KCTC 7219), *C. intermedia* (KCTC 7935), *C. melibiosica* (KCTC 7003), *C. rugosa* (KCTC 7288), *C. tropicallis* (KCTC 7212), *T. beigelii* (KCTC 7707) *Salmonella typhimurium* (KCTC 1926) and *Bacillus subtilis* (KCTC 1998) were obtained from the Korea Collection for Type Culture. *S. aureus* (CCARM 0027), *S. aureus* (CCARM 3018), *S. aureus* (CCARM 3089), *S. aureus* (CCARM 3090), *S. aureus* (CCARM 3114), *S. aureus* (CCARM 3126) and *S. aureus* (CCARM 3708) were obtained from the Culture Collection of Antibiotics Resistant Microbes at Seoul Women's University, Korea. *P. aeruginosa* 1162, 4007, 4891 and 5018 were resistant strains isolated from patients with otitis media at Chunnam National University Hospital.

2. Methods

2.1 Peptide synthesis

Linear peptides used in this study were prepared using solid-phase methods with Fmoc(n-(9-flurenyl) methoxy carbonyl)-protected amino acids using a Liberty microwave peptide synthesizer (CEM Co. Matthews, NC). 4-(2′,4′-Dimethoxypheny

l-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin (100-200 mesh, Novabiochem) (0.59 mmol/g) were employed to create the amidated peptides.

Dendrimer peptides synthesized with same method. and I use the Fmoc-Lys(Fmoc)-OH (Novabiochem) linker for dendrimer peptides [30].

After peptide synthesis, the crude peptides were purified on a Waters preparative HPLC system using Waters 15 μm Deltapak C_{18} column (39 x 300 mm) using a 0 - 60% acetonitrile gradient in water with 0.1% trifluoroacetic acid. The purity of the isolated peptides was determined using a Shimadzu analytical HPLC system equipped with a Vydac C_{18} column (4.6 x 250 mm, 300Å, 5 μm). The molecular masses of the peptides were confirmed using a matrix-assisted laser desorption ionization mass spectrometer (MALDI II, Kratos Analytical Ins.).

2.2 Purification of synthetic peptides

Aliquots of the ultrafiltrates were injected into a reverse phase C_{18} column (5 μ m, 300 Å 4.6 × 250 mm; Vydac, Hesperia, CA, USA) on an HPLC system (Shimadzu, Kyoto, Japan). Peptides dissolved in 0.1% (v/v) TFA in HPLC grade water (solvent A) and were then loaded onto a C_{18} RP-HPLC column in equilibrated with 0.1% TFA. Peptides were separated using a gradient of 10 - 60% acetonitrile for 50 min at a flow rate of 1 ml/min. Elutes were monitored by measuring the absorbance at 214 nm. Each fraction was pooled and dried in a freeze-dryer.

2.3 Mass Spectrometry

MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) analysis was performed in linear mode using a Voyager DE RP instrument (Perseptive Biosystems, Framingham, MA) as described by Pouvreau et al [20].

2.4 Antibacterial assay

The antibacterial activity of the synthetic peptides was determined by microdilution assay. Synthetic peptides solutions were prepared by serial dilution using PBS (1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 135 mM NaCl, pH 7.2) and SP (10 mM sodium phosphate, pH 7.4) supplemented with 10% appropriate culture media. Peptides with concentrations ranging from 0.5 to 64 μg achieved by serial dilution were added to sterile 96-well plates, after which aliquots of the cell suspension (5 x 10^5 CFU/ml) were added to each well. After incubation for 24 h, the turbidity of the suspensions was measured at a wavelength of 600nm using a VERSAmax microplate reader (Molecular Devices Co., Sunnyvale, CA, USA) to determine the minimal inhibitory concentration (MIC). The lowest peptide concentration that completely inhibited growth was defined as the MIC [21].

2.5 Antifungal assay

The fungal strains [*C. albicans*, *C. catenulate*, *C. glabrate*, *C. intermedia*, *C. melibiosica*, *C. rugosa*, *C. tropicallis*, *C. neoformans*, *T. beigelii*] were evaluated for antifungal activity by XTT assay. Briefly, fungal cells (5 × 10^5 CFU/ml) that were grown in 100 μl of yeast peptone-dextrosemedia (yeast extract 0.5%, peptone 1%, dextrose 2%, pH 5.0 - 5.5) were seeded in each well of a microtiter plate containing 100 μl of 2-fold serially diluted peptides in buffer (as described earlier). The plate was then incubated for 24 h at 28 ℃. After incubation, 5 μl of MTT solution [5 mg/ml of XTT in PBS, pH 7.4] was added to each well, after which the plates were further incubated at 37 ℃ for 4 h. Next, 30 μl of 20% SDS (w/v) containing 0.02 M HCl was added, and the plates were subsequently incubated [22].

2.6 Hemolytic assay

Fresh human red blood cells (hRBCs) from a healthy donor were centrifuged at 800 μl and washed with PBS until the supernatant was clear. The hRBCs [8% (v/v) of final concentration] were then added 2-fold serially diluted peptides in

PBS. After incubation with mild agitation for 1 h at 37 ℃, the samples were centrifuged at 800 μl for 10 min and absorbance of the supernatant was then measured at 414 nm. One hundred per cent hemolysis was defined as the absorban ce of hRBCs containing 1% Triton X-100, where 0% hemolysis consisted of hRBCs suspended in PBS. Each measurement was conducted in triplicate, and the percentage of hemolysis was calculated using the following equation [22].

2.7 Cell culture and cytotoxicity assay

To examine the cytotoxic effects of the synthetic peptides, HaCats (human keratinocytes) were grwon in Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics (100 U/μl of penicillin, 100 μg/ml of streptomycin) and 10% heat-inactivated fetal bovine serum (FBS) (v/v) at 37 ℃ in a humidified chamber containing 5% CO_2. Growth inhibition was evaluated by XTT assay to determine cell viability. A total of 2 x 10^4 cells/well was seeded into a 96-well plate, which was incubated for 24h. Peptides serially diluted two-fold with DMEM were then added to the plate, followed by incubation for 24 h at 37 ℃. Then, 10 μl of XTT (5 μg/ml) was added to each well, followed by incubation for an additional 4h. Absorbance was measured at a wavelength of 450nm using a microtiter reader (Molecular Devices Emax, CA, USA) [23]

2.8 Time-killing kinetics

Suspensions of *S. aureus* (5 × 10^5 cells/ml) were added to peptide solutions of the same concentration as these used in the membrane depolarization experiment. The bacteria were exposed to the peptides at the MIC for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, or 30 min, after which they were diluted 2-fold, plated on Tryptone soya brothagar (TSA), and incubated overnight before counting colonies [24].

2.9 SYTOX green uptake assay

S. aureus cells were grown to mid-logarithmic phase at 37 °C, washed, and suspended in SP buffer (1×10^7 cells/ml) containing 10% TSB medium, after which they were incubated with 1 μM SYTOX green for 15 min in the dark. After addition of peptides at the indicated concentrations, the time-dependent increase in fluorescence caused by binding of the cationic dye to intracellular DNA was monitored. The excitation and emission wavelengths were 485 nm and 520 nm, respectively [25].

2.10 Preparation and visualization of giant unilamellar vesicles (GUVs)

GUVs were prepared using the electro formation method originally developed by Angelova and Dimitrov [26, 27]. Briefly, phospholipid mixtures were prepared in chloroform. For this, 250 μl of the lipid mixture was deposited onto a 25 x 35 x 1.1 mm indiumthnoxide (ITO)-coated glass slide (Sigma- Aldrich, St, Louis, MO, USA), followed by spin-coating at 600 rpm for 2 min. the residual organic solvents (one containing a film of lipids) were separated using a poly (dimethylsiloxane) spacer the form an electroformation chamber (25 x 25 x 1mm). The chamber was then filled with 5 mM HEPERS buffer containing 0.1 M sucrose through a hole in the poly (dimethylsiloxane) spacer. The ITO electrodes were immediately subjected to a 1.7 V (peak to PEak), 10 Hz AC field using a function generator (Agilent 33220A, Agilent Technology, US). After 1 h, the electronic field was switched to 4 V, 4 Hz for 10 min in order to detach the formed liposomes form the electrode. The liposome solution in the electroformation chamber was gently removed, and aliquots were diluted with 5 mM HEPERS buffer contanining 0.1 M glucose. The liposome suspension (usually 10 μl) was then deposited and allowed to settle on the bottom of the microscope slides for 1 min due to the density difference between the sugar solutions inside and outside the liposomes. Contrast was enhanced using

an inverted fluorescence phase contrast microscope (IX71, Olympus, Tokyo, Japan). Images were recorded using a digital CCD camera (DP71, Olympus) equipped with a video recorder and analyzed using the software provided by the manufacturer .

2.11 Scanning electron microscopy (SEM)

S. aureus and *E. coli* bacteria at 1 x 10^7 CFU and LIN1-2, DEN1-2, DEN1-3 concentration (MIC) for each strain were incubated for 15 min. After concentration, the pellets were fixed in 500 µl of (v.v) 5% glutaraldehyde, followed by dehydration in 50, 60, 70, 80, 90 and 100% ethanol. After gold coating, the samples were examined using a scanning electron microscope (Hitachi S-2400N, Japan)

Ⅲ. Results and Discussion

1. Sequence and characterization of synthetic peptides

Dendrimer peptides designed and synthesized with KLWK repeat sequence by linker. The repeat times were 2, 4, 8. Also, linear peptides synthesized KLWK repeat linear structure. Repeat array, increased the amount of positive charge on the retention time of 18 min, 27 min or 37 min was increased. However, the dendrimer peptides, DEN1-1, DEN1-2 and DEN1-3, increased of positive charge, but 16 min, 18 min, 20 min, combined with linear an increase in peptide retention time were less than (Table 1). I showed RP-HPLC profile of synthetic peptides in Figure 1. The sample was injected into an HPLC system eguipped with a vydac C_{18} column. Elution was achieved using a linear gradient of acetonitrile in 0.1% TFA (Figure 1). Molecular masses were determined as follows using MALDI-TOF/MS: DEN1-1 1257.4 Da, DEN1-2 2627.5 Da, DEN1-3 5502.8 Da, LIN1-1 1128.8 Da, LIN1-2 2241.3 Da and LIN1-3 4462.2 Da (Figure 2).

Table 1. Sequence and physicochemical parameters of synthetic peptides

Name	Sequence	Molecular mass (Da)	Net charge	Retention time (min)[a]
DEN1-1	**$(KLWK)_2[K]$-NH_2**	**1257.4**	**+5**	**16.53**
DEN1-2	**$(KLWK)_4[K_2K]$-NH_2**	**2627.5**	**+9**	**18.59**
DEN1-3	**$(KLWK)_8[K_2K]_2K$-NH_2**	**5502.8**	**+17**	**20.82**
LIN1-1	**$(KLWK)_2$-NH_2**	**1128.8**	**+5**	**18.28**
LIN1-2	**$(KLWK)_4$-NH_2**	**2241.3**	**+9**	**27.67**
LIN1-3	**$(KLWK)_8$-NH_2**	**4462.2**	**+17**	**34.69**

[a] Samples were injected into a C_{18} RP-HPLC system and were run using a linear gradient of 10~60% acetonitrile containing 0.75% trifluoroacetic acid for 50 min.

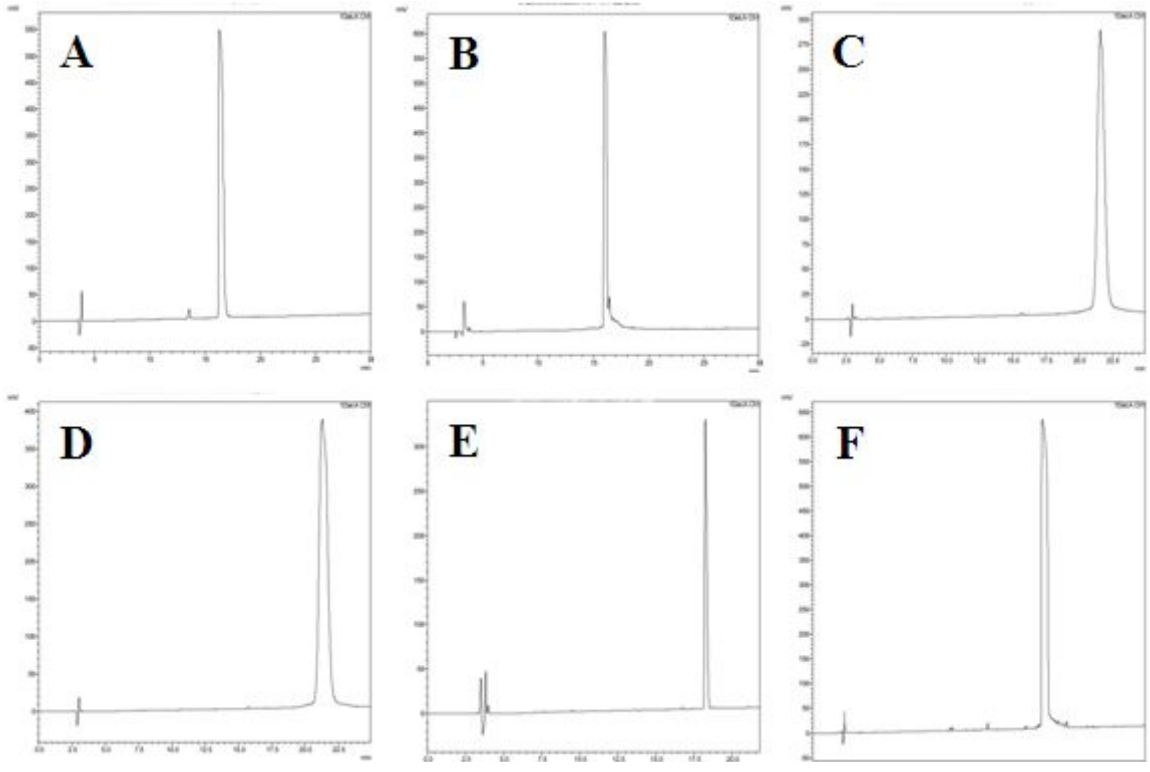

Figure 1 RP-HPLC profile of synthetic peptides (A. DEN1-1. B. DEN1-2, C. DEN1-3, D. LIN1-1, E. LIN1-2, F. LIN1-3). Sample was injected into an HPLC system equipped with a vydac C_{18} column. Elution was achieved with a linear gradient of acetonitrile in 0.1% TFA.

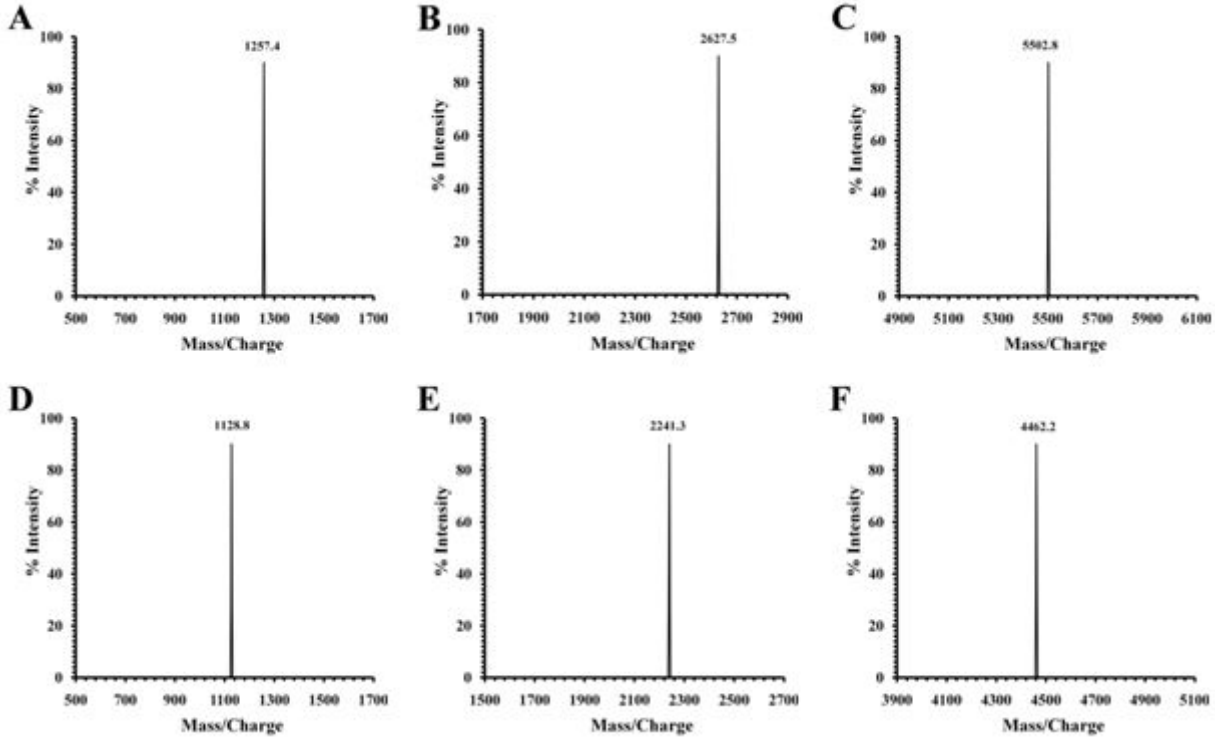

Figure 2 Mass spectrum of synthetic peptides (A. DEN1-1. B. DEN1-2, C. DEN1-3, D. LIN1-1, E. LIN1-2, F. LIN1-3). Purified peptides were run on MALDI TOF/MS.

2. Biological activity of synthetic peptides

To determine the antibacterial activity of synthetic peptides, both microdilution assay was performed in low (10 mM sodium phosphate. pH 7.2) or high (phosphate-buffered saline, PBS) ionic strength buffer against two Gram-positive and Gram-negative bacteria (Table 2). Low ionic strength buffer was used, LIN1-2 and DEN1-2 displayed superior antibacterial activity against all tested strains at concentrations ranging from 2.5 to 10 μg. In addition, LIN1-1, DEN1-1 and DEN1-3 displayed antibacterial activity against at concentrations ranging from 10 to 80 μg, both LIN1-3 showed antibacterial activity 160 μg. But high ionic strength buffer was used LIN1-2, DEN1-2 and DEN1-3, came up with similar antibacterial activity results. On the other hand, LIN1-2 and DEN1-1 were antibacterial activity decreased, but LIN1-3 was antibacterial activity results improved.

E. coli cells were incubated for 4 h at 37 ℃ in the absence or presence of peptides. Bacterial morphological changes were visualized under inverted microscopy. DEN1-3 and LIN1-3 were after treated peptide, morphology of *E. coli* was elongated and expended. LIN1-2 was after treated peptide morphology of *E. coli* cells were aggregated (Figure 3A). As well as, *S. aureus* cells were incubated for 4 h at 37 ℃ in the absence or presence of MIC peptides. Bacterial morphological changes were visualized under inverted microscopy. DEN1-2 and DEN1-3 were after treated peptide, morphology of *S. aureus* was expanded into a round shape. LIN1-3 was after treated peptide, morphology of *S. aureus* was elongated and aggregated (Figure 3B).

The antiungal activity of synthetic peptides. both microdilution assay was performed in RPMI 1640 containing MOPS (pH 7.2) against 9 strains yeast and fungal (Table 3). Antifungal activity against synthetic peptides confirmed the results LIN1-2 and DEN1-2 showed superior antifungal activity against all tested strains at concentrations ranging from 1.25 to 40 μg.

The hemolytic effect of peptides were test against hRBCs (Figure 4A). DEN1-3 caused 15% hemolysis 10 μg, respectively, while no hemolysis showed in the presence of 400 μg LIN1-1, DEN1-1 and DEN1-2, comparing to high hemolytic activity of LIN1-2 and LIN1-3. This indicated that they exerted a selective antibacterial action. As well as, The cytotoxic effect of peptides were test against HaCat (Figure 4B). Lin1-2 caused 20% cell survivals 200 μg, repectively, while no cytotoxic showed in the presence of 200 μg DEN1-2.

As a result, linear peptides and dendrimer peptides on the activity of bacteria and fungi show similar results. However, human red blood cells (hRBC) and mammalian cells for the cytoxicity , but a and b indicate the toxicity does not appear toxic, LIN1-3 compared to the more toxic DEN1-3 shows a very low position. when you modify a to b to maintain the activity and toxicity for human red blood cells (hRBC) and mammalian cells are not represented.

Table 2. Antibacterial activity of synthetic peptides

Peptide	MIC (μg/mL)[a]			
	Gram (-) bacteria		Gram (+) bacteria	
	E. coli	*S. typhimurium*	*S. aureus*	*B. subtilis*
DEN1-1	**20 (>160)**	**10 (160)**	**80 (>160)**	**40 (>160)**
DEN1-2	**5 (10)**	**2.5 (2.5)**	**10 (20)**	**10 (10)**
DEN1-3	**10 (40)**	**10 (2.5)**	**10 (10)**	**5 (5)**
LIN1-1	**20 (>160)**	**40 (160)**	**80 (>160)**	**40 (>160)**
LIN1-2	**10 (2.5)**	**5 (2.5)**	**10 (2.5)**	**5 (5)**
LIN1-3	**>160 (20)**	**160 (10)**	**160 (40)**	**160 (20)**

[a] MICs were determined with 10 mM sodium phosphate (pH 7.4) containing 10% TSB medium (low ionic strength buffer) and PBS containing 10% TSB medium (high ionic strength buffer, number in parenthesis).

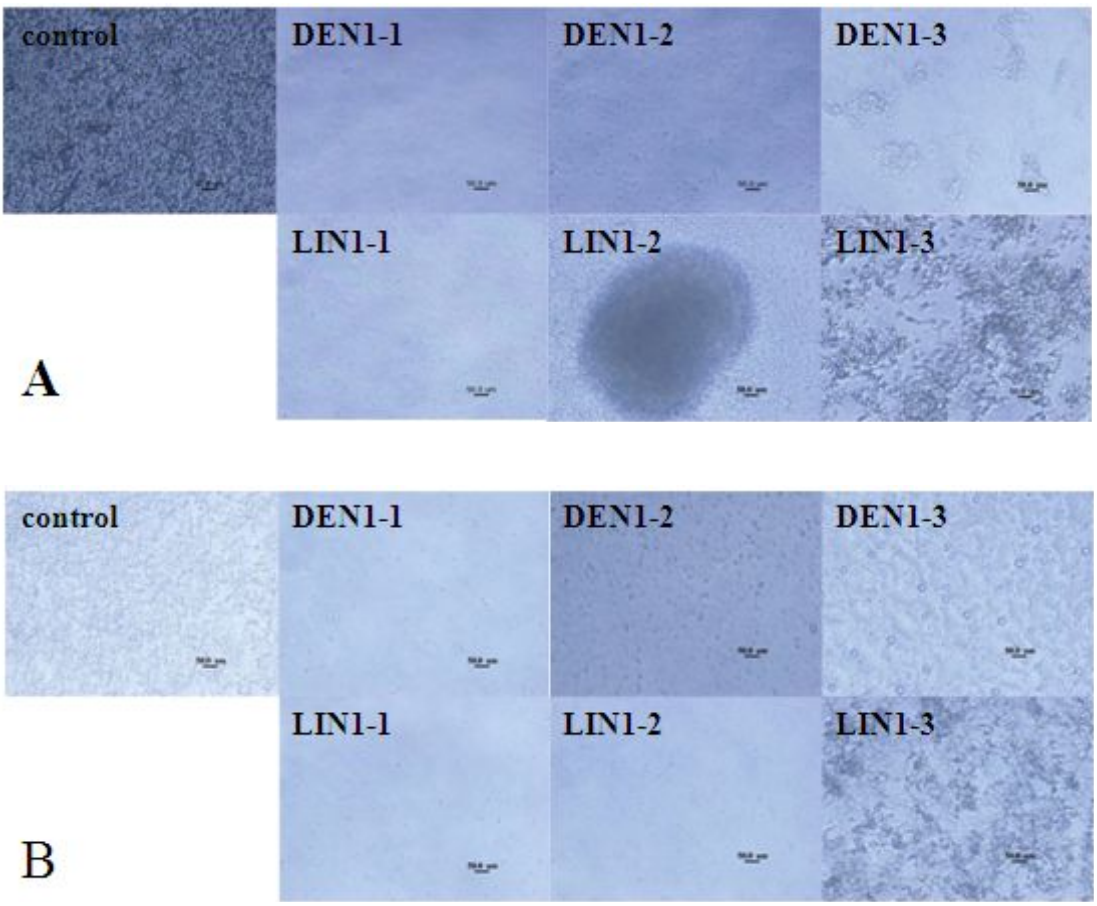

Figure 3. Micrographs of *E. coli* and *S. aureus* cells incubated with synthetic peptides *E. coli* (A) and *S. aureus* (B) cells were incubated for 4 h at 37℃ in the presence or absence of MIC peptides. Bacterial morphological changes were visualized by inverted microscopy. Bar is sized to 50 ㎛

Table 3. Antifungal activity of synthetic peptides against yeast and fungal

Peptide	MIC (μg/mL)[a]								
	C. albicans	*C. catenulate*	*C. glabrate*	*C. intermedia*	*C. melibiosica*	*C. rugosa*	*C. tropicallis*	*C. neoformans*	*T. beigelii*
DEN1-1	**>160**	**>160**	**>160**	**>160**	**>160**	**>160**	**40**	**160**	**10**
DEN1-2	**40**	**20**	**40**	**20-40**	**<1.25**	**40**	**2.5-5**	**10**	**<1.25**
DEN1-3	**160**	**2.5**	**80**	**>160**	**40-80**	**80**	**<1.25**	**10**	**<1.25**
LIN1-1	**>160**	**>160**	**>160**	**160**	**160**	**>160**	**40**	**80**	**20**
LIN1-2	**5**	**<1.25**	**10**	**5**	**2.5**	**10**	**2.5-5**	**5**	**<1.25**
LIN1-3	**80**	**5**	**5**	**80-160**	**80**	**2.5**	**10**	**40**	**20**

[a] MICs were determined with RPMI 1640 containing MOPS (pH 7.2).

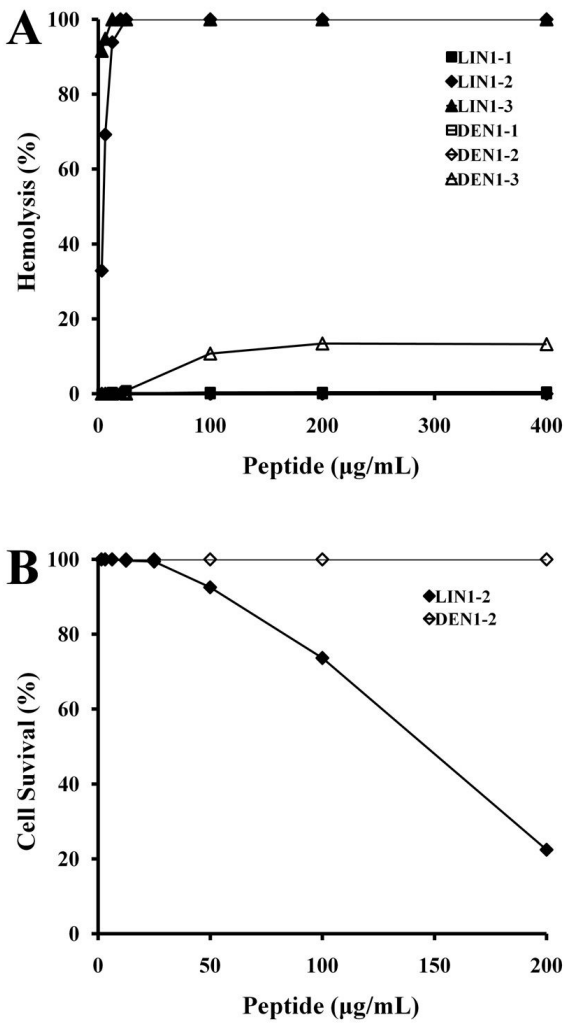

Figure 4. Hemolytic and cytotoxic effect of synthetic peptides

(A) Hemoglobin release activity was dose-dependently measured after incubation for 60 min with human erythrocytes (8% hematocrit). Absorbance of the supernatant was measured at 414 nm. (B) Cytotoxicities of the two peptides were detemined by XTT assay. HaCat cells (2 x 10^4 cells/well) were exposed to DEN1-2 and LIN1-2 for 24 h. XTT (50 μg) was added to each well, and the plate incubated for 4 h. Absorbance of the supernatant was measured at 450 nm.

3. Drug-resistant activity of synthetic peptides

To determine the drug resistance bacteria activity of synthetic peptides. Drug-resistant *S. aureus* 7 strains used (Table 4). LIN1-2, DEN1-2 and DEN1-3 displayed superior antibacterial activity against all tested strains at concentrations ranging from 4 to 16 μg. In addition, LIN1-3 displayed antibacterial activity against at concentrations ranging from 16 to 64 μg, both LIN1-1 and DEN1-1 showed antibacterial activity 64 μg.

Anti-biofilm activity of synthetic peptides against non-drugresistant and drugresistant *P. aeruginosa* 4 strins planktonic cells. LIN1-2 and DEN1-2 displayed superior antibacterial activity against all tested strains at concentrations ranging from 20 to 80 μg. In addition, DEN1-3 displayed antibacterial activity against at concentrations ranging from 40 to 80 μg, both LIN1-1, LIN1-3 and DEN1-3 showed antibacterial activity 160 μg (Table 5). Inhibition of biofilm formation of synthetic peptides against drug-resistance *P. aeruginosa*. Cells were incubated for 12 h at 37 ℃ of treated peptides. The result show that LIN1-2, DEN1-2 and DEN1-3 caused 100% activity at a concentration range 20 to 40 μg (Figure 5). Biofilm reduction activity of synthetic peptides against drug-resistance *P. aeruginosa*. Cells were incubated for 24 h at 37 ℃ and treated peptides incubated 12 h. The result show that DEN1-2 and DEN1-3 caused 60% activity at a concentration 160 μg (Figure 6).

As a result, linear peptides and dendrimer peptides on the activity of drug-resistant bacteria showed similar results. However, biofilm formation inhibition and biofilm reduction activity tested results. Activity of the dendrimer peptide than linear peptides were immiscible [7].

Table 4. Antibacterial activity of synthetic peptides against drug-resistant *S. aureus* strains

Peptide	MIC (μg/mL)[a]						
	S. aureus 0027	*S. aureus* 3018	*S. aureus* 3089	*S. aureus* 3090	*S. aureus* 3114	*S. aureus* 3126	*S. aureus* 3708
LIN1-1	**64**	**64**	**16**	**16**	**>64**	**2**	**64**
LIN1-2	**4**	**4**	**4**	**4**	**8**	**4**	**8**
LIN1-3	**16**	**32**	**32**	**32**	**>64**	**32**	**>64**
DEN1-1	**32**	**>64**	**>64**	**64**	**>64**	**8**	**16**
DEN1-2	**8**	**16**	**4**	**16**	**>64**	**8**	**2**
DEN1-3	**8**	**8**	**8**	**8**	**>64**	**8**	**4**

Drug-resistant *S. aureus* strains were distributed from Culture Collection of Antibiotics Resistance Microbes at the Seoul Women`s University, Korea

Table 5. Antibacterial activity of synthetic peptides against drug-resistant *P. aeruginosa* strains

Peptide	MIC (μg/mL)[a]				
	P. aeruginosa ATCC 15692	*P. aeruginosa* 1162	*P. aeruginosa* 4007	*P. aeruginosa* 4891	*P. aeruginosa* 5018
DEN1-1	**>160**	**>160**	**>160**	**>160**	**>160**
DEN1-2	**20**	**20-40**	**80**	**40**	**20-40**
DEN1-3	**40**	**40**	**80**	**80**	**80**
LIN1-1	**>160**	**>160**	**>160**	**>160**	**>160**
LIN1-2	**40**	**20-40**	**20**	**40**	**20-40**
LIN1-3	**>160**	**160**	**160**	**160**	**160**

P. aeruginosa 1162, 4007, 4891 and 5018 were resistant strains isolated from patients with otitis media in a Chunnam National University Hospital.
P. aeruginosa (ATCC 15692) were obtained from American Type Culture Collection.

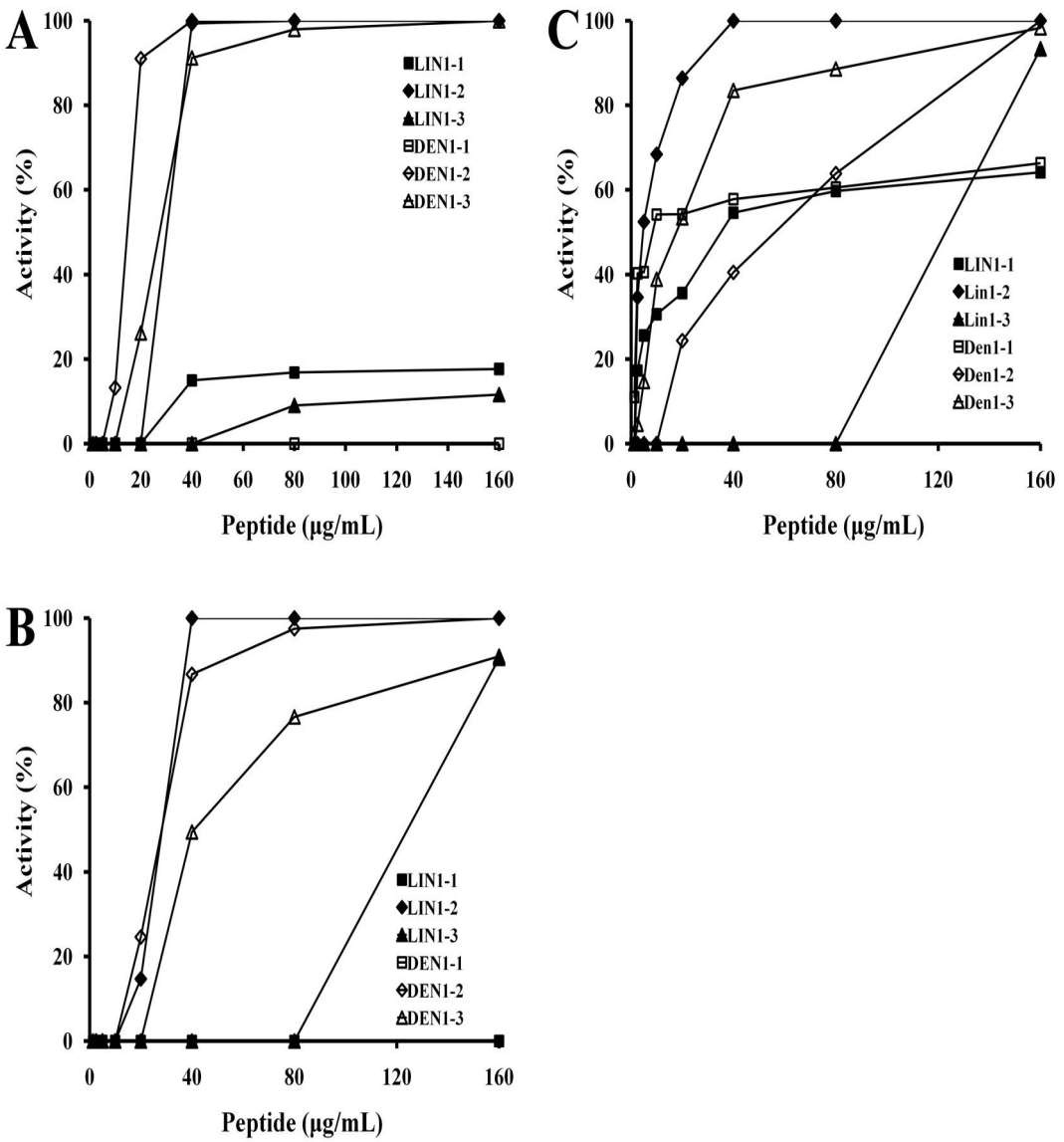

Figure 5. Biofilm inhibition of synthetic peptides against non-resistant and drug-resistant *P. aeruginosa* strains Cells were incubated for 12 h at 37 ℃ with synthetic peptides (A. *P. aeruginosa* 15692 B. *P. aeruginosa* 1162 C. *P. aeruginosa* 5018). The absorbance of the supernatant was measured at 450 nm.

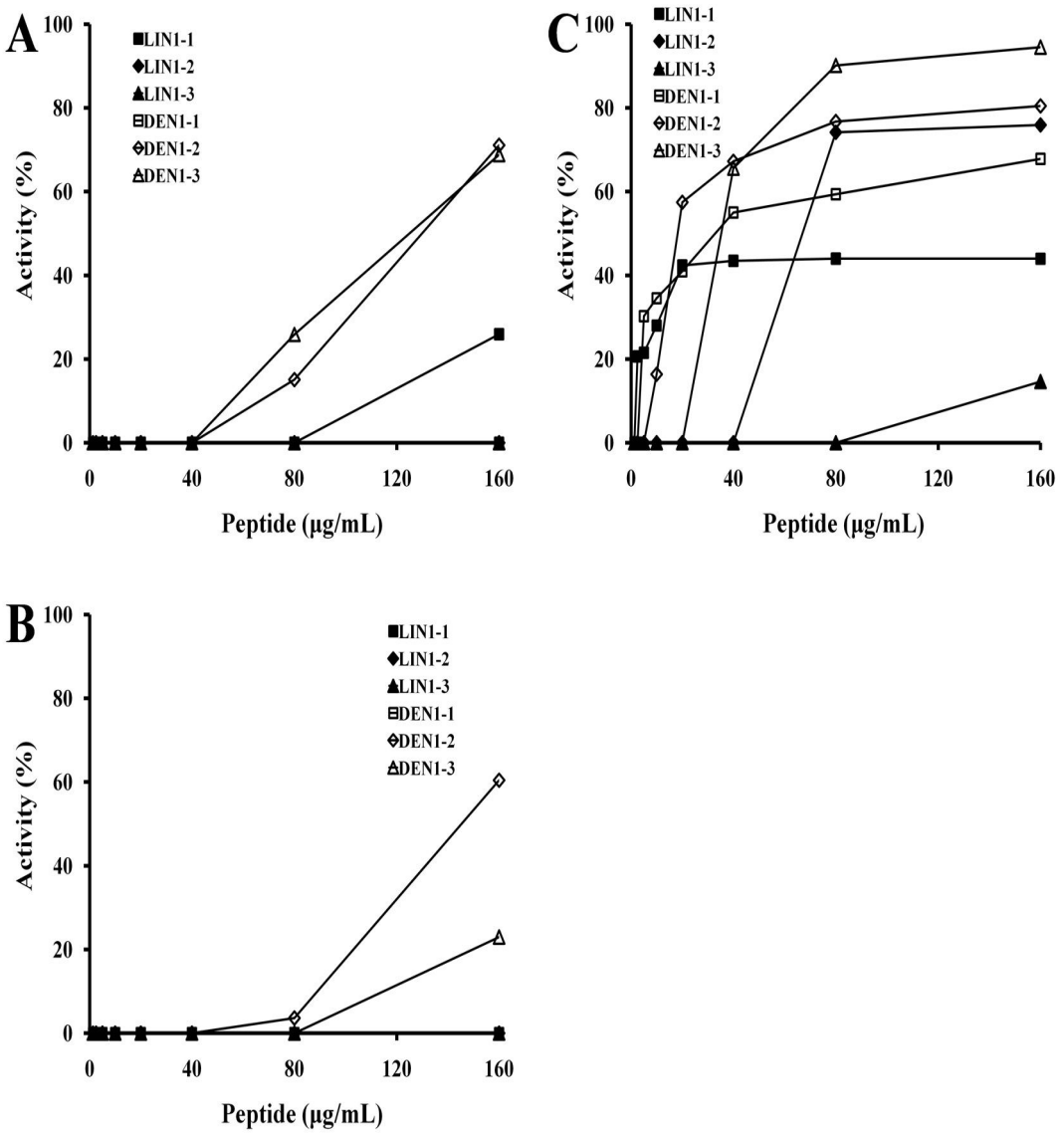

Figure 6. Biofilm reduction of synthetic peptides against non-resistant and drug-resistant *P. aeruginosa* strains Cells were incubated for 24 h at 37 ℃ with synthetic peptides incubated 12 h. (A. *P. aeruginosa* 15692 B. *P. aeruginosa* 1162 C. *P. aeruginosa* 5018). Absorbance of the supernatant was measured at 450 nm.

4. Membrane-permeable action in *S. aureus*

Bactericidal activity of the peptide was evaluated in a time-dependent manner. At the indicated time, *S. aureus* (ATCC 25923) cells were exposed to LIN1-2, DEN1-2 and plated on an agar plate. In Figure 7, LIN1-2 and DEN1-2 completely killed bacteria within at the MIC for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 30 min. The results show that the number of CFUs drastically decreased. This further suggests that the peptides interactied with the bacteral membrane. Using SYTOX green uptake assay. which is based on the interaction between the dye and nucleic acids. we investigated whether or not the peptide affents the bacterial inner membrane. The SYTOX green uptake assay showed results between treated with MIC of the peptides. The result show fluorescence increased (Figure. 7). These results indicate that synthetic peptides rapidly interacts with Gram-positive bacteria at the inner membrane.

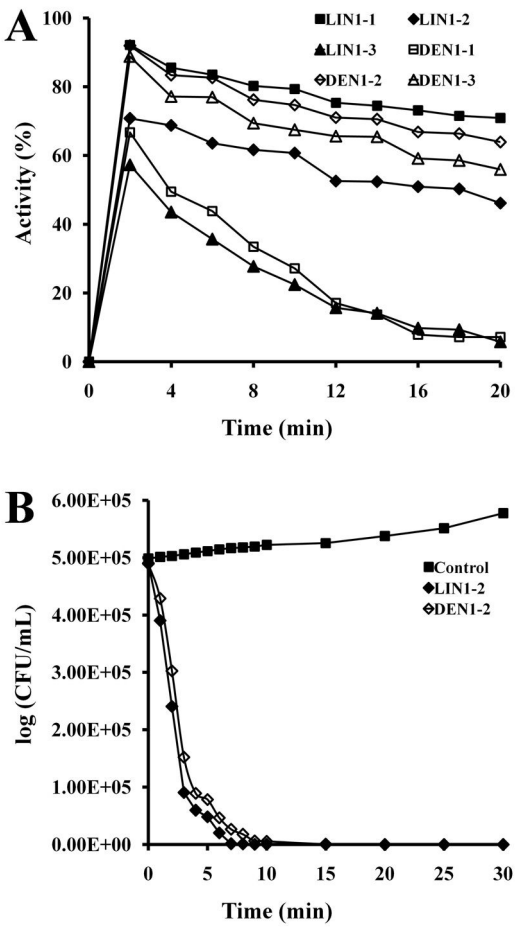

Figure 7. Time-killing kinetic and flouresence of synthetic peptides against *S. aureus* (ATCC 25923) cells. (A) To examine the interaction of synthetic peptides with the inner bacterial membrane, *S. aureus* (ATCC 25923) cells were incubated with 1 μM SYTOX green for 15 min in the dark. synthetic peptide was added when basal fluorescence reached a constant value, and the increase in fluorescence was monitored at a corresponding time. Excitation and emission wavelengths were 485 nm and 520 nm, respectively. (B) To examine time-kill kinetic of LIN1-2 and DEN1-2, *S. aureus* (ATCC 25923) cells were incubated with 2 μM of peptides (MIXs) and transferred to an agar plate at the indicated time.

5. Preparation and visualization of giant unilamellar vesicles (GUVs)

GUVs were prepared to observe the effects of synthetic peptides on artificial lipid vesicles. The results of GUVs LIN1-2 and DEN1-2 acts on the cell membrane. After additon of the LIN1-2 and DEN1-2, Both LIN1-2 and DEN1-2 in 10 seconds was the membrane action (Figure. 8). GUV debris then settled to the bottom of the slide glass [8]. Therefore, the peptides interacted with and lysed the liposomal membrane.

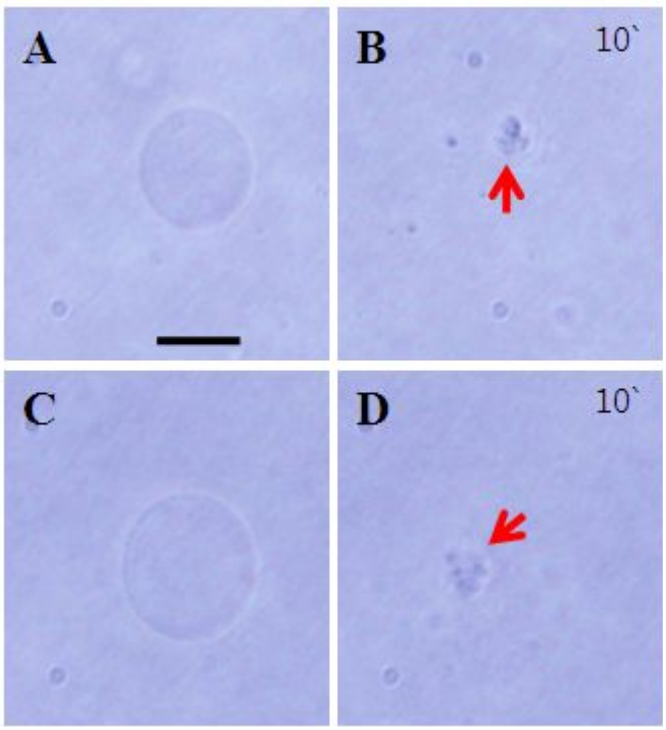

Figure 8. Microscopic images of liposomes (PE/PG) Morphological changes in PE/PG liposome in the absence (A and B) or presence of synthetic peptide (C. LIN1-2, D. DEN1-2) were examined using a digital camera (DP71, Olympus). Side bar 50 μm

6. Scanning electron microscopy (SEM)

In order to investigate the action of synthetic peptides, the peptides were for incubated 15 min with *S. aureus* at MIC. Scanning electron microscopy (SEM) confirmed that DEN1-2. That peptides were action by similar method. The membrane of *S. aureus* was formed blebs by DEN1-2.

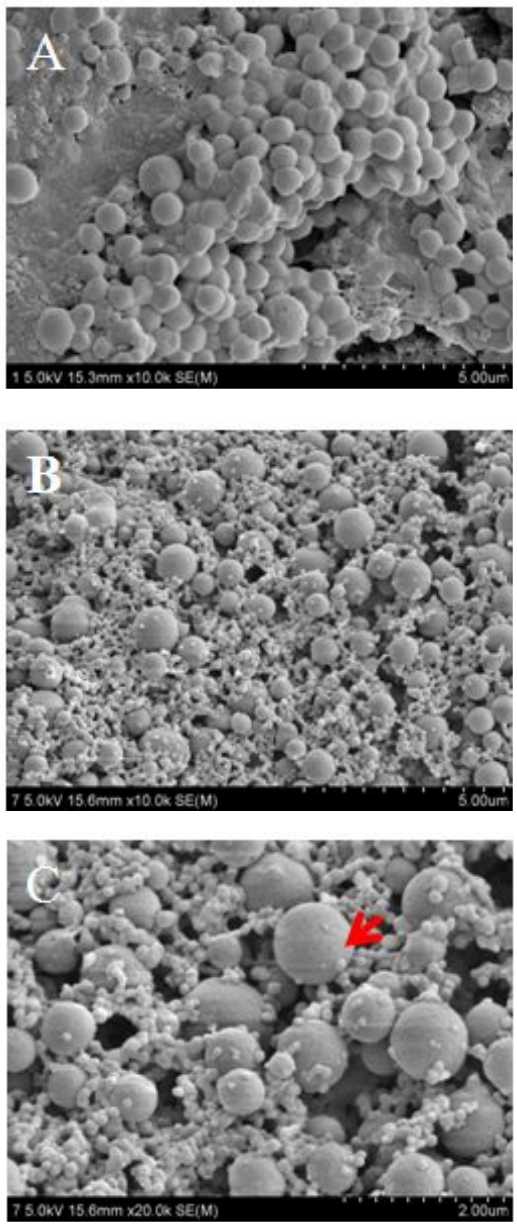

Figure 9 Morphological changes *of S. aureus* by scanning electron microscopy (SEM) *S. aureus* were incubatied with peptide for 15 min at MIC A: only *S. aureus*, B: celsl with DEN1-2 10 μg, x5000 C: cells with DEN1-2 10μg x10000

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