교육학석사학위논문

프롤린으로 꺾인 양면성 알파나선구조 펩타이드의 그람 음성균 외막 섭동에 의한 다양한 항생제와의 시너지 항균효과 향상에 관한 연구

Proline hinged amphipathic α-helical peptide enhances synergistic antimicrobial activity with various antibiotics by perturbing outer membrane of gram-negative bacteria

2016년 12월

서울대학교 대학원

과학교육과 화학전공

최 윤 화

Abstract

Proline hinged amphipathic α-helical peptide enhances synergistic antimicrobial activity with various antibiotics by perturbing outer membrane of gram-negative bacteria

Yunhwa Choi

Department of Science Education

(Major in Chemistry)

The Graduate School

Seoul National University

The proline hinge is a frequently observed conformation in antimicrobial peptides with high α-helical propensity. Since it disrupts intra-molecular hydrogen bonding cascade, hinged peptides have lower α-helicity and significantly reduce membrane-disrupting ability of host cells, affording alleviated toxicity. Furthermore, some hinged peptides give improved bactericidal effects. Thus, specific proline hinge might be quite important for

increasing selective antimicrobial activity. In order to address this matter, a small proline-scanning library was made by using amphipathic 14-aa long LK and KL model peptides that are comprised with lysine and leucine. After measuring minimum inhibitory concentrations against E.coli, S.aureus and hemolytic activity against red blood cell, the hinged peptides could be categorized into three groups. One group showed complete loss of hemolytic activity and antimicrobial activity against S. aureus, while retained activity against E.coli. Since these peptides show the anticipated selectivity, further investigation for peptides in the group was done. KL-L9P, one of the peptides, showed the best synergy with antibiotics, which were not used against gram-negative bugs because of impermeability of the drug against outer membrane. Sytox Green staining and NPN assay showed that the peptide mainly perturbs outer membrane, while it does not demolish or penetrate into inner membrane. Consequently, three important standards; weak hemolytic activity, high OM perturbing ability and weak IM demolishing ability are found and they might be important factors to design synergistic antimicrobial peptide.

Keywords: Proline hinged antimicrobial peptide, synergistic effect, Inner membrane demolishing, Outer membrane perturbing,

Student number: 2015-21625

Contents

Abstract i

Contents iii

List of tables iv

List of figures v

Introduction **1**

Experimental Section **3**

1. Peptide synthesis 4
2. Hemolysis assay 13
3. Minimum inhibitory concentration 13
4. Flow cytometry analysis 14
5. Inner membrane mechanism study (Sytox Green staining) 15
6. Outer membrane mechanism study (NPN assay) 16
7. AMP antibiotic interaction assay 16
8. Time klling assay..... 17

Results and Discussion **18**

1. Hemolytic activity and MIC of Pro hinged peptides 18
2. Cell penetrating specificity of Pro hinged peptides to Hela and E.coli 24
3. Inner membrane and Outer membrane mechanism study 26
4. Synergistic effect of KL-L9P with sevral antibiotic combinations 30
5. Difference of synergistic effect between melittin and group 1, 2, 3 Pro mutants with

erythromycin 37

6. Summary 40

References 42

Abstract (in Korean) 44

List of tables

Table 1. MIC and hemolytic activities and hydrophobicity of LK Pro mutants 21

Table 2. MIC and hemolytic activities and hydrophobicity of KL Pro mutants 22

Table 3. MIC of antibiotics against gram-negative, gram-positive bacteria 32

Table 4. Combination MIC of KL-L9P with antibiotics and FICI 34

Table 5. Combination MIC and FICI of erythromycin with melittin and group 1, 2, 3 Pro mutants 39

List of figures

Figure 1. Helical wheel diagram of LK and KL peptides …..…… 20

Figure 2. Hemolytic activity of LK, KL Pro mutant peptides …… 23

Figure 3. Cellular uptake of LK, KL Pro mutant peptides in Hela and E.coli …… 25

Figure 4. Comparison of inner membrane demolishing ability; group 1, 2, 3 Pro mutant peptides …… 28

Figure 5. NPN assay; Outer membrane perturbation of group 1, 2, 3 Pro mutants..… 29

Figure 6. Peptide with antibiotic novel interaction assay ...…… 33

Figure 7. Time killing assay of KL-L9P and antibiotic combinations …… 35

Introduction

After a successful period of generating various antibiotics against bacteria in 1950-1980, we are now confronting an era of a worrying prospect: the drug resistant bacteria are enormously diverse and prevalent, while new drugs against them are only a few. Especially, there are several new antibiotics in clinical trials against gram-positives, whereas no new pharmacopore approved by US FDA against gram-negative bacteria in recent 3 decades. Therefore, any kind of antibiotics or synergistic sensitizing molecules are urgently needed against gram-negative bacteria, irrespective of their known or new pharmacophores.

An apparent reason for no new pharmacophore against gram-negatives is its formidable nature of outer membrane (OM).[1] The lipid composition of the OM is very asymmetric. The inner leaflet is composed of phospholipids (PL), consisting of mainly phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) and the outer leaflet is composed of glycolipids. Furthermore, end of the outer leaflet of OM consist of heavily glycosylated lipid known as lipopolysaccharides (LPS), giving different environments from rest of the membrane. These membrane structures surely are barriers of some antibiotics that are only effective against gram-positives and not effective against gram-negatives.[2]

A few sensitized or permeable substances against gram-negative bacteria have been investigated. Most of them are general metal binder[3] or acids[4] that interrupt divalent ion binding to membrane components and general detergent-like molecules.[5] Recently non-natural AMPs were reported to possess ability of anion

clustering or altering package and reorganization of the lipids.[6, 7] In contrast to general sensitizers, these peptides possess selective permeability against gram-negatives, rendering its cytotoxicity against host cells. Even though the α-helical propensity is one of the essential factors for antimicrobial activities, recent data from advance researches suggest that a deviation from a perfect helicity and bent or kinked conformation gives more antimicrobial potency. Furthermore, a deviation from the perfect helical structure is one of strategies to increase therapeutic index (or selectivity) of the antimicrobial peptides, because amphipathic peptides with high helical propensity give disruption of eukaryotic host cells.

Even though there are numerous chemical ways to make bent or kinked helicity, nature uses proline (Pro) residues by breaking a series of hydrogen bonds cascade. There are several examples of natural antimicrobial peptides that contain Pro residue in the middle of helical structures to make a hinge or kinked conformation; fowlicidin from chicken has the central cationic kink region[8] and melittin,[9] from bee, has Pro residues with extended helices. Both peptides showed improved selectivity against pathogens, suggesting that the Pro hinge is nature's strategy to reduce cytotoxicity of the host cell. Reduction of N- nor C-terminal helical length is not sufficient to confer antimicrobial function, suggesting that bent helical conformation is essential for the function. However, antimicrobial peptides with proline hinges might possess different mode(s) of action than membrane disruption. Buforin II, an analogue of histone 2A protein with the proline hinge, penetrated cell membrane by making small holes.[10] Cecropin-magainin hybrid peptide with a

proline hinge also forms holes in bacterial cell membrane.[11] Improved antimicrobial activity with a hinged helical peptide was also observed in another histone-derived antimicrobial peptide, DesHDAP1, by inducing more translocation of the peptide targeting internal DNA.[12] Recently, Pro-containing peptide with a bent structure can stay in OM and induces ion clustering to loosen membrane.[13] Even though there are several Pro-containing natural antimicrobial peptides, the anti-bacterial mode of action by the proline hinge containing peptides are still a controversial issue whether it improves membrane disruption or it create another mode of action such as translocation through membrane or just activating membrane.

Proline hinge might be a plausible α-helical breaker of amphipathic peptide that gives a distinctive mechanism without membrane disruption. The conformational changes might affect antimicrobial activity by activating outer membrane of gram-negative pathogen. In this communication, libraries of proline scanning peptide were generated using model amphipathic LK and KL peptides and their antimicrobial and toxic activities against pathogens and host cells were measured, respectively. One kind of proline-hinge peptide, KL-L9P, showed outer membrane-perturbing but neither penetrating nor demolishing ability. The ability gave a synergistic effect with antibiotics that were otherwise could not get into the membrane and has no antimicrobial activity against gram-negative E.coli. This peptide, here, works as a sensitizer to increase efficacy of antibiotics for gram-positive bacteria which cannot go through LPS layer of gram-negative bacteria, making potent and selective antimicrobial combination therapy against gram-negative bacteria.

Experimental Section

1. Peptide synthesis

A. General

Peptides were synthesized by standard Fmoc solid-phase peptide synthesis method on Discover SPS Microwave Peptide Synthesizer (CEM). Rink Amide MBHA resin (Novabiochem, 0359 mmol/g) was used and synthesized in 50 mg (29.5 μmol) scale. Fmoc-protected amino acids (Novabiochem) were used. Fmoc protecting group was deprotected by 20 % piperidine in DMF (Avantor) and resin was rinsed by DMF and DCM (Daejung) with vacuum aspirator(Promega). 6 eq of Fmoc-protected amino acid, PyBOP (Novabiochem) and DIPEA were dissolved in DMF to react couping reaction with SPS Microwave.

Cleavage cocktail was composed of 950 μL TFA (Sigma), 25 μL TIS (Sigma) and 25 μL distilled water (TFA : TIS : DW = 95 : 2.5 : 2.5). Resin was moved to peptide column and 1 mL cleavage coctail solution was added. Cleavage reaction was done by shaker 500 – 700 rpm for 2 hours. TFA solution was moved to 15 mL conical tubes and TFA was removed by nitrogen gas until small amount of TFA remained. Crystallization solution (n-hexane : diethyl ether = 1 : 1) stored at – 20 ℃ was added to TFA solution and voltexed for 2 – 3 min. Peptide solutions were centrifuged at 5000 rpm, 4 ℃ and supernatant was removed. DMSO (Deajung) 200-300 μL was added and filterized by 0.45 μm filter. Peptide in DMSO was purified by HPLC

with Zorbax C18 column. Binary solvent system composed of solvent A (distilled water, 0.1 % v/v TFA) and solvent B (acetonitrile, 0.1 % v/v TFA) was used for seperating peptide DMSO solution. Gradient was 0 - 5 min, 5 %, 5-70 % of buffer B for 5 - 30 min, and 70-100 % of boffer B for 30 - 40 min. The peptides purified with HPLC were lyophilized by freeze dryer (Operon). Concentration of synthesized peptides were measured by IR spectrometer (Direct detect™ spectrometer, Merk millipore).

B. Acetylation of N-terminal

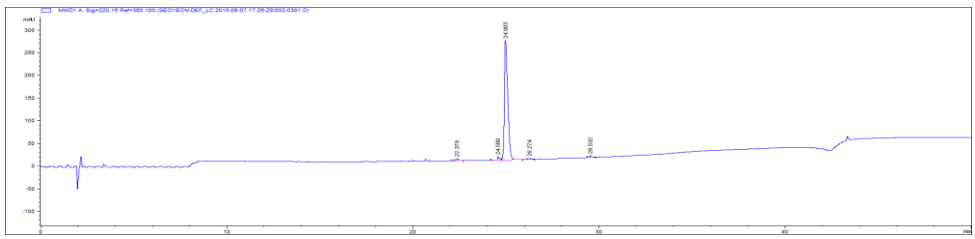
After deprotection of N-terminal Fmoc group, 20 % piperidine. resin was rinsed with DMF 3 times, DCM 5 times and DMF 3 times. 10 eq of acetic anhydride (Sigma), 10 eq of 1-hydroxybenzotriazole hydrate (HOBt, Fluka) were dissolved in 10% v/v DCM in DMF. Acetylation was reacted on SPS Microwave.

C. N-terminal conjugation of 5(6)-Carboxytetramethylrhodamine (TAMRA)

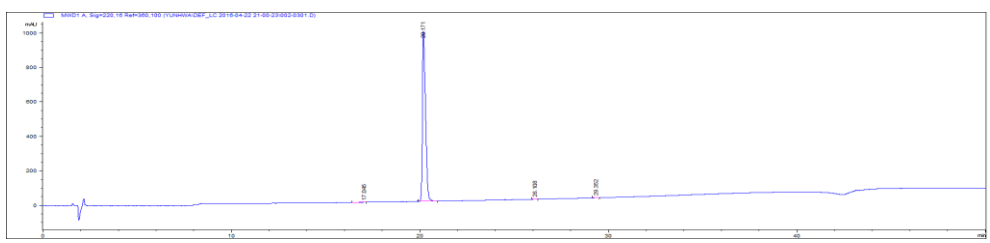
After deprotection of N-terminal Fmoc group, 20 % piperidine. resin was rinsed with DMF 3 times, DCM 5 times and DMF 3 times. 3 eq of 5(6)-TAMRA, 3 eq of *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, Novabiochem.), 3 eq of HOBt and 3 eq of DIPEA were dissolved in DMF. The reaction was carried out for 2 h at room temperature by rotator mixer.

D. Purification of peptides

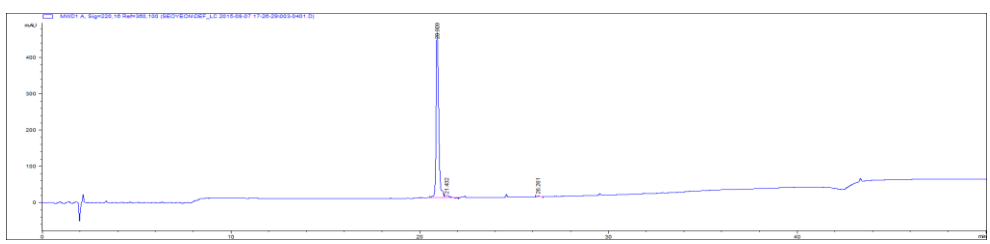
LK (Ac-LKKLLKLLKKLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1733.28 (calcd), 1734.05 (obsd). The HPLC chromatogram of LK is shown below (95 % purity).



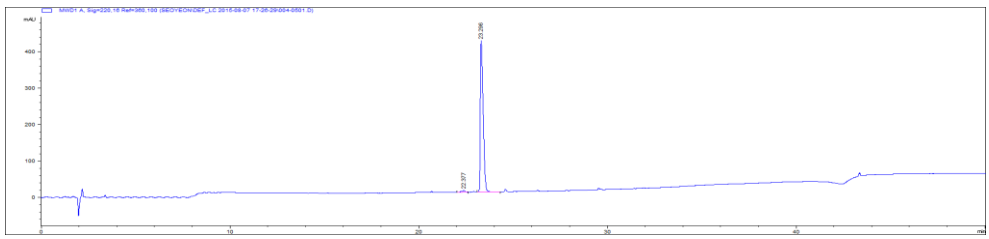
LK-L4P (Ac-LKKPLKLGKKLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.12 (obsd). The HPLC chromatogram of LK-L4P is shown below (>99 % purity).



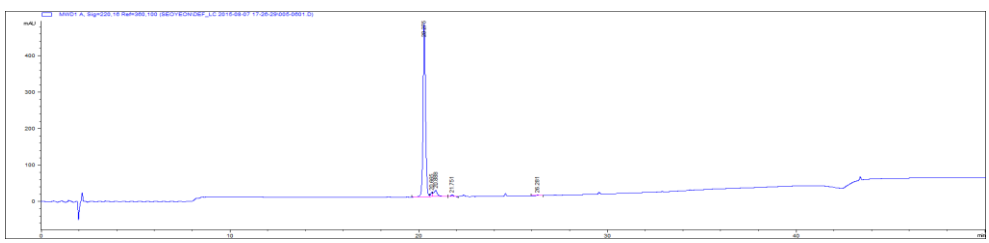
LK-L5P (Ac-LKKLPKLSKKLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1719.23 (obsd). The HPLC chromatogram of LK-L5P is shown below (97 % purity).



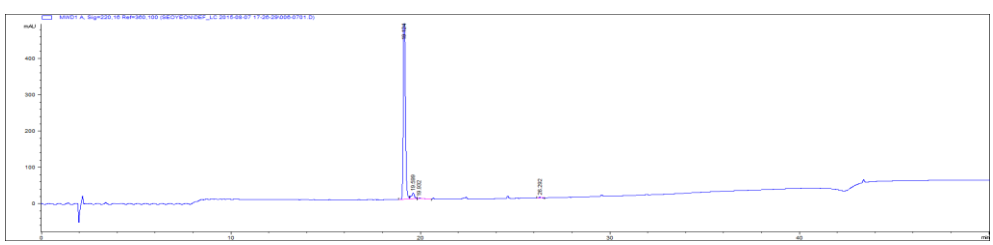
LK-K6P (Ac-LKKLLPLLKKLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1702.24 (calcd), 1703.07 (obsd). The HPLC chromatogram of LK-K6P is shown below (>99 % purity).



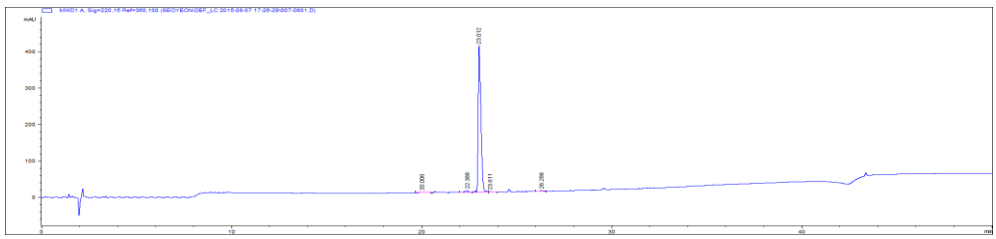
LK-L7P (Ac-LKKLLKPLKKLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.33 (obsd). The HPLC chromatogram of LK-L7P is shown below (91 % purity).



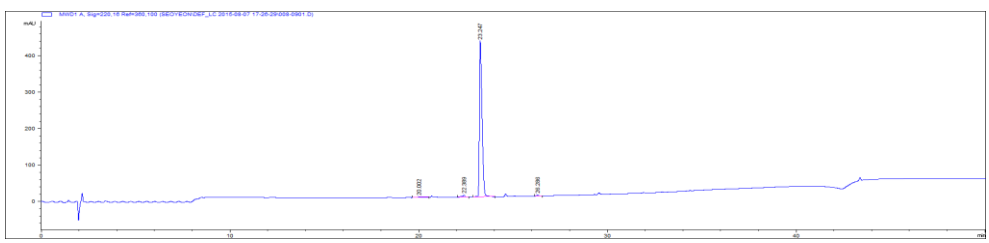
LK-L8P (Ac-LKKLLKLPKKLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.48 (obsd). The HPLC chromatogram of LK-L8P is shown below (95 % purity).



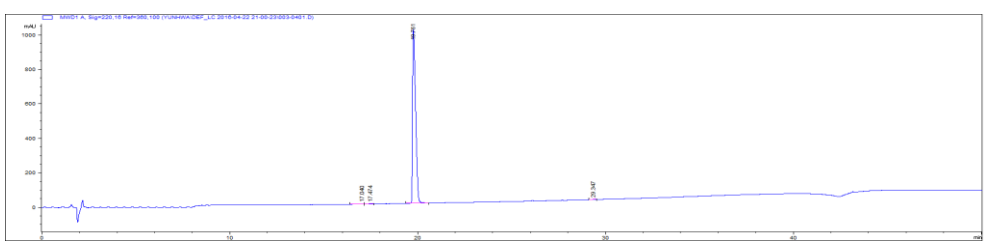
LK-K9P (Ac-LKKLLKLLPKLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1702.24 (calcd), 1703.29 (obsd). The HPLC chromatogram of L8D is shown below (97 % purity).



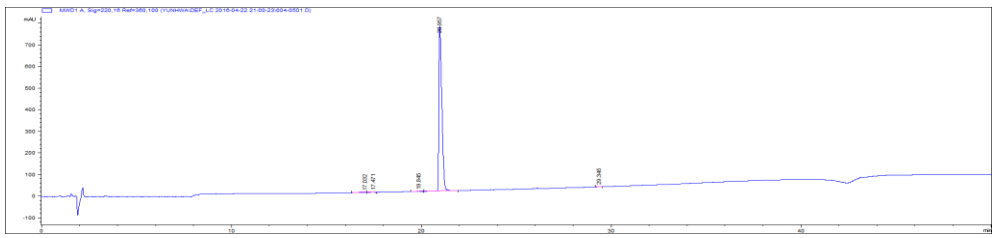
LK-K10P (Ac-LKKLLKLLKPLLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1702.24 (calcd), 1703.43 (obsd). The HPLC chromatogram of LK-K10P is shown below (97 % purity).



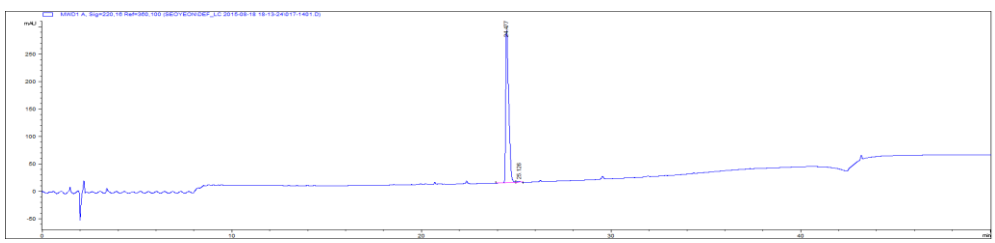
LK-L11P (Ac-LKKLLKLLKKPLKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1719.43 (obsd). The HPLC chromatogram of LK-L11P is shown below (>95 % purity).



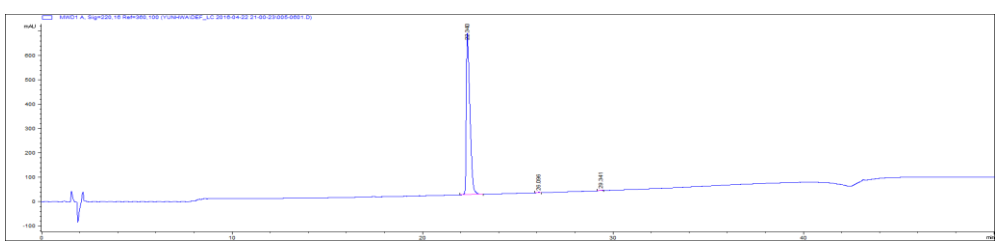
LK-L12P (Ac-LKKLLKLLKKLPKL-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.43 (obsd). The HPLC chromatogram of LK-L12P is shown below (>99 % purity).



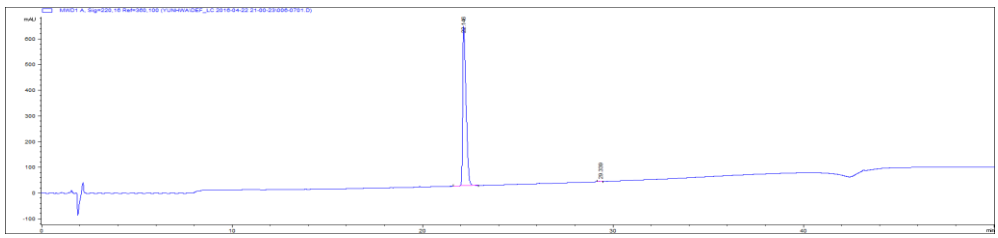
KL (Ac-KLLKLLKKLLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1733.28 (calcd), 1734.32 (obsd). The HPLC chromatogram of KL is shown below (>99 % purity).



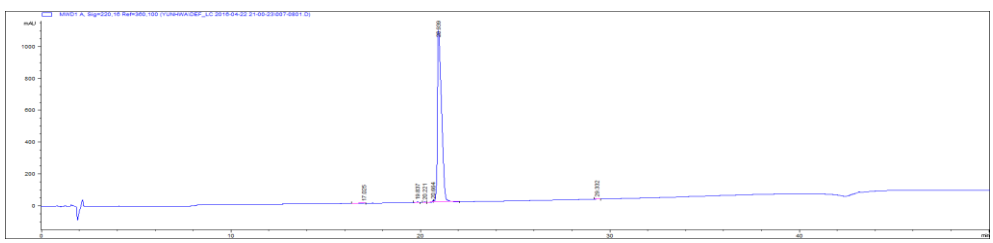
KL-L2P (Ac-KPLKLLKKLLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.17 (obsd). The HPLC chromatogram of KL-L2P is shown below (>95 % purity).



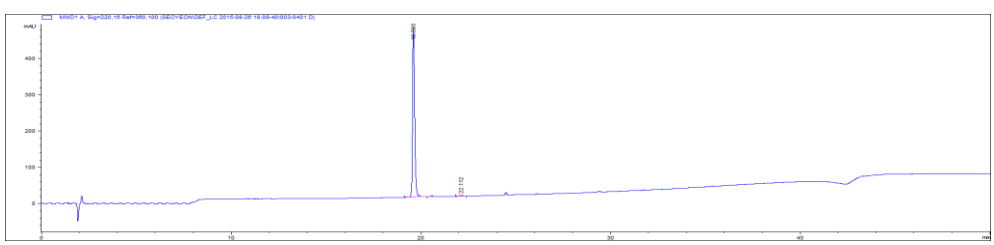
KL-L3P (Ac-KLPKLLKKLLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.37 (obsd). The HPLC chromatogram of KL-L3P is shown below (>95 % purity).



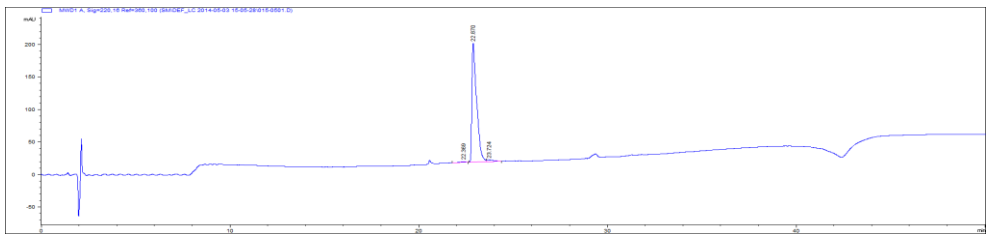
KL-L5P (Ac-KLLKPLKKLLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.26 (obsd). The HPLC chromatogram of KL-L5P is shown below (>99 % purity).



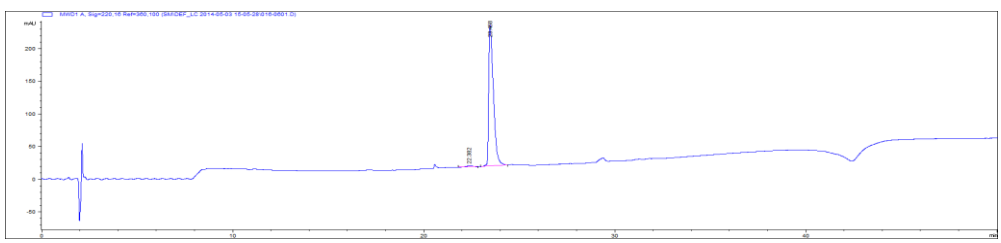
KL-L6P (Ac-KLLKLPKKLLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.57 (obsd). The HPLC chromatogram of KL-L6P is shown below (>99 % purity).



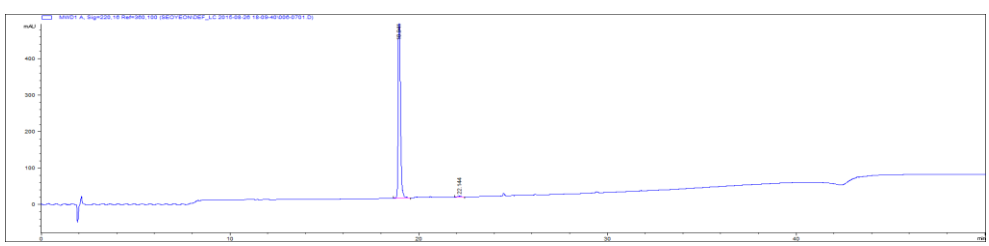
KL-K7P (Ac-KLLKLLPKLLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1702.24 (calcd), 1703.37 (obsd). The HPLC chromatogram of KL-K7P is shown below (97 % purity).



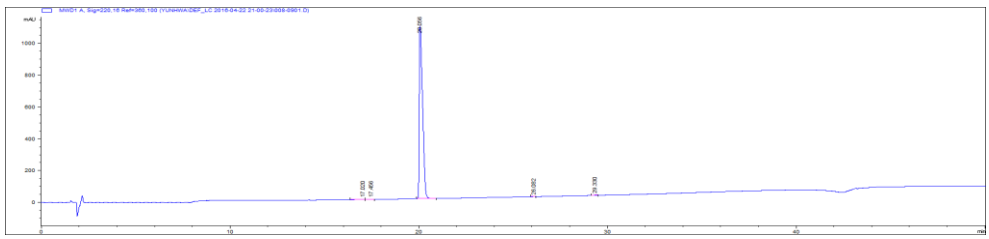
KL-K8P (Ac-KLLKLLKPLLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1702.24 (calcd), 1703.47 (obsd). The HPLC chromatogram of KL-K8P is shown below (>99 % purity).



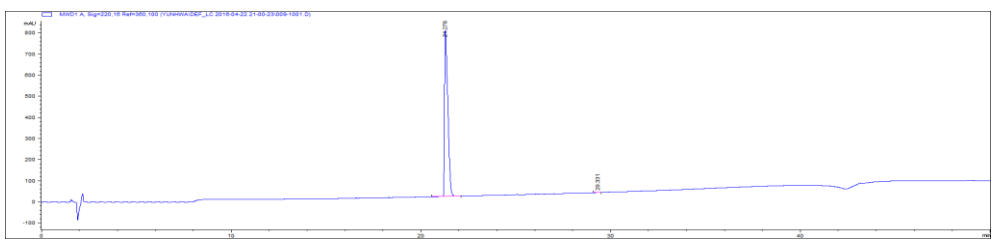
KL-L9P (Ac-KLLKLLKKPLKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.50 (obsd). The HPLC chromatogram of KL-L9P is shown below (>99 % purity).



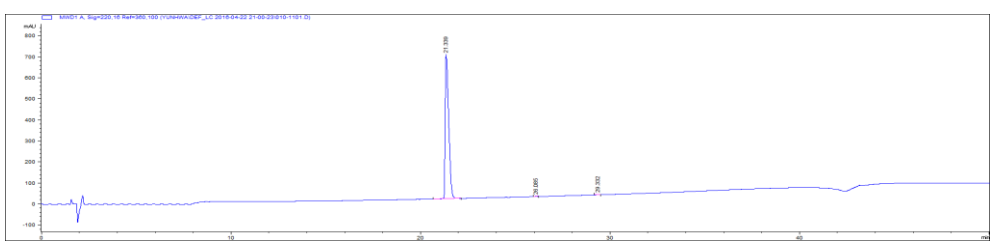
KL-L10P (Ac-KLLKLLKKLPKLLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.47 (obsd). The HPLC chromatogram of KL-L10P is shown below (>99 % purity).



KL-L12P (Ac-KLLKLLKKLLKPLK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.46 (obsd). The HPLC chromatogram of KL-L12P is shown below (>99 % purity).



KL-L13P (Ac-KLLKLLKKLLKLPK-NH_2) was obtained as a white powder. MS $[M+H]^+$: 1717.25 (calcd), 1718.61 (obsd). The HPLC chromatogram of KL-L13P is shown below (>99 % purity).



2. Hemolysis assay

Minimum hemolytic concentration (MHC) of peptides was measured by concentration of peptide giving 10 % hemolysis of red blood cells compared to positive control; distilled water. Human blood was washed three times with PBS centrifuged by 500g for 5 min. Human red blood cells were suspended in phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, pH 7.4) with 5 volume percent to make 5 % hematocrit. Peptides were diluted by two-fold serial dilution method with PBS and 200 μL of peptide solution was loaded to v shaped 96 well plate. 50 μL of 5 % hematocrit (2.5×10^8 hRBCs) was added to each well. Positive control sample is distilled water for 100% hemolysis and negative control sample is PBS. Samples were incubated 4 hours ar 37 ℃. After 4h incubation, they were centrifuged with 1400 rpm 5 min and 180 μL of supernatants were moved to clear bottom 96 well plate. Hemolytic activity was measured by 405/600 nm absorbance. Melittin is used as control peptide. Experiment was performed in triplicate.

3. Minimum Inhibitory concentration

Minimum inhibitory concentration (MIC) was measured by Broth microdilution susceptibility testing. As gram-negative and positive bacteria representative strain, Escerichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 were used. Peptides and antibiotics were prepared by 2-fold microdilution technique. Peptides were diluted with distilled water and antibiotics having poor solubility in water were

diluted with DMSO. DMSO volume percent was kept below 5 % which does not affect bacteria growth. 10 μL of 2 x 10^7 CFU/mL bacteria suspension was added to 200 μL samples to prepare 5 x 10^5 CFU/mL. As CLSI guide lines, Muller Hinton Broth Ⅱ cation adjusted (Difco) was used and incubated at 37 ℃ for 18 hours. MIC was determined by optical density (OD 600).

4. Flow cytometry analysis

Hela cells were seeded at a density of 1 x 10^5 cells per well in serum containing DMEM (Hycolne). Hela cells were incubated 24 hours at 37 ℃, 5 % CO_2. After 24 hours incubation DMEM was removed and Hela cells were treated with TAMRA-labelled peptide in DMEM and 4 h incubated at 37 ℃, 5 % CO_2. Cells were washed with PBS and trypsinzed for 5 min to remove any peptides bind to cellular surface. DMEM 200 μL were added to each sample and moved to 1.5 mL e-tube. Samples were centrifuged at 14000 rpm, 4 ℃ for 1 min and washed by ice-cold PBS buffer 300 μL to wash peptides which bind to cellular surface and cells were suspended by ice-cold PBS buffer 300 μL finally. Cellular uptake of peptides was evaluated by Fluorescence-activated cell sorting machine (BD Accuri C6, USA). 488 nm laser source and FL2 detection filter was used. 1 x 10^4 gated cells were collected for analysis.

E.coli was inoculated to Muller Hinton Ⅱ cation adjusted broth and incubated 37 ℃, 150 rpm overnight. TAMRA-labeled peptides were diluted by Muller Hinton Ⅱ cation adjusted broth. 4 x 10^7 CFU/mL E.coli 10 μL was added to 200 μL

TAMRA-labeled peptides containing solution; final concentration of E.coli was 2 x 10^6 CFU/mL. Each samples was incubated at 37 ℃, 4 hours. After 4 hr incubation, samples were centrifuged at 13000 rpm, 4℃ for 10 min and washed by ice-coled PBS buffer two times to remove peptides bind to cellular surface. After 2 times of PBS wash, E.coli was suspended by ice-cold PBS 300 μL. E.coli is smaller than mammalian cells so thresh hold value should be adjusted; FSC 12000, SSC 10000. Cellular uptake of peptides was evaluated in the same manner.

5. Sytox Green staining

E.coli ATCC 25922 was inoculated to 2 mL of Muller-Hinton broth Ⅱ cation adjusted and incubated at 37 ℃ shaker incubator with 150 rpm during overnight. E.coli (4 x 10^8 CFU/mL) was centrifuged 13000 rpm at RT. E.coli pellet was washed and suspended with phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, pH 7.4). 2x PBS peptide solutions 100 μL were moved to 96 well plate and 2x E.coli PBS suspesion (4 x 10^8 CFU/mL) 100 μL was added and incubated 1hr at 37 ℃. Sytox Green nuceleic acid stain; 5 mM solution in DMSO (lifetechnologies) was diluted with PBS (100 μM). Sytox Green solution 10 μL was added to each sample (Sytox Green final concentration 5 μM). Samples was incubated 10 min at room temperature. Samples were moved to 1.5 mL e-tube and centrifuged at RT, 13000 rpm, 10 min. supernatants were discarded and resuspended with 300 μL ice cold PBS. Each samples were analyzed by by Fluorescence-activated cell sorter (BD Accuri C6, USA). Thresh hold; FSC was adjusted to 12000 and SSC was adjusted

to 10000. Blue laser (488 nm) source and FL-1 detector was used. Each sample, 1 x 10^4 gated cells were collected for analysis of Mean fluorescence intensity (MFI). Triplicates were done.

6. NPN assay

E.coli ATCC 25922 was inoculated to 2 mL of Muller-Hinton broth Ⅱ cation adjusted and incubated at 37 ℃ shaker incubator with 150 rpm during overnight. E.coli 2 x solution (4 x 10^8 CFU/mL) was washed and suspended by 5 mM HEPES buffer (pH 7.2). 250 μL of E.coli 2x suspension solution and 250 μL of 5 mM HEPES buffer pH (7.2) were added to quartz cell. 1000 μM NPN (TCI, in DMSO) solution 10 μL was added. Fluorescence intensity was measured by LS-55 Fluorometer. 100 x peptide solution 5 μL was treated to E.coli NPN mixture and fluorescence intensity was measured immediately. (excitation wavelength 355 nm, emission wavelength 370 - 600 nm, scan mode, excitation slit width 3.5 nm, emission slit width 7 nm, scan speed 400 nm/min, 3 scans per one sample to gain average fluorescence intensity) Peptides were treated by their fractional MIC concentration; 1/4 MIC of peptide and 1/2 MIC of peptide. Triplicates were done.

7. AMP with antibiotic interaction assay

Antimicrobial peptides and antibiotics were prepared to 3 x MIC concetration. Peptides and antibiotics were 2/3 diluted serially by microdilution technique. Peptides and antibiotics were mixed by 1:1 ratio (1.5 x MIC : 1.5 x MIC). Fractional

Inhibitory concentration (FICI) was calculated by the formula.

$$\text{FICI} = \frac{MIC_A\ combination}{MIC_A\ alone} + \frac{MIC_B\ combination}{MIC_B\ alone}$$

(FICI < 1.0 synergistic effect, FICI = 1 ideal additivity, FIC = 2 ideal independence)

MIC value of MICi combination or MICi alone (i = A or B) was calculated by mean value of duplicate sample. FICI value was calculated by mean MIC value of duplicate sample. Four individual plates were done. (total N = 8)

8. Time killing assay

E.coli ATCC 25922 was inoculated to 2 mL of Muller-Hinton broth Ⅱ cation adjusted and incubated at 37 ℃ shaker incubator with 150 rpm during overnight. 10^5 – 10^6 CFU/mL. E.coli were incubated with or without 2.5 μM KL-L9P (1/8 MIC of KL-L9P) and 0.32 μM tetracycline, 5 μM erythromycin, 40 μM linezolid and 80 μM bacitracin (1/8 MIC of antibiotics) in Muller-Hinton broth Ⅱ cation adjusted. Total amount of sample was 2 mL. At 0, 2, 4, 8, 16, 24 hr, 100 μL samples were 10-fold diluted with MHCA and moved to MH agarplate. Solution was spreaded by spreader. MH agarplates were incubated at 37 ℃ for 20 – 24 hours.

Results and Discussion

1. Hemolytic activity and MIC of Pro hinged peptides

Two α-helical LK and KL peptides, sequenced as LKKLLKLLKKLLKL and KLLKLLKKLLKLLK are used for this study as amphiphathic AMP templates (Figure 1). Since these peptides possess high α-helical propensity at membrane condition, they have lytic ability, especially against eukaryotic membrane. Proline residue was introduced at LK and KL peptide by proline-scanning.[14] Peptides were synthesized by standard Fmoc chemistry and purified by HPLC. Values of MIC, MHC and hydrophobicity of the library of proline scanned LK and KL peptides were measured and tabulated in Table 1 and Table 2, respectively.

All peptides with a Pro residue were categorized into three groups in terms of hemolytic activities (Figure 1). Group 1 peptides have improved MIC values against gram-negative and –positives, while they maintains most of high hemolytic activity. All K to P mutants and two L to P mutants KL-L2P, KL-L3P belong to this group. These peptides do not have selective membrane activity. Second group showed much improved MIC values against gram-negative, but still they showed moderate MHC values. These peptides are still involved in membranes of both eukaryotes and gram-negative bacteria, thus, have no selectivity. The last group showed nearly abolished hemolytic activity and bactericidal activity against gram-positive bugs, but still remained or even improved activity against gram-negative bugs. Even though the second and third categorized mutants have all L to P mutations, the last group showed

a possible membrane selectivity against gram negative bugs. Thus, third group peptides, LK-L8P, LK-L11P, KL-L6P, and KL-L9P, were chosen for further study. Even though bactericidal activity against gram-positives and hemolytic activity of all peptides are totally vanished, interestingly, their MIC values against gram-negative bugs showed somewhat different antimicrobial activities.

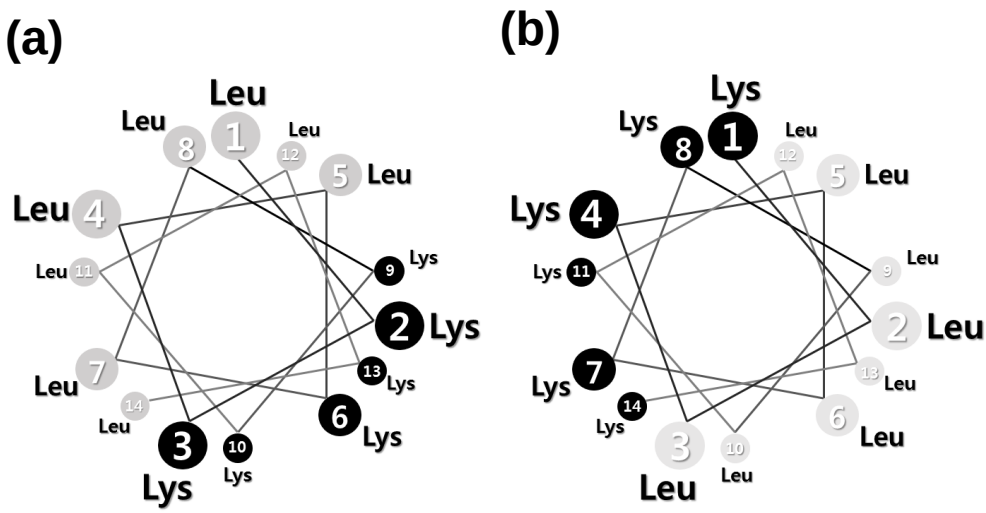


Figure 1. Helical wheel diagram of LK peptide (a) and KL peptide (b). Hydrophobic residues are colored gray and hydrophilic residues black.

Table 1. MIC, hemolytic activity and hydrophobicity of LK and LK Pro mutants

MIC, MHC and hydrophobicity of LK-derivative Proline mutant peptides				
Peptide[a]	MIC [µM][b] *E.coli ATCC 25922*	MIC [µM][b] *S.aureus ATCC 25923*	MHC [µM][c]	t_R [min][e]
LK	20 – 80	10 – 20	5	24.96
LK-L4P	1.3-2.5	40	80	20.17
LK-L5P	2.5	10 – 20	80	20.91
LK-K6P	5 – 10	2.5	1.25	23.30
LK-L7P	2.5 – 5	20	80	20.28
LK-L8P	10	>80	640	19.12
LK-K9P	5	2.5	5	23.01
LK-K10P	5	2.5	5	23.25
LK-L11P	2.5 – 5	40	320	19.76
LK-L12P	1.3 - 2.5	10	40	20.96
Amikacin	5 - 10	5 - 10	-	-

[a] Sequence of LK peptide is Ac-LKKLLKLLKKLLKL-NH_2. [b] Minimum inhibitory concentration. N = 4 [c] Minimum hemolytic concentration. MHC is defined as concentration required to produce 10% hemolysis. [d] HPLC conditions: buffer A (water with 0.1% v/v TFA) and buffer B (acetonitrile with 0.1% v/v TFA); 0 min, 5% B followed by linear gradient 70% B over 30 min and 100% B over 40 min. Flow rate was 1 ml/min.

Table 2. MIC, hemolytic activity and hydrophobicity of KL and KL Pro mutants

MIC, MHC and hydrophobicity of KL-derivative Proline mutant peptides				
Peptide[a]	MIC [µM][b] *E.coli ATCC 25922*	MIC [µM][b] *S.aureus ATCC 25923*	MHC [µM][c]	t_R [min][e]
KL	40 – 80	10 – 20	0.625	24.48
KL-L2P	2.5 – 10	5 – 10	5	22.34
KL-L3P	10	10	10	22.15
KL-L5P	5	20 – 40	80	20.94
KL-L6P	10	>80	640	19.59
KL-K7P	5 – 10	5	5	22.87
KL-K8P	10	2.5 – 5	2.5	23.46
KL-L9P	20	>80	>1280	18.94
KL-L10P	2.5	40	80	20.06
KL-L12P	2.5 – 5	10	40	21.28
KL-L13P	2.5 - 5	10	40	21.34
Amikacin	5-10	5 - 10	-	-

[a] Sequence of KL peptide is Ac-KLLKLLKKLLKLLK-NH_2. [b] Minimum inhibitory concentration. N = 4. [c] Minimum hemolytic concentration. MHC is defined as concentration required to produce 10% hemolysis. [d] HPLC conditions: buffer A (water with 0.1% v/v TFA) and buffer B (acetonitrile with 0.1% v/v TFA); 0 min, 5% B followed by linear gradient 70% B over 30 min and 100% B over 40 min. Flow rate was 1 ml/min.

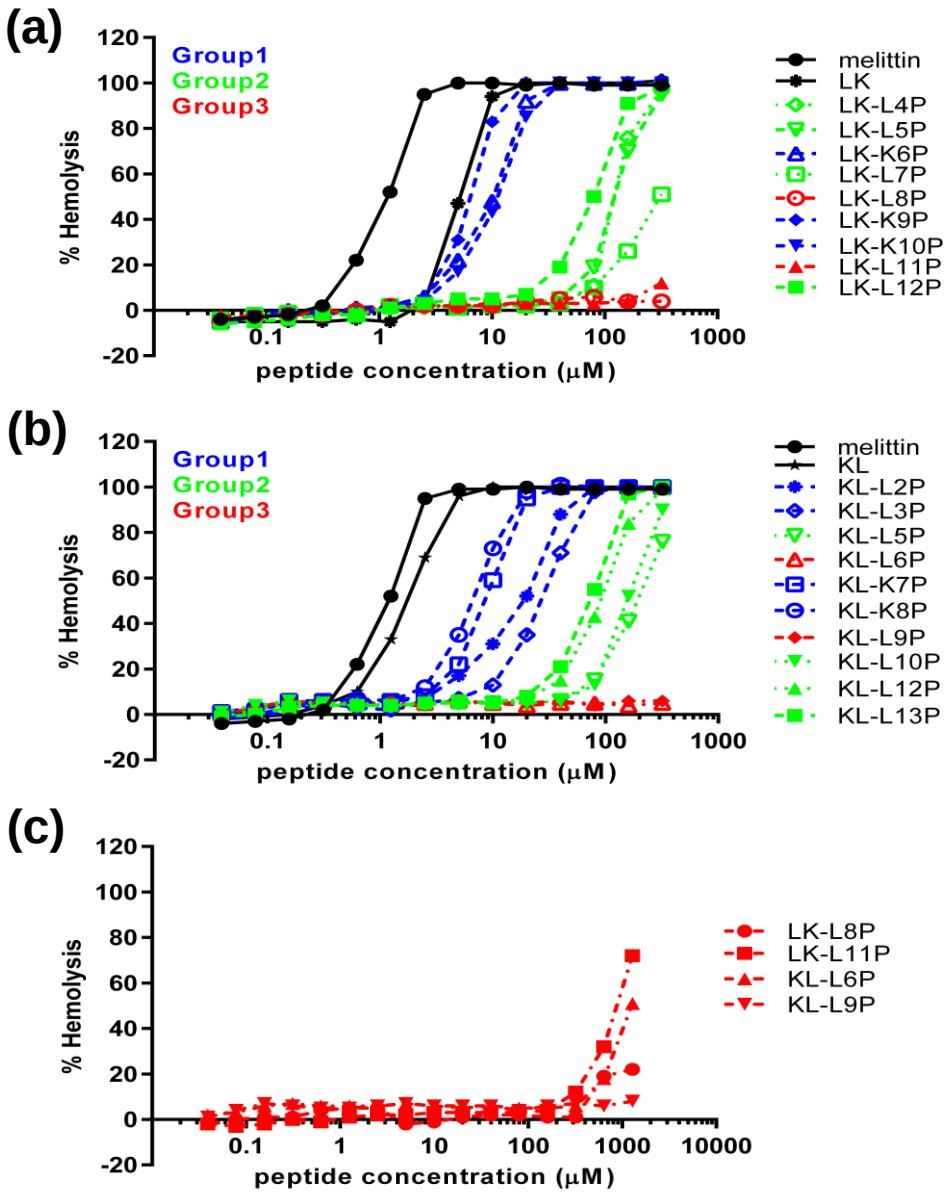


Figure 2. Hemolytic activity of LK, LK Pro mutant peptides (a) KL, KL Pro mutant peptides (b). and Group3 LK, KL Pro mutant peptides at high peptide concentration.

2. Cell penetrating specificity of Pro hinged peptides to Hela and E.coli

In order to study difference of cellular uptake between mammalian cell and gram-negative bug, TAMRA was conjugated to N-terminal of LK, KL and group 1, 2, 3 Pro mutant peptides. LK-K9P, LK-L12P were chosen as representatives of group 1 and group 2 Pro mutants respectively. In Hela, even though incubated 4 hours TAMRA labeled peptides showed a good cell penetrating efficiency except most of group 3 peptides LK-L8P, KL-L6P and KL-L9P. On the other hand, LK-L8P, KL-L6P and KL-L9P show high cellular uptake percents in E.coli. This reversed cellular uptake percent between Hela and E.coli seems to have relation with hemolytic activity for peptides. Low hemolytic activity of group 3 peptides might mean poor interaction with membrane of Hela. Though LK-L11P belongs to group 3, it have relatively high MHC value, 320 μM compared with other group 3 Pro mutant peptides (Figure 1. c). Because of relative high hemolytic activity among group 3 Pro mutants, LK-L11P might have better interaction than other group 3 mutants. This peptide shows high cell penetrating ability both Hela and E.coli. In terms of cell penetrating selectivity for mammalian cell and gram-negative bug, LK-L8P, KL-L6P, KL-L9P have high selectivity than other Pro mutants.

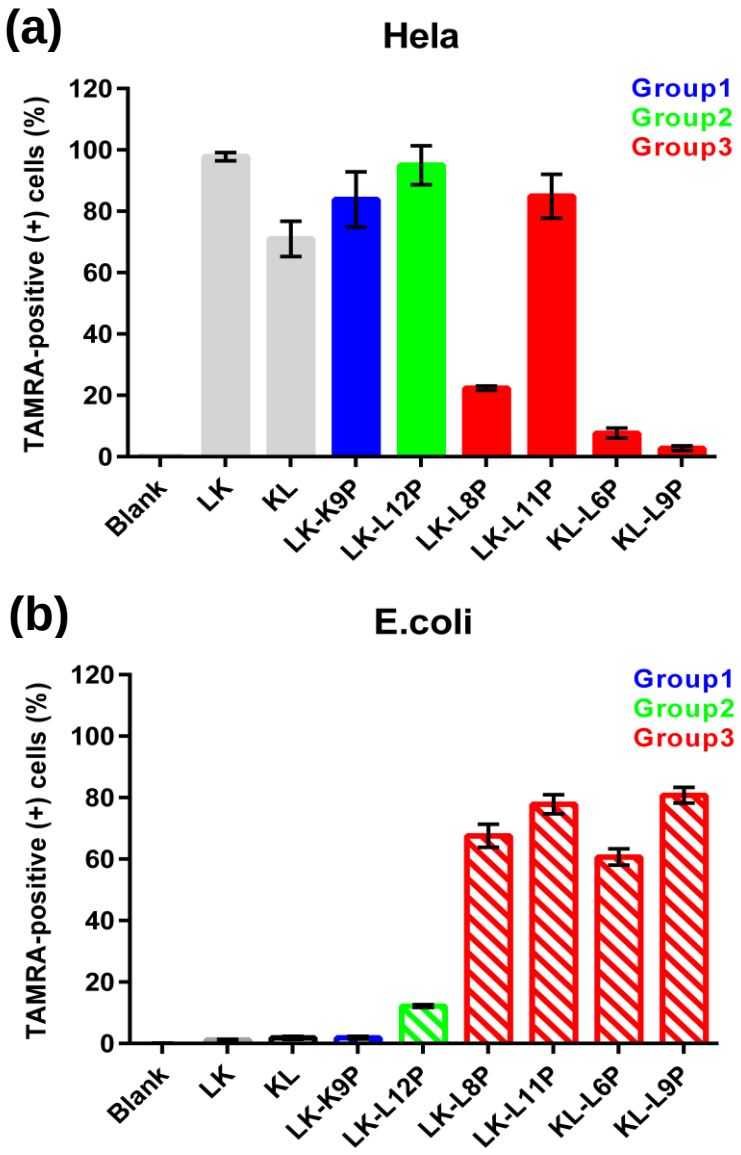


Figure 3. Cellular uptake of LK, KL Pro mutant peptides in Hela and E.coli; FACS analysis with TAMRA labeled peptide. Hela (a) and E.coli (b). Cellular uptake of TAMRA labeled peptides was evaluated by TAMRA positive cells (%) compared to blank.

3. Inner membrane and Outer membrane mechanism study

For mechanism study of LK, KL Pro mutant peptides against gram-negative bacteria, Inner membrane demolishing and outer membrane perturbing properties were measured by Sytox Green staining and NPN assay. Sytox Green is cell impermeable dye. When inner membrane of E.coli is demolished by antimicrobial peptide, it can bind with DNA and emits green fluorescence. Melittin known for pore-forming antimicrobial peptide was used to control peptide.[15] Melittin, LK-K9P showed similar inner membrane demolishing ability and both of them are saturated at 5 μM. (Fig. 4.) LK-L12P, group 2 Pro mutant peptide has weaker inner membrane demolishing abillity less than 10 μM compared to melittin and LK-K9P, but it shows stronger inner membrane demolishing ability above 10 μM. All group 3 peptides have nearly no inner membrane breaking property but therer is small difference among them. Despite of different phospholipid composition between mammalian cell (PE, PC) and inner membrane of E.coli (PE, PG), the order of hemolytic activity is matching up with inner membrane breaking activity (LK-L11P > KL-L6P > LK-L8P > KL-L9P).

When NPN can interact with hydrophibic part of phospholipid, fluorescence intensity of NPN is amplified largely compared to that of NPN alone.[16] To evaluate outer membrane perturbing ability of peptide fairly, fluorescence intensity was measured along the fractional minimal inhibitory concentration of peptides; 1/2 peptide and 1/4 peptide means half MIC of peptide and quarter MIC of peptide. Melittin showed high OM purterbing activity compared to group 1 and group 2 Pro

mutant peptides. (Fig. 5.) Interestingly, OM purterbing ability of group 3 Pro mutant peptides is comprable to that of melittin except LK-L11P. LK-L11P showed similar fluorescence intensity with LK-K9P and LK-L12P. Among the LK-L8P, KL-L6P and KL-L9P, KL-L9P show strongest OM purterbing ability. As an outer membrane sensitizer, KL-L9P is the best one.

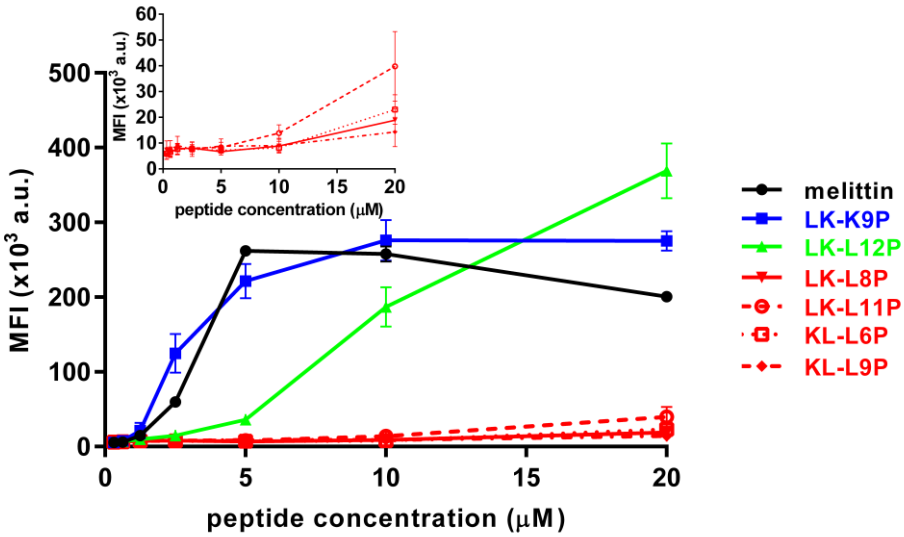


Figure 4. Sytox Green staining. Comparison of inner membrane demolishing ability; group 1, 2, 3 Pro mutants against E.coli ATCC 25922.

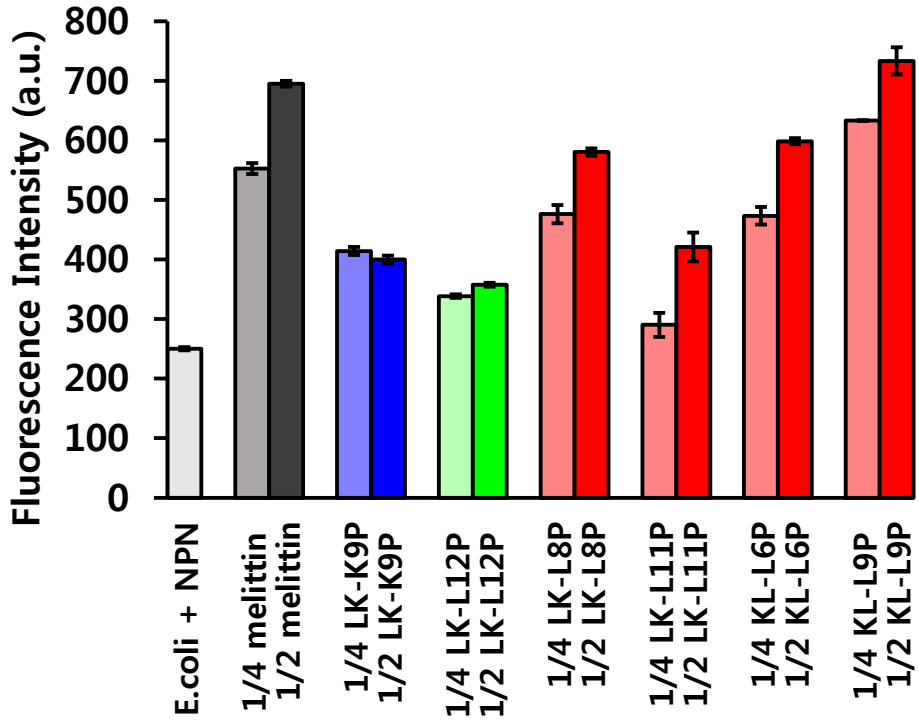


Figure 5. NPN assay. Outer membrane perturbation of Group 1, 2, 3 Pro mutant peptides were measured by NPN fluorescence intensity change. 1/2, 1/4 means fractional minimum inhibitory concentration.

4. Synergistic effect of KL-L9P and antibiotics

Though melittin sensitized outer membrane of E.coli strongly, it was reported that melittin have no synergistic effect with ampicillin, streptomycin and ciprofloxacin.[17] Other membrane permeablizing antimicrobial peptides, LK-K9P and LK-L12P, also shows nearly no synergistic effect with chemical antibiotics. Membrane permeablizing mechanism essentially contains breaking innner membrane of gram-negative bugs.

This study find out new type of outer membrane sensitizer, KL-L9P. This new type of outer membrane sensitizer has nearly no inner membrane demolishing character and perturb outer membrane strongly. Traditional checkerboard assay have statistical weakness so we used novel antibiotic interaction assay to calculate Fractional inhibitory concentration index exactly (Fig. 6). Firstly, MIC of antibiotics were measured by 2-fold microdilution techniques. After 3 x MIC of KL-L9P (60 μM) and antibiotics were confirmed, we carried out novel antibiotic interaction assay with various antibiotics against E.coli ATCC 25922.

Table 4. shows that KL-L9P has synergistic effect with 18 antibiotics and especially shows strong synergistic effect (FICI < 0.5) with 10 antibiotics. This amazing synergistic effect was maximized particulary for some antibiotics only used to gram-positive bugs because of their poor permeability for LPS layer of gram-negative bugs, so called innate resistance. For example, novobiocin[18], erythromycin[1] is well known antibiotic because of their poor permeability for OM of gram-negative

bacteria. MIC of novobiocin for E.coli and S.aureus was 40 – 80 μM and 1.3 μM respectively. By mixing 1.0 μM KL-L9P with novobiocin, MIC of novobiocin decreased to 2.0 μM, similar MIC value (1.3 μM) of S.aureus. MIC of erythromycin for E.coli and S.aureus was 40 – 80 μM and 1.3 μM respectively. By mixing 1.0 μM KL-L9P with novobiocin, MIC of erythromycin decreased to 2.0 μM, similar MIC value (1.3μM) of S.aureus.

Time killing assay confirms synergistic effect of KL-L9P with antibiotics. Synergistic effect of two antibiotics is defined as greater than 2-log decrease compared with its most active constituent alone after 24 hr incubation.[19] 1/8 MIC of KL-L9P and 1/8 MIC of each antibiotics and combinations were treated to E.coli. In tetracycline, Synergistic effect was not observed because FICI of tetracycline was measured 0.80 by novel antibiotic interaction assay which is larger than 0.25. Other three antibiotics show synergistic effect between KL-L9P, erythromycin, linezolid have a bactericidal activity (7-log decrease at 24 hr) and bacitracin has a bacteriostatic activity (6-log decrease at 24 hr). Result of time killing assay comfirms validity of novel antibiotic interaction assay.

Table 3. MIC of antibiotics against gram-negative and gram-positive bacteria

MIC of antibiotics		
Antibiotic	MIC [µM][a]	
	E.coli ATCC 25922	*S.aureus ATCC 25923*
amikacin	5.0-10	5.0-10
ampicillin	5.0-10	5.0
azithromycin	2.5-10	1.3-2.5
bacitracin	640	5.0-20
chloramphenicol	10	10-20
ciprofloxacin	0.039	1.3
cloxacillin	320-640	0.63
colistin	0.31-0.63	80-160
erythromycin	40-80	1.3
levofloxacin	0.078-0.16	0.63
linezolid	320	10
novobiocin	40-80	1.3
penicillin G	80-160	2.5-5.0
polymyxin B	0.31-0.63	20-40
rifampicin	5.0-10	0.16-1.3
tetracyclin	2.5	1.3-2.5
trimethoprim	0.63	5.0-20
vancomycin	80-160	0.31-0.63

[a] Minimum inhibitory concentration. N=4.

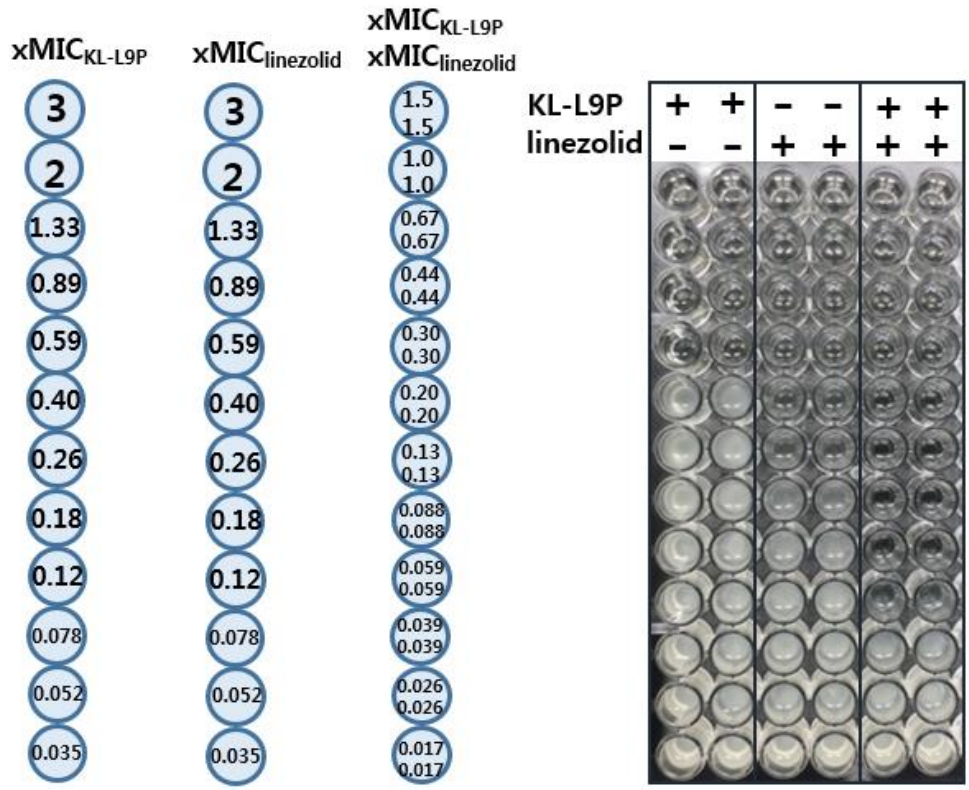


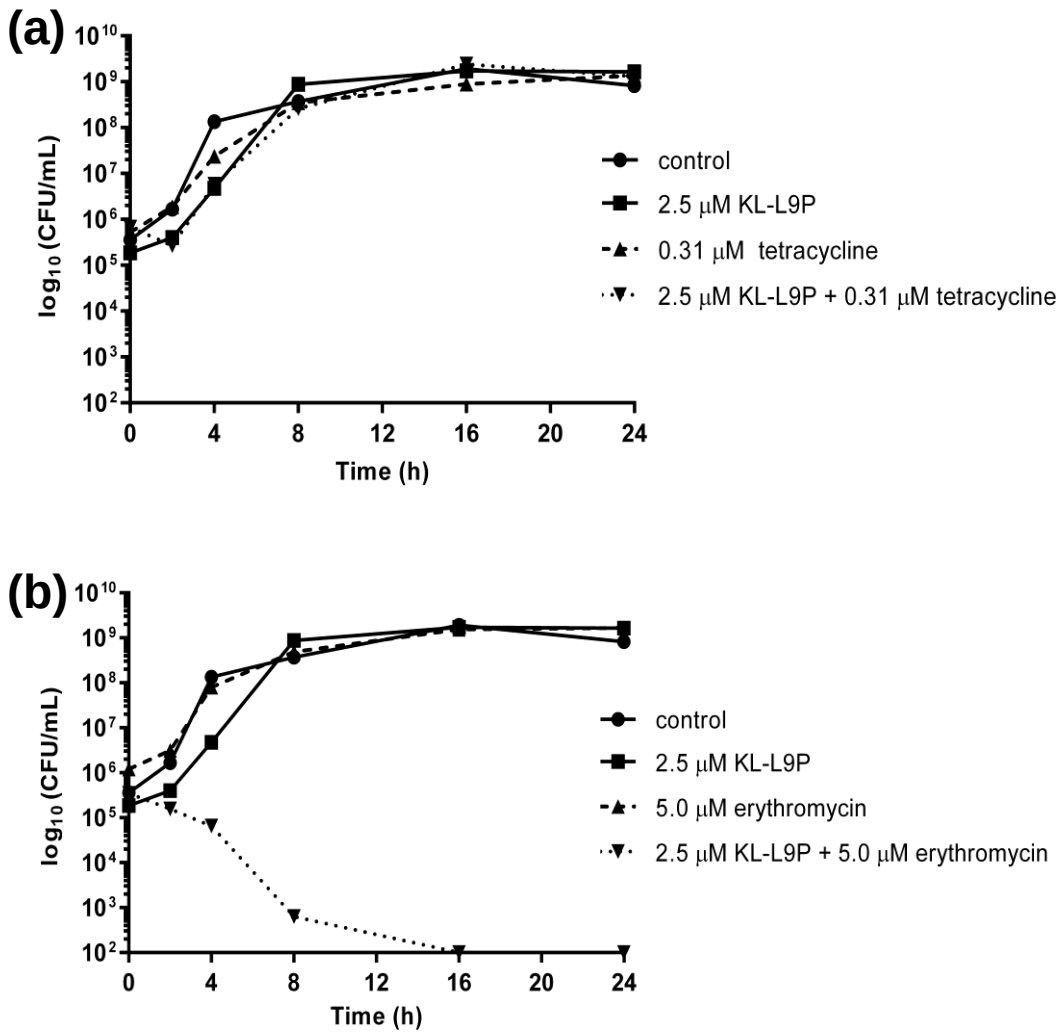
Figure 6. KL-L9P with linezolid interaction assay.

Table 4. Combination MIC of KL-L9P with antibiotics and FICI

Table 4. KL-L9P with antibiotics combination MIC and FICI

Antibiotic	MIC [μM][a] of KL-L9P against *E.coli ATCC 25922*	MIC [μM][a] of antibiotics against *E.coli ATCC 25922*	combination MIC [μM][a] against *E.coli ATCC 25922*		FICI[b]
			KL-L9P	antibiotic	
amikacin	24 (± 4)	9.0 (± 0.9)	12 (± 2)	3.1 (± 0.6)	0.84 (± 0.03)
ampicillin	18 (± 1)	7.0 (± 1.5)	5.9 (± 0.1)	3.0 (± 0.1)	0.76 (± 0.04)
azithromycin	15 (± 6)	8.5 (± 7.2)	0.98 (± 0.23)	0.49 (± 0.11)	0.16 (± 0.06)
bacitracin	20 (± 4)	1000 (± 220)	2.3 (± 0.4)	74 (± 13)	0.19 (± 0.02)
chloramphenicol	27 (± 1)	8.1 (± 1.4)	7.8 (± 1.4)	3.9 (± 0.7)	0.71 (± 0.16)
ciprofloxacin	22 (± 5)	0.046 (± 0.016)	5.9 (± 0.1)	0.023 (± 0.001)	0.81 (± 0.10)
cloxacillin	20 (± 6)	560 (± 110)	1.5 (± 0.3)	25 (± 5)	0.15 (± 0.02)
colistin	18 (± 1)	0.30 (± 0.08)	6.0 (± 1.6)	0.094 (± 0.026)	0.66 (± 0.18)
erythromycin	15 (± 3)	54 (± 29)	1.0 (± 0.2)	2.0 (± 0.4)	0.13 (± 0.03)
levofloxacin	18 (± 7)	0.074 (± 0.012)	5.9 (± 0.1)	0.023 (± 0.001)	0.81 (± 0.02)
linezolid	16 (± 6)	320 (± 66)	1.8 (± 0.1)	28 (± 1)	0.22 (± 0.03)
novobiocin	20 (± 4)	45 (± 19)	1.0 (± 0.2)	2.0 (± 0.5)	0.09 (± 0.01)
penicillin G	14 (± 5)	42 (± 1)	1.6 (± 0.4)	13 (± 4)	0.43 (± 0.10)
polymyxin B	21 (± 5)	0.40 (± 0.05)	5.0 (± 1)	0.078 (± 0.019)	0.44 (± 0.08)
rifampicin	13 (± 2)	6.3 (± 1.0)	1.2 (± 0.2)	0.57 (± 0.12)	0.18 (± 0.02)
tetracyclin	21 (± 5)	2.5 (± 0.5)	8.1 (± 0.9)	1.0 (± 0.1)	0.80 (± 0.07)
trimethoprim	20 (± 4)	0.39 (± 0.07)	4.9 (± 0.8)	0.15 (± 0.03)	0.64 (± 0.08)
vancomycin	14 (± 5)	100 (± 28)	2.5 (± 0.7)	9.9 (± 2.6)	0.30 (± 0.04)

[a] Minimum inhibitory concentration. (N=8) [b] Fractional Inhibitory concentration, Fractional Inhibitory concentration (FICI) was calculated by the formula. $\mathrm{FICI} = \frac{MIC_A\ combination}{MIC_A\ alone} + \frac{MIC_B\ combination}{MIC_B\ alone}$ (FICI < 1.0 synergistic effect, FICI = 1 ideal additivity, FIC = 2 ideal independence)


(a)
log10 (CFU/mL)
Time (h)
control
2.5 μM KL-L9P
0.31 μM tetracycline
2.5 μM KL-L9P + 0.31 μM tetracycline
(b)
log10 (CFU/mL)
Time (h)
control
2.5 μM KL-L9P
5.0 μM erythromycin
2.5 μM KL-L9P + 5.0 μM erythromycin

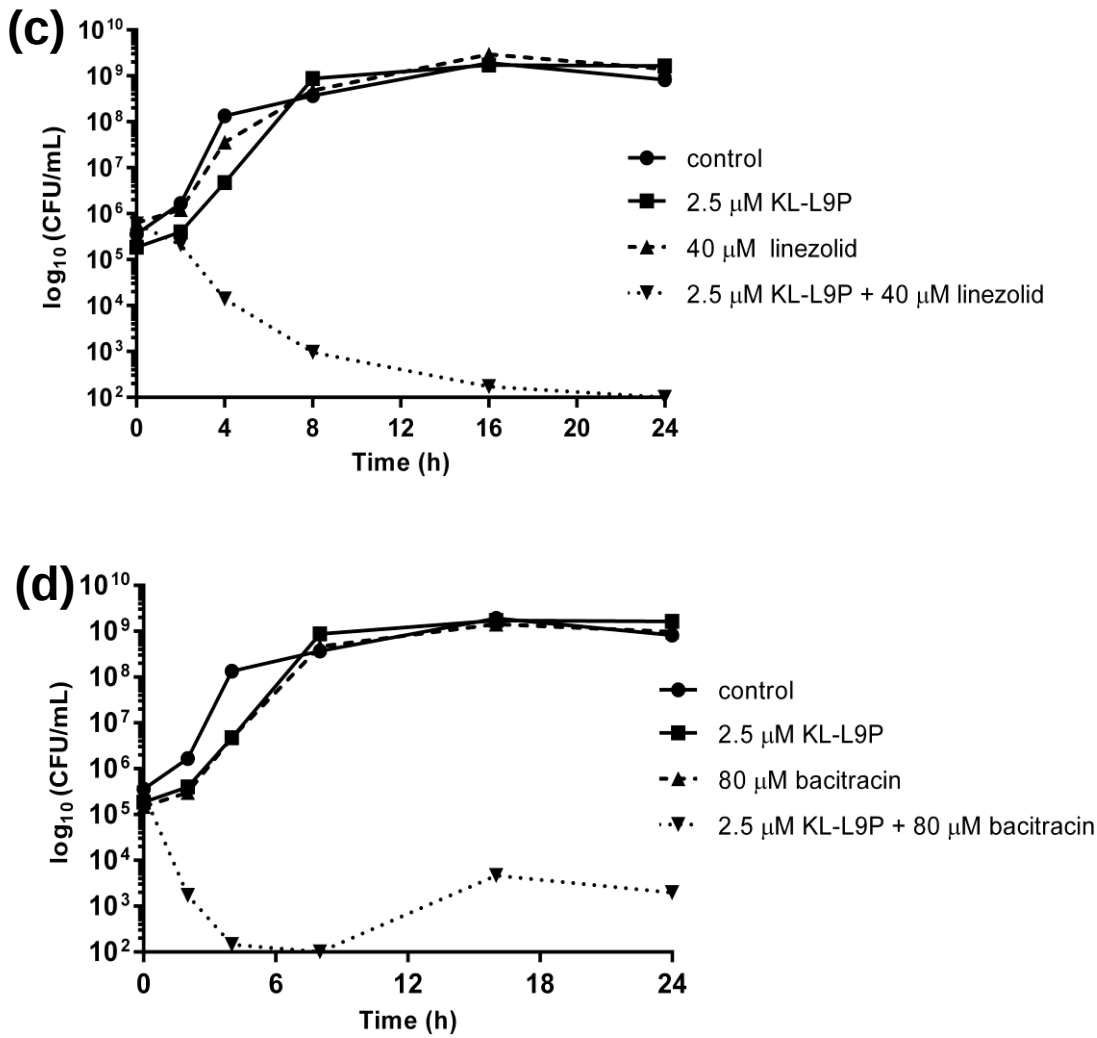


Figure 6. Time killing assay of KL-L9P with antibiotic combinations, tetracycline and KL-L9P (a), erythromycin and KL-L9P (b), linezolid and KL-L9P (c) and bacitracin and KL-L9P (d) against *E.coli ATCC 25922.*

5. Difference of Synergistic effect between melittin and group 1, 2, 3 Pro mutants and erythromycin.

KL-L9P is selected for 1) low hemolytic activity, 2) selective cell penetrating ability, 3) low IM demolishing activity and 4) high OM perturbing ability but other group 3 Pro mutant peptides also have these properties. Synergistic effect with erythromycin was investigated further by novel antibiotic interactoin assay. Erythromycin was chosen because it gives a good synergistic effect against *E.coli ATCC 25922*.

Table 5, all group 3 Pro mutant peptides have a strong synergy (FICI < 0.5) with erythromycin against E.coli. Traditional membrane permeablizing peptides, melittin, LK-K9P and LK-L12P, have nearly no synergistic effect with erythromycin corresponding to preceding research.[17] FICI value and combination MIC reaveal the order of synergy with erythromycin; KL-L9P > LK-L8P > KL-L6P > LK-L11P. KL-L9P is the strongest OM sensitizer (Fig. 5) and have the weakest IM demolishing activity (Fig. 4) and lowest hemolytic activity (Fig. 1). LK-L8P, KL-L6P sensitize OM similarly but IM demolishing activity and hemolytic activity of KL-L6P is larger than those of LK-L8P. So LK-L8P has better synergistic effect than KL-L6P. LK-L11P shows weakest synergistic effect among group 3 Pro mutant peptides because LK-L11P is the weakest OM sensitizer and have the strongest IM demolishing activity, hemolytic activity. Order of selective cell penetrating ability toward gram negative bug is slightly different from that of synergy with erythromycin; KL-L9P > KL-L6P > LK-L8P > LK-L11P. This discrepency might be derived from different cell penetrating ability of LK and KL peptides in Hela. Consequently, valid

standards for screening synergistic Pro mutant peptide are turned out to be three of four; 1) low hemolytic activity 3) low IM demolishing activity 4) high OM perturbing ability excepting 2) selective cell penetrating ability.

Table 5. Combination MIC and FICI of erythromycin with melittin and group 1, 2, 3 Pro mutants.

Erythromycin with melittin and Group 1.2. 3 Pro mutants MIC and FICI					
Peptide	MIC [μM][a] of KL-L9P against *E.coli ATCC 25922*	MIC [μM][a] of erythromycin against *E.coli ATCC 25922*	combination MIC [μM][a] against *E.coli ATCC 25922*		FICI[b]
			erythromycin	peptide	
Melittin	8.1 (± 3.0)	40 (± 8)	3.3 (± 0.1)	27 (± 1)	1.1 (± 0.1)
LK-K9P	4.4 (± 0.1)	38 (± 6)	2.2 (± 0.1)	18 (± 1)	0.98 (± 0.05)
LK-L12P	1.8 (± 0.4)	44 (± 10)	1.1 (± 0.1)	18 (± 1)	1.1 (± 0.2)
LK-L8P	9.4 (± 1.6)	42 (± 9)	0.88 (± 0.01)	3.5 (± 0.1)	0.18 (± 0.01)
LK-L11P	3.2 (± 0.8)	42 (± 9)	0.91 (± 0.15)	7.2 (± 1.2)	0.47 (± 0.07)
KL-L6P	8.9 (± 0.1)	38 (± 6)	1.5 (± 0.3)	5.9 (± 1.2)	0.33 (± 0.05)
KL-L9P	15 (± 3)	47 (± 9)	1.2 (± 0.2)	2.5 (± 0.4)	0.12 (± 0.01)

[a] Minimum inhibitory concentration. (N=4) [b] Fractional Inhibitory concentration, Fractional Inhibitory concentration (FICI) was calculated by the formula.

$\mathrm{FICI} = \frac{MIC_A\ combination}{MIC_A\ alone} + \frac{MIC_B\ combination}{MIC_B\ alone}$ (FICI < 1.0 synergistic effect, FICI = 1 ideal additivity, FIC = 2 ideal independence)

6. Summary

Two libraries of proline scanning peptide using model amphipathic LK and KL peptides were made and their antimicrobial and toxic activities against pathogens and host cells were cartegorized into three groups. Group 1 Pro mutant peptides showed improved bactericidal activity both gram-negative and gram-positive bugs with retained hemolytic activity. Group 2 Pro mutant peptides showed improved bactericidal activity for gram-negative bug and decreased MIC of gram-positive bug and reduced hemolytic activity. Group 3 Pro mutant peptide have improved MIC of E.coli and nearly no bactericidal activity for gram-positive bacteria and minimized hemolytic activity.

Group 3 Pro mutant peptides have selectivity between Hela and E.coli for cellular uptake experiment. Sytox Green staining showed IM demolishing activity of group 3 Pro mutant peptides is negilgible compared to group 1 and group 2 Pro mutant peptides. In NPN assay, group 3 Pro mutant peptides were perturbing OM much stronger than group 1 and group 2 Pro mutant peptides excepting LK-L11P.

One of group 3 Pro mutant peptides, KL-L9P, best synergistic antimicrobial peptide, was screened from four parameters; 1) low hemolytic activity, 2) selective cell penetrating ability, 3) low IM demolishing activity and 4) high OM perturbing ability. KL-L9P have good synergistic effect with 10 antibiotics (FICI < 0.5). We evaluate 4 criterion by investigating synergistic effect with group 3 Pro mutant peptides with linezolid. Three of four standards were valid except selective cell penetrating ability among the group 3 Pro mutant peptides. KL-L9P was turned out to most synergistic one with erythromycin among melittin and group 1, 2, 3 Pro mutant peptides. In

order to develop synergistic antimicrobial peptide, high OM perturbing ability and low IM demolishing properties might be considered as important criteria.

7. References

1. Delcour, A.H., *Outer membrane permeability and antibiotic resistance.* Biochim Biophys Acta, 2009. **1794**(5): p. 808-16.
2. Zgurskaya, H.I., C.A. López, and S. Gnanakaran, *Permeability barrier of Gram-negative cell envelopes and approaches to bypass it.* ACS infectious diseases, 2015. **1**(11): p. 512-522.
3. Alakomi, H.L., M. Saarela, and I.M. Helander, *Effect of EDTA on Salmonella enterica serovar Typhimurium involves a component not assignable to lipopolysaccharide release.* Microbiology, 2003. **149**(Pt 8): p. 2015-21.
4. Alakomi, H.-L., et al., *Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane.* Applied and environmental microbiology, 2000. **66**(5): p. 2001-2005.
5. Alakomi, H.-L., et al., *Weakening effect of cell permeabilizers on gram-negative bacteria causing biodeterioration.* Applied and environmental microbiology, 2006. **72**(7): p. 4695-4703.
6. Lakshminarayanan, R., et al., *Branched Peptide, B2088, Disrupts the Supramolecular Organization of Lipopolysaccharides and Sensitizes the Gram-negative Bacteria.* Scientific reports, 2016. **6**.
7. Wang, J., et al., *High specific selectivity and Membrane-Active Mechanism of the synthetic centrosymmetric α-helical peptides with Gly-Gly pairs.* Scientific reports, 2014. **5**: p. 15963-15963.
8. Xiao, Y., et al., *The central kink region of fowlicidin-2, an α-helical host defense peptide, is critically involved in bacterial killing and endotoxin neutralization.* Journal of Innate Immunity, 2008. **1**(3): p. 268-280.
9. Čeřovský, V., et al., *Melectin: a novel antimicrobial peptide from the venom of the cleptoparasitic bee Melecta albifrons.* ChemBioChem, 2008. **9**(17): p. 2815-2821.
10. Lee, J.K., et al., *A proline-hinge alters the characteristics of the amphipathic α-helical AMPs.* PloS one, 2013. **8**(7): p. e67597.
11. Shin, S., et al., *Antibacterial, antitumor and hemolytic activities of α-helical antibiotic peptide, P18 and its analogs.* The Journal of Peptide Research,

2001. **58**(6): p. 504-514.

12. Pavia, K.E., S.A. Spinella, and D.E. Elmore, *Novel histone-derived antimicrobial peptides use different antimicrobial mechanisms.* Biochimica et Biophysica Acta (BBA)-Biomembranes, 2012. **1818**(3): p. 869-876.
13. Wadhwani, P., et al., *Membrane-active peptides and the clustering of anionic lipids.* Biophysical journal, 2012. **103**(2): p. 265-274.
14. Proline scanning at 1-4 and 11-14 positions was omitted because the impact of proline hinges might be negligible in helix formation near the end of both N- and C-terminus.
15. Torcato, I.M., et al., *The Antimicrobial Activity of Sub3 is Dependent on Membrane Binding and Cell-Penetrating Ability.* Chembiochem, 2013. **14**(15).
16. Helander, I. and T. Mattila-Sandholm, *Fluorometric assessment of Gram-negative bacterial permeabilization.* Journal of applied microbiology, 2000. **88**(2): p. 213-219.
17. He, J., C.G. Starr, and W.C. Wimley, *A lack of synergy between membrane-permeabilizing cationic antimicrobial peptides and conventional antibiotics.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2015. **1848**(1, Part A): p. 8-15.
18. Tamaki, S., T. Sato, and M. Matsuhashi, *Role of lipopolysaccharides in antibiotic resistance and bacteriophage adsorption of Escherichia coli K-12.* Journal of bacteriology, 1971. **105**(3): p. 968-975.
19. Belley, A., et al., *Assessment by time-kill methodology of the synergistic effects of oritavancin in combination with other antimicrobial agents against Staphylococcus aureus.* Antimicrobial agents and chemotherapy, 2008. **52**(10): p. 3820-3822.

국문초록

최윤화

과학교육과 화학 전공

서울대학교

프롤린 꺾임은 높은 알파나선 경향을 갖는 항균 펩타이드에서 빈번하게 발견되는 구조이다. 이것은 분자내 연속적인 수소결합을 파괴하기 때문에 꺾인 펩타이드들은 낮은 알파 나선구조를 갖게되고 완화된 독성을 주며 숙주세포에 대한 세포막 파괴를 크게 감소시킨다. 게다가 몇몇 꺾인 펩타이드들은 향상된 향균효과를 준다. 따라서 특정한 프롤린 꺾임은 항균효과를 향상시키는데 아마도 중요한 역할을 할 수 있다. 이를 입증하기 위해서 우리는 류신과 라이신으로 구성된 14개 아미노산 길이의 양면성 LK, KL 모델 펩타이드들을 사용해서 작은 프롤린 스캐닝 라이브러리를 만들었다. E.coli와 S.aureus에 대한 최소 저해 농도와 적혈구에 대한 용혈 현상을 측정한 후에 꺾인 펩타이드들은 세가지 그룹으로 나눠졌다. 이중 한 그룹은 E.coli에 대한 최소 저해농도가 유지되거든 조금 줄어든 반면 용혈 현상과 S.aureus에 대한 항균 능력이 거의 없어졌다. 이 펩타이드들이 기대한 선택성을 보였기 때문에 우리는 이 그룹에 대한

연구를 더 진행했다. 이 그룹에 속한 펩타이드들 중 하나인 KL-L9P는 특히 그람 음성균에는 외막을 통과하지 못하기 때문에 쓰이지 않았던 항생제들에 대해 가장 강한 시너지효과를 보였다. Sytox Green 염색 실험과 NPN 어세이는 이 펩타이드가 주로 외막을 섭동하여 항생제들이 세포막을 통해 들어오게하는 반면 세포 내막을 투과하거나 부서뜨리지 않는다는 것을 보여주었다. 결과적으로 우리는 세가지 중요한 기준들; 낮은 숙주세포 독성, 높은 외막 섭동 능력, 낮은 내막 파괴 능력이 시너지 효과가 있는 항균 펩타이드를 디자인 하는데 중요한 기준이 될 수 있다.

주요어: 프롤린 꺾인 항균 펩타이드, 시너지 효과, 내막 붕괴, 외막 섭동

학번: 2015-21625