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Conversion of Antimicrobial Peptides Toward Cell Penetrating Peptides; An Introduction of Specific Glutamine and Lysine to the Alpha-Helical Peptide Deletes Membrane Disrupting Ability but Remains Cell Penetrating Ability

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Abstract

Conversion of Antimicrobial Peptides Toward Cell Penetrating Peptides; An Introduction of Specific Glutamine and Lysine to the Alpha-Helical Peptide Deletes Membrane Disrupting Ability but Remains Cell Penetrating Ability

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Higher vertebrates produce antimicrobial peptides (AMPs) as a result of immune response and main mode of action for AMPs is disrupting the membrane. However, penetration of AMPs into cells followed by interact with intracellular targets might be other mechanism. They also have severe toxicity that might come from disruption of eukaryotic membrane as well as modulation of internal targets through membrane translocation. Though AMPs have both membrane disrupting and penetrating mechanism simultaneously, we tried to make and select mutant(s) in which cell disrupting ability is deleted but the penetrating ability is left over among systematically mutants peptide(s) from an amphipathic α-helical model peptide, sequenced as LKKLLKLLKKLLKLAG. Most of mutants showed correlations between their cell penetrating ability and cell disrupting ability. In contrast, L8Q-
and L8K-mutants in which hydrophobic interactions are ruined by hydrophilic groups in Q or K showed cell penetrating ability at low micromolar concentrations, while their membrane disrupting ability were totally abolished. Mutant peptides with unnatural amino acids with shorter and longer carbon chains of Q- and K-analogues were also generated and their membrane disrupting and penetrating abilities were also investigated. While longer carbon chain (homoQ and homoK) mutants gave similar cell penetrating ability, shorter carbon chain mutants (N, Dab and Orn) showed total loss of cell penetrating ability as well as membrane demolishing ability. MTX-conjugated peptides of all cell penetrating peptides selected (Q and K) gave much severe toxicity in comparison with the corresponding non-conjugated mutants, suggesting that internalized MTX might be a culprit of toxicity. Positional Q- and K-mutation of a natural AMP, LL37, gave total loss of cell disrupting ability while be left cell penetrating ability. Taken together, Q and K mutation might be a strategy in nature to be left over the cell penetrating ability while deleting membrane disrupting ability of AMPs.

**Keywords**: antimicrobial peptide, cell penetrating peptide, hemolytic activity, drug delivery activity

**Student number**: 2014-20957
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**Introduction**

In nature, higher eukaryotes produce antimicrobial peptides (AMPs) as a result of immune response[1][2] and main mode of action for AMPs is disrupting the membrane.[3] But in some cases, they can penetrate into cells without disruption of membrane and interact with the internal targets.[3][4][5] For this cell penetrating ability of AMPs and other similarity to cell penetrating peptides (CPPs) like length of peptide, charge and ability to interact with membrane, they can be used as a drug delivery vector for eukaryotic cells, if the disrupting ability to membrane is deleted.[3][5]

However, measuring membrane disrupting and penetrating ability separately is not easy, as minimum inhibitory concentration (MIC) is the only reliable value of the death of bacteria[6], which can be derived from membrane disruption or modulation of internal targets. But in eukaryotic cells, there are several biological effects that could be separately measured from intracellular targeting. One of easily measurable membrane disruption in eukaryotic cells is hemolysis of red blood cells (RBCs). Thus, the effect of membrane disruption could be easily differentiated from other biological effects in eukaryotic cells.

We have been thoroughly investigated RBC’s hemolytic activity of a model amphipathic LK peptide and its mutants, since the model peptide has strong membrane disrupting ability even though it has a reasonable antimicrobial activity. The aim of the previous study was to reduce hemolytic activity in host cells, affording antimicrobial peptide(s) with improved therapeutic index.[7][8] Some mutations that interrupt interactions of hydrophobic face by a polar residue gave total loss of hemolytic activity of RBC, suggesting that breaking up hydrophobic interactions is essential for reducing the membrane disrupting ability.[7][8] Generally, both membrane disrupting and cell penetrating abilities behaves simultaneously.[9] Therefore, mutants with total loss of membrane disrupting ability are expected to lose its cell penetrating ability. But we also found out some mutants still remain membrane disrupting ability, although the cell penetrating ability decreases slightly. We further thought that the anticipated cell penetrating ability might be found in those mutants. It
might be general mutation strategy to convert AMP to CPP if we find out one.

Previous results in our group suggest the most sensitive Leu position in the model LK peptide for eukaryotic membrane disruption.[7] In this manuscript, we describe their cell penetrating ability against eukaryotic cell membrane by generating fluorescent labeled mutant peptides based on the specific Leu position and observing. Among many mutants in hydrophobic face, glutamine (Q) and lysine (K) mutations were deviated from their membrane disruption ability, which was measured by Minimum hemolytic concentration (MHC) of RBC. In other words, theses mutants remain most of cell penetrating ability, while they lose eukaryotic membrane disrupting ability in general. As a “proof-of-concept” experiment, we made methotrexate conjugation of these mutant peptides. As we was anticipated, they gave much severe toxicity in comparison with the corresponding MTX-free mutants, suggesting that internalized MTX might be a culprit of the extra cytotoxicity. Among glutamine (Q) and lysine (K) mutations of LL-37 peptide, cell penetrating ability is maintained or even improved, while their membrane disrupting ability is much reduced, suggesting it is a general way to put Q or K to convert AMP to CPP.
Experimental Section

1. Peptide synthesis

A. General

Peptides were synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc) active ester chemistry on SPS Microwave Peptide Synthesizer (CEM). Rink Amide MBHA resin (Novabiochem) was used for solid-phase peptide synthesis (SPPS) method and synthesized in 50 mg (29.5 µmol) scale. For peptide synthesis, 9-fluorenylmethyloxycarbonyl (Fmoc)-protected alanine, leucine, serine, proline, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, ornithine and diaminobutylic acid were purchased from Novabiochem. Fmoc-protected homo glutamine and homo lysine were purchased from Iris biotech and Fmoc-protected aminoadipic acid was purchased from Bachem.

Deprotection of Fmoc was performed by using 20 % piperidine in N,N-dimethylformamide (DMF, Avantor) and then resin was washed using DMF and dichloromethane (DCM, Daejung). Fmoc-protected amino acid (6 eq.), benzotriazol-1-yl oxytripyrroldinophosphonium hexafluorophosphate (PyBOP, 6 eq. Novabiochem) and N,N-diisopropylethylamine (DIPEA, 6 eq. Sigma) dissolved in DMF were used for coupling of amino acids.

For cleavage of synthesized peptide, cleavage solution contained 950 µL of Trifluoroacetic acid (TFA, Sigma), 25 µL of Triisopropylsilane (TIS, Sigma) and 25 µL of distilled water was used and shaken for 2 h. After cleavage step, resins were filtered and peptide was precipitated using mixture combined with n-hexane (Daejung) and diethyl ether (Avantor). Peptides cleaved from resin were dissolved in methyl sulfoxide (DMSO, Sigma) and purified with HPLC using Zorbax C18 column (3.5 µm, 4.6 x 150 mm) as a stationary phase. For mobile phase, mixture of buffer A (distilled water with 0.1 % v/v TFA) and buffer B (acetonitrile with 0.1 % v/v TFA) was used at a flow rate of 1 mL/min. (Condition of mobile phase: 0-5
min, 5% of buffer B followed by linear gradient 5-70% of buffer B over 25 min). The peptides purified with HPLC were lyophilized by freeze dryer (Operon). Concentration of synthesized peptide including acetyl capped, fluorescence labeled and Methotrexate conjugated peptide was calculated by a spectrometer (Direct detect™ spectrometer, Merk millipore).

B. Acetylation of N-terminal

After the coupling of final amino acid at N-terminal, 20% piperidine in DMF was treated for deprotection of Fmoc and resin was washed with DMF and DCM. For acetylation of N-terminal, mixture contained acetic anhydride (Sigma), 1-hydroxybenzotriazole hydrate (HOBt, Fluka) and 10% v/v DCM in DMF was used and reaction was performed on SPS Microwave Peptide Synthesizer.

C. Conjugation of Methotrexate (MTX)

Methotrexate (MTX) was coupled to the N-terminus of Fmoc deprotected peptide with mixture of MTX (3 eq. Sigma), HOBt (1.5 eq.), O-(benzotriazol-1-yl)-N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU, Sigma, 3 eq.), DIPEA (6 eq.) in DMF. The reaction was performed for 2 h at room temperature.

D. Conjugation of 5(6)-Carboxytetramethylrhodamine (TAMRA)

5(6)-Carboxytetramethylrhodamine (TAMRA, Sigma) was conjugated to the peptide for flow cytometry analysis. For conjugation, amino group at N-terminal of peptide coupled to 5(6)-TAMRA with mixture of 5(6)-TAMRA (2 eq.), O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, Novabiochem, 2eq.), HOBt (2 eq.) and DIPEA (4 eq.) in DMF. The reaction was carried out for 2 h at room temperature.
E. Purification of peptides

**LK** (Ac-LKKLLKLKLLKLAG) was obtained as a white powder (9.2 mg, 17 % yield). MS [M+H]^+:
1861.34 (calcd), 1861.06 (obsd). The HPLC chromatogram of LK is shown below (>95 % purity).

**L8G** (Ac-LKKLLKLGKKLLKLAG) was obtained as a white powder (10.5 mg, 49 % yield). MS [M+H]^+:
1805.28 (calcd), 1806.15 (obsd). The HPLC chromatogram of L8G is shown below (>95 % purity).

**L8S** (Ac-LKKLLKLSKKLLKLAG) was obtained as a white powder (9.5 mg, 44 % yield). MS [M+H]^+:
1835.29 (calcd), 1836.33 (obsd). The HPLC chromatogram of L8S is shown below (>95 % purity).
**L8P** (Ac-LKKLLLPKKLKLKLAG) was obtained as a white powder (13.4 mg, 24 % yield). MS [M+H]^+: 1845.31 (calcd), 1844.78 (obsd). The HPLC chromatogram of L8P is shown below (>95 % purity).

**L8N** (Ac-LKKLLKNKKLKLKLAG) was obtained as a white powder (18.1 mg, 33 % yield). MS [M+H]^+: 1862.30 (calcd), 1861.74 (obsd). The HPLC chromatogram of L8N is shown below (>95 % purity).

**L8Q** (Ac-LKKLLQLQKLLLKLAG) was obtained as a white powder (17.0 mg, 31 % yield). MS [M+H]^+: 1876.31 (calcd), 1875.92 (obsd). The HPLC chromatogram of L8Q is shown below (>95 % purity).
**L8D** (Ac-LKKLLKLDKLLKLAG) was obtained as a white powder (11.7 mg, 21 % yield). MS [M+H]⁺: 1863.28 (calcd), 1863.05 (obsd). The HPLC chromatogram of L8D is shown below (>93 % purity).

![HPLC Chromatogram of L8D](image)

**L8E** (Ac-LKKLLKLEKLLKLAG) was obtained as a white powder (10.3 mg, 19 % yield). MS [M+H]⁺: 1877.30 (calcd), 1876.86 (obsd). The HPLC chromatogram of L8E is shown below (>95 % purity).

![HPLC Chromatogram of L8E](image)

**L8K** (Ac-LKKLLKLLKLKLAG) was obtained as a white powder (9.6 mg, 43 % yield). MS [M+H]⁺: 1876.35 (calcd), 1875.10 (obsd). The HPLC chromatogram of L8K is shown below (>95 % purity).

![HPLC Chromatogram of L8K](image)
**L8R** (Ac-LKKLLKLRKLLLKLAG) was obtained as a white powder (10.6 mg, 47 % yield). MS [M+H]+: 1904.36 (calcd), 1905.33 (obsd). The HPLC chromatogram of L8R is shown below (>95 % purity).

![HPLC Chromatogram of L8R](image)

**L8H** (Ac-LKKLLKHLKKLLLKLAG) was obtained as a white powder (13.1 mg, 59 % yield). MS [M+H]+: 1885.31 (calcd), 1884.26 (obsd). The HPLC chromatogram of L8H is shown below (>95 % purity).

![HPLC Chromatogram of L8H](image)

**L8hQ** (Ac-LKKLLKLhQKLLLKLAG) was obtained as a white powder (22.9 mg, 68 % yield). MS [M+H]+: 1890.33 (calcd), 1889.91 (obsd). The HPLC chromatogram of L8hQ is shown below (>95 % purity).

![HPLC Chromatogram of L8hQ](image)
**L8Aad** (Ac-LKKLLKLAadKKLLKLAG) was obtained as a white powder (10.8 mg, 48 % yield). MS [M+H]+: 1891.31 (calcd), 1892.20 (obsd). The HPLC chromatogram of L8Aad is shown below (>95 % purity).

![HPLC chromatogram of L8Aad](image)

**L8hK** (Ac-LKKLLKlhKKLLKLAG) was obtained as a white powder (21.1 mg, 63 % yield). MS [M+H]+: 1890.36 (calcd), 1890.04 (obsd). The HPLC chromatogram of L8hK is shown below (>95 % purity).

![HPLC chromatogram of L8hK](image)

**L8Dab** (Ac-LKKLLKLDabKKLLKLAG) was obtained as a white powder (31.2 mg, 95 % yield). MS [M+H]+: 1848.32 (calcd), 1848.17 (obsd). The HPLC chromatogram of L8Dab is shown below (>95 % purity).

![HPLC chromatogram of L8Dab](image)
**L8Orn** (Ac-LKKLLKOrrKKLLKLAG) was obtained as a white powder (30.2 mg, 92 % yield). MS [M+H]^+: 1862.33 (calcd), 1862.14 (obsd). The HPLC chromatogram of L8Orn is shown below (>95 % purity).

![HPLC chromatogram of L8Orn](image1)

**MTX-LK** (MTX-LKKLLKLLKKLLKLAG) was obtained as a yellow powder (12.4 mg, 55 % yield). MS [M+H]^+: 2257.50 (calcd), 2255.35 (obsd). The HPLC chromatogram of MTX-LK is shown below (>95 % purity).

![HPLC chromatogram of MTX-LK](image2)

**MTX-L8Q** (MTX-LKKLLQKKLLKLAG) was obtained as a yellow powder (10.9 mg, 48 % yield). MS [M+H]^+: 2272.48 (calcd), 2270.24 (obsd). The HPLC chromatogram of MTX-L8Q is shown below (>95 % purity).

![HPLC chromatogram of MTX-L8Q](image3)
**MTX-L8E** (MTX-LKKLLKLEKKLLKLAG) was obtained as a yellow powder (11.3 mg, 50 % yield).

MS [M+H]^+: 2273.46 (calcd), 2271.28 (obsd). The HPLC chromatogram of MTX-L8E is shown below (>95 % purity).

**MTX-L8K** (MTX-LKKLLKLKKLLKLAG) was obtained as a yellow powder (14.4 mg, 54 % yield).

MS [M+H]^+: 2272.51 (calcd), 2272.14 (obsd). The HPLC chromatogram of MTX-L8K is shown below (>95 % purity).

**Rho-L8G** (Rho-LKKLL KgKKLLKLAG) was obtained as a red powder (2.2 mg, 17 % yield). MS [M+H]^+: 2176.41 (calcd), 2176.15 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8G are shown below (>95 % purity).
**Rho-L8S** (Rho-LKKLKLSSKKLLKLAG) was obtained as a red powder (2.8 mg, 22 % yield). MS [M+H]^+: 2206.42 (calcd), 2206.25 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8S are shown below (>95 % purity).

![HPLC chromatogram of Rho-L8S](image)

**Rho-L8P** (Rho-LKKLKLPPKKLLKLAG) was obtained as a red powder (5.1 mg, 49 % yield). MS [M+H]^+: 2216.45 (calcd), 2215.57 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8P are shown below (>95 % purity).

![HPLC chromatogram of Rho-L8P](image)
Rho-L8N (Rho-LKKLLKLNNKLLKLAG) was obtained as a red powder (2.3 mg, 22 % yield). MS [M+H]+: 2233.44 (calcd), 2233.34 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8N are shown below (>95 % purity).

Rho-L8Q (Rho-LKKLLKLQKLLKLAG) was obtained as a red powder (2.3 mg, 22 % yield). MS [M+H]+: 2247.45 (calcd), 2246.45 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8Q are shown below (>90 % purity).
**Rho-L8D** (Rho-LKKLLKLDKLLKLAG) was obtained as a red powder (2.3 mg, 22 % yield). MS [M+H]⁺: 2234.42 (calcd), 2233.87 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8D are shown below (>95 % purity).

![HPLC Chromatogram of Rho-L8D](image)

**Rho-L8E** (Rho-LKKLLKLEKLLKLAG) was obtained as a red powder (2.2 mg, 21 % yield). MS [M+H]⁺: 2248.44 (calcd), 2247.67 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8E are shown below (>95 % purity).

![HPLC Chromatogram of Rho-L8E](image)
**Rho-L8K** (Rho-LKKLLKLKKLLKLAG) was obtained as a red powder (4.3 mg, 32 % yield). MS [M+H]^+: 2247.49 (calcd), 2247.57 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8K are shown below (>95 % purity).

![HPLC Chromatogram of Rho-L8K](image1)

**Rho-L8R** (Rho-LKKLLKLRKKLLKLAG) was obtained as a red powder (11.2 mg, 83 % yield). MS [M+H]^+: 2275.49 (calcd), 2275.84 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8R are shown below (>90 % purity).

![HPLC Chromatogram of Rho-L8R](image2)
**Rho-L8H** (Rho-LKKLLKHKKLLKLAG) was obtained as a red powder (2.6 mg, 20 % yield). MS [M+H]+: 2256.45 (calcd), 2256.28 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8H are shown below (>90 % purity).

![HPLC Chromatogram of Rho-L8H](image1)

**Rho-L8hQ** (Rho-LKKLLKHLQKLLKLAG) was obtained as a red powder (6.3 mg, 47 % yield). MS [M+H]+: 2261.47 (calcd), 2262.80 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8hQ is shown below (>95 % purity).

![HPLC Chromatogram of Rho-L8hQ](image2)
**Rho-L8Aad** (Rho-LKKLLKAadKKLLKLAG) was obtained as a red powder (2.2 mg, 16 % yield). MS [M+H]^+: 2262.45 (calcd), 2262.44 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8Aad is shown below (>95 % purity).

![HPLC Chromatogram of Rho-L8Aad](image)

**Rho-L8Dab** (Ac-LKKLLKDabKKLLKLAG) was obtained as a red powder (7.3 mg, 56 % yield). MS [M+H]^+: 2219.46 (calcd), 2219.69 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8Dab is shown below (>90 % purity).

![HPLC Chromatogram of Rho-L8Dab](image)
**Rho-L8Orn** (Rho-LKKLLKLOrnKKLLKLAG) was obtained as a red powder (6.7 mg, 51% yield). MS [M+H]^+ : 2233.47 (calcd), 2233.48 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8Orn is shown below (>95% purity).

![HPLC Chromatogram of Rho-L8Orn](image1)

**Rho-L8hK** (Rho-LKKLLKKhKKLLKLAG) was obtained as a red powder (7.1 mg, 53% yield). MS [M+H]^+ : 2261.50 (calcd), 2263.35 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-L8hK is shown below (>95% purity).

![HPLC Chromatogram of Rho-L8hK](image2)
**Lt** (Ac-FKRIVQRIKDFLR) was obtained as a white powder (10.5 mg, 51 % yield). MS [M+H]^+: 1760.07 (calcd), 1759.82 (obsd). The HPLC chromatogram of Lt is shown below (>95 % purity).

**Lt-F1Q** (Ac-QKRVQRIKDFLR) was obtained as a white powder (8.0 mg, 39 % yield). MS [M+H]^+: 1741.06 (calcd), 1740.88 (obsd). The HPLC chromatogram of Lt-F1Q is shown below (>95 % purity).

**Lt-I4Q** (Ac-FKRQVQRIKDFLR) was obtained as a white powder (10.5 mg, 50 % yield). MS [M+H]^+: 1775.05 (calcd), 1774.83 (obsd). The HPLC chromatogram of Lt-I4Q is shown below (>95 % purity).
Lt-V5Q (Ac-FKRIQQRIKDFLR) was obtained as a white powder (11.2 mg, 53% yield). MS [M+H]^+: 1789.06 (calcd), 1788.95 (obsd). The HPLC chromatogram of Lt-V5Q is shown below (>95 % purity).

Lt-I8Q (Ac-FKRIVQRQKDFLR) was obtained as a white powder (17.2 mg, 82 % yield). MS [M+H]^+: 1775.05 (calcd), 1775.01 (obsd). The HPLC chromatogram of Lt-I8Q is shown below (>95 % purity).

Lt-F11Q (Ac-FKRIVQRIKDQLR) was obtained as a white powder (8.3 mg, 40 % yield). MS [M+H]^+: 1741.06 (calcd), 1740.89 (obsd). The HPLC chromatogram of Lt-F11Q is shown below (>95 % purity).
**Lt-L12Q** (Ac-FKRIVQRIKDFQR) was obtained as a white powder (13.2 mg, 65 % yield). MS [M+H]^+:
1775.05 (calcd), 1774.95 (obsd). The HPLC chromatogram of Lt-L12Q is shown below (>95 % purity).

**Lt-F1K** (Ac-KKRIVQRIKDFLR) was obtained as a white powder (20.0 mg, 65 % yield). MS [M+H]^+:
1741.10 (calcd), 1742.80 (obsd). The HPLC chromatogram of Lt-F1K is shown below (>95 % purity).

**Lt-I4K** (Ac-FKRKVQRIKDFLR) was obtained as a white powder (12.8 mg, 41 % yield). MS [M+H]^+:
1775.08 (calcd), 1776.69 (obsd). The HPLC chromatogram of Lt-I4K is shown below (>95 % purity).
**Lt-V5K** (Ac-FKRIKRIKDFLR) was obtained as a white powder (15.0 mg, 47 % yield). MS [M+H]⁺: 1789.10 (calcd), 1790.82 (obsd). The HPLC chromatogram of Lt-V5K is shown below (>95 % purity).

**Lt-I8K** (Ac-FKRIVRKKDFLR) was obtained as a white powder (9.6 mg, 31 % yield). MS [M+H]⁺: 1775.08 (calcd), 1776.78 (obsd). The HPLC chromatogram of Lt-I8K is shown below (>95 % purity).

**Lt-F11K** (Ac-FKRIVQRIKDKLR) was obtained as a white powder (18.0 mg, 58 % yield). MS [M+H]⁺: 1741.10 (calcd), 1742.82 (obsd). The HPLC chromatogram of Lt-F11K is shown below (>95 % purity).
**Lt-L12K** (Ac-FKRVQRIKDFKR) was obtained as a white powder (8.8 mg, 28 % yield). MS [M+H]+: 1775.08 (calcd), 1776.82 (obsd). The HPLC chromatogram of Lt-L12K is shown below (>95 % purity).

![HPLC chromatogram of Lt-L12K](image1)

**Rho-Lt** (Rho-FKRVQRIKDFLR) was obtained as a red powder (3.4 mg, 27 % yield). MS [M+H]+: 2131.21 (calcd), 2131.65 (obsd). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. The HPLC chromatogram of Rho-Lt is shown below (>95 % purity).

![HPLC chromatogram of Rho-Lt](image2)

**Rho-Lt-F1Q** (Rho-QKRVQRIKDFLR) was obtained as a red powder (2.9 mg, 23 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]+: 2112.20 (calcd), 2112.86 (obsd). The HPLC chromatogram of Rho-Lt-F1Q is shown below (>95 % purity).

![HPLC chromatogram of Rho-Lt-F1Q](image3)
Rho-Lt-I4Q (Rho-FKRVQVRIKDFLR) was obtained as a red powder (4.6 mg, 36 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+: 2146.19 (calcd), 2146.89 (obsd). The HPLC chromatogram of Rho-Lt-I4Q is shown below (>95 % purity).

Rho-Lt-V5Q (Rho-FKRIQVQRIKDFLR) was obtained as a red powder (3.0 mg, 24 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+: 2160.20 (calcd), 2160.89 (obsd). The HPLC chromatogram of Rho-Lt-V5Q is shown below (>95 % purity).

Rho-Lt-I8Q (Rho-FKRIVQRQKDFLR) was obtained as a red powder (0.9 mg, 7 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+: 2146.19 (calcd), 2146.30 (obsd). The HPLC chromatogram of Rho-Lt-I8Q is shown below (>95 % purity).
**Rho-Lt-F11Q** (Rho-FKRIVQRIKDQLR) was afforded as a red powder (0.4 mg, 3 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+ : 2112.20 (calcd), 2112.31 (obsd). The HPLC chromatogram of Rho-Lt-F11Q is shown below (>95 % purity).

![HPLC chromatogram of Rho-Lt-F11Q](image)

**Rho-Lt-L12Q** (Rho-FKRIVQRIKDQR) was obtained as a red powder (0.8 mg, 6 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+ : 2146.19 (calcd), 2147.10 (obsd). The HPLC chromatogram of Rho-Lt-L12Q is shown below (>95 % purity).

![HPLC chromatogram of Rho-Lt-L12Q](image)

**Rho-Lt-F1K** (Rho-KKRIVQRIKDFLR) was obtained as a red powder (1.9 mg, 10 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+ : 2112.24 (calcd), 2112.03 (obsd). The HPLC chromatogram of Rho-Lt-F1K is shown below (>95 % purity).

![HPLC chromatogram of Rho-Lt-F1K](image)
**Rho-Lt-I4K** (Rho-FKRKVQRIKDFLR) was obtained as a red powder (11.6 mg, 61 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+: 2146.22 (calcd), 2145.90 (obsd). The HPLC chromatogram of Rho-Lt-I4K is shown below (>95 % purity).

**Rho-Lt-V5K** (Rho-FKRIKQRIKDFLR) was obtained as a red powder (15.5 mg, 81 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+: 2160.24 (calcd), 2160.26 (obsd). The HPLC chromatogram of Rho-Lt-V5K is shown below (>95 % purity).

**Rho-Lt-I8K** (Rho-FKRIVQRKDFLR) was obtained as a red powder (3.9 mg, 21 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]^+: 2146.22 (calcd), 2147.50 (obsd). The HPLC chromatogram of Rho-Lt-I8K is shown below (>95 % purity).
**Rho-Lt-F11K** (Rho-FKRVQRIKDKLR) was obtained as a red powder (4.2 mg, 22 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]+: 2112.24 (calcd), 2113.75 (obsd). The HPLC chromatogram of Rho-Lt-F11K is shown below (>95 % purity).

![HPLC chromatogram of Rho-Lt-F11K](image)

**Rho-Lt-L12K** (Rho-FKRVQRIKDFKR) was obtained as a red powder (4.6 mg, 24 % yield). Isomers of peptide were separated to each isomer by HPLC and one of isomers was used in experiment. MS [M+H]+: 2146.22 (calcd), 2146.53 (obsd). The HPLC chromatogram of Rho-Lt-L12K is shown below (>90 % purity).

![HPLC chromatogram of Rho-Lt-L12K](image)
3. Circular dichroism

Secondary structure of peptide was measured by Chirascan plus Circular Dichroism detector (Applied Photophysics, United Kingdom) and alpha helicity was calculated using CDNN program (version 2.1). Two conditions (10 mM Potassium phosphate buffer pH 7.4 and 50 % 2,2,2-trifluoroethanol (TFE) in same buffer at room temperature) are used in order to measure secondary structure of peptides. Concentration of peptide was 100 µM and CD spectra of peptides was measure from 190 nm to 260 nm.

4. Hemolysis assay

Hemolytic activity of peptides was represented by concentration of peptide which produces 10 % hemolysis of red blood cells. 5 % hematocrit of human red blood cell (hRBC) in phosphate buffered saline (PBS, HyClone) was used and two-fold serial dilution method was performed to dilute the peptides. For calculation of hemolytic activity of peptide, PBS and distilled water condition were used as negative and positive control, respectively. hRBC was incubated with peptide for 6 h at 37 °C and supernatant of each well was used for detection at 540 nm.

5. Cell culture

MDA-MB-231 cells, the human breast cancer cell line, were used for this research. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, HyClone, Thermo) containing 10 % v/v FBS (HyClone, Thermo) and 1 % v/v Penicillin/Streptomycin (HyClone, Thermo) at 37 °C.
6. Flow cytometry analysis

MDA-MB-231 cells were incubated with TAMRA labelled peptides in order to measure the cell penetrating activity of peptides. Cells were seeded at a density of 5 x 10^4 cells per well in serum containing DMEM. The following day, DMEM was removed and cells were treated with TAMRA labelled peptide in serum containing DMEM. After 24 h incubation, cells were trypsinized and collected in microcentrifuge tubes. Collected cells were centrifuged at 14000 rpm for 1 min and resuspended in PBS.

Cellular uptake of peptides was evaluated by Fluorescence-activated cell sorter (BD Accuri C6, BD Biosciences, USA). Cells were analyzed at 488 nm laser and 1 x 10^4 cells were used for each experiment.

7. Mechanism study

To investigate the cell penetrating mechanism of the peptides in MDA-MB-231 cells, cells were seeded at a density of 5 x 10^4 cells per well in serum containing DMEM. After overnight incubation, each treatment for inhibition was performed.

Cells were incubated at 4 °C for 30 min to inhibit the energy dependent mechanism and TAMRA labelled peptides in serum free DMEM were treated for 2 h 30 min. Inhibition of energy dependent endocytosis was performed by treating specific endocytosis inhibitors. 15 μg/mL of EIPA (Sigma), 25 μg/mL of nystatin (Sigma) or 50 μg/mL of wortmannin (Sigma) were incubated with cells for 30 min and serum free DMEM containing TAMRA labelled peptide at appropriate concentration was treated for 2 h 30 min. Cellular uptake of peptides was analyzed by FACS and calculated using TAMRA positive cells (%).
8. Cell viability assay (WST-1 assay)

Viability of cells treated with peptides was measured using WST-1 Assay and EZ-CYTOX (Dogen) was used in this experiment.

MDA-MB-231 cells were seeded at a density of 1.5 x 10³ cells per well in serum containing DMEM. After overnight incubation, DMEM was removed and peptides dissolved in serum containing DMEM was treated for 72 h at 37 °C. After 72 h incubation, the reagent was added to the each well and incubated for 45 min in the incubator. Absorbance at 450 nm of each well was recorded by a 96-well microplate reader (UVM 340, Biochrom).
Results and Discussion

1. Cell penetrating ability of mutant peptides.

We used LK model peptide, LKKLLKLLKLLKLAG (Figure 1A),[10] as base peptide to synthesize systematic mutant peptides. As we already knew that there are many advantages to use a model LK peptide instead of natural AMPs, from results done previously in our group. As shown in sequence, since LK peptide only consists of Leu for hydrophobic surface and Lys for hydrophilic surface, its mutation to other amino acids could give significant effects than natural AMPs that had already evolved to be optimized.

For amphipathic peptides, we already elucidated that the tilted or kinked peptides could be generated by either introducing a polar residue or internal disulfide bonds to disrupt hydrophobic interaction of the region.[7][8] The tilted or kinked peptides generally gave reduced membrane disrupting ability in eukaryotic cell membrane, affording reduced hemolytic activity. After trials with position scanning peptides, we found out that the Ala mutations at 8th position of the LK peptide gave the least hemolytic activity.[7] Furthermore, many mutants at this position gave a whole spectrum of hemolytic activities. This suggests that it might be the most sensitive position to be affected the translocating ability as well as the membrane disrupting ability, because the two abilities co-exist in various AMPs and CPPs.

In order to prove above assumption, we have tried to synthesize peptides with mutations at 8th position. The ten mutant peptides were synthesized using a standard solid phase and Fmoc chemistry in excellent yields (Table 1). Next, hemolytic activities of the peptides were measured using RBC. Measuring hemolytic activity is one of the most sensitive and reliable method for eukaryotic membrane disruption, we did use MHC_{10} values instead of IC_{50} (or LD_{50}) values from cell viability using the same cell line for observation of cell penetrating ability. When we compared the hemolytic activity of LK and LK mutant peptides, all mutant peptides show lower hemolytic activity. Especially, some mutant peptides (Pro, Asn, Gln, Asp, Glu,
Lys and Arg) have reduced hemolytic activity but not thoroughly (64-fold or less reduction), while some mutant (Asp and Glu) showed total loss of hemolytic activity.

Next, to confirm the cell penetrating ability of the peptides, tetramethylrhodamine (TAMRA) dye was conjugated to each mutant peptide. Then, flow cytometry experiments were carried out with the fluorescent labeled peptides using MDA-MB-231 cell. A glance of cell penetrating ability by percent points of TAMRA-positive cell (Figure 2A) reveals that the original LK peptide penetrates into cells most efficiently even in sub-micromolar concentration, while mutants could be categorized into two groups. Those G-, S-, Q-, K-, R-, and H mutants gave reasonable cell penetrating ability at 5 µM concentrations, P-, N-, D- and E-mutants gave almost no cell penetrating ability at the same concentrations. But judged by cell penetrating ability by fluorescent intensity at 5 µM concentration, the abilities are more differentiated, affording that G- and S-mutants have the largest ability, Q-, K-, R-, and H mutants have median ability, P-, N-, D- and E-mutants have no cell penetrating ability (Figure 2B). Since the cell penetrating ability of mutants was more differentiated by the by the latter method, we continuously use these parameters as the cell penetrating ability of each mutant.

α-Helicity of these mutants, measured both in aqueous and membrane conditions by CD spectroscopy, however, showed no correlation with their cell penetrating abilities (Figure 3A and 3B). Probably those delicate changes of mutants for cell penetrating ability was so small that they were not reflected in the helical propensity. A rough correlation between cell penetrating ability and hydrophobicity measured by the retention time of each mutant in HPLC chromatogram was observed instead (Figure 3C). The loss of hydrophobicity, represented by the earlier retention time, can tell how efficiently interactions in the hydrophobic face are disrupted by the hydrophilic or charged side chain that was introduced by mutation. The more disruption then gave poorer cell penetrating ability.

To compare the membrane disrupting and the cell penetrating abilities of peptides, we tried to make a
correlation chart between cell penetrating ability (either percent point of TAMRA positive cells or fluorescence intensity at 5 µM) and membrane disrupting ability (1/ MHC₁₀) in Figure 4A or 4B. Most of mutants were in a correlated trend that can be pointed around the correlation line or curve. But the two mutations, L8Q and L8K, were deviated from the correlation line or curve. Since L8Q and L8K consistently showed more cell penetrating but low disrupting activity, we decided to further study on Q and K mutations.
Figure 1. Helical wheel diagram of LK peptide (A) and Lt peptide (B). Hydrophobic residues are shown as grey and hydrophilic residues as dark grey.
Table 1. Sequences, structures and hemolytic activities of LK and LK mutant peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MHC [μM][a]</th>
<th>α-helicity [%][b]</th>
<th>( t_\text{R} ) [min][c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LK</td>
<td>LKKLLKLLKKLLKLAG</td>
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<tr>
<td>L8G</td>
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<td>40</td>
<td>16.9/37.4</td>
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<td>80</td>
<td>17.0/38.0</td>
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<tr>
<td>L8P</td>
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<td>1280</td>
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<td>19.7</td>
</tr>
<tr>
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<td>17.1/38.8</td>
<td>18.8</td>
</tr>
<tr>
<td>L8Q</td>
<td>LKKLLKQLKKLLKLAG</td>
<td>640</td>
<td>18.0/49.1</td>
<td>19.7</td>
</tr>
<tr>
<td>L8D</td>
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<td>&gt;1280</td>
<td>16.7/33.9</td>
<td>19.5</td>
</tr>
<tr>
<td>L8E</td>
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<td>&gt;1280</td>
<td>16.7/39.6</td>
<td>19.5</td>
</tr>
<tr>
<td>L8K</td>
<td>LKKLLKLKKLLKLAG</td>
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<td>19.2</td>
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<td>L8H</td>
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<td>160</td>
<td>17.5/40.1</td>
<td>18.7</td>
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</table>

[a] Minimum hemolytic concentration (MHC) is evaluated as the peptide concentration that produces 10% hemolysis. [b] α-helicity of peptides was calculated in 10 mM potassium phosphate buffer (first value) or in the same buffer with 50% 2,2,2-trifluoroethanol (TFE) (second value). [c] Retention time of peptides. Conditions of HPLC elute: buffer A (0.1% v/v TFA in water) and buffer B (0.1% v/v TFA in acetonitrile); 0-5% (B %) over 5 min, 5-70% (B %) over 25 min; detected at UV, 220nm; column, Zorbax C18 column (3.5 μm, 4.6 x 150 mm)
Figure 2. Cellular uptake of the peptides in MDA-MB-231 cell at 1 μM (grey) and 5 μM (black). Uptake was represented by number of tetramethylrhodamine (TAMRA) positive cells (A) and mean of fluorescence intensity (B). All of the peptides were labeled with TAMRA dye and used to evaluate cell penetrating abilities in the experiments.
Figure 3. Comparison of cell penetrating ability and α-helicity or hydrophobicity of peptides. Cell penetrating ability was measured at 5 μM and α-helicity was measured in 10 mM potassium phosphate buffer (A) or same buffer containing 50% 2,2,2-trifluoroethanol (TFE) condition (B). HPLC retention time was used to compare cell penetrating activity with hydrophobicity (C).
Figure 4. Comparison of cell penetrating ability and hemolytic activity of peptides. Cell penetrating ability was measured at 5 μM and inverse of MHC$_{10}$ were used to compare cell penetrating activity with hemolytic activity. Trend line is $y=29.6\ln(x)+18.8$ ($R^2=0.860$) (A) and $y=2240x+1370$ ($R^2=0.989$) (B)
2. Confirmation of cell penetrating ability of Q and K mutations by generating peptides with unnatural amino acids

We next questioned that only Q mutation showed significant cell penetrating ability but N- or E- mutant that has only one less carbon with amide functionality or has carboxyl functionality with the same carbon number, did not. Also, K mutation gave the best cell penetrating ability with the longest carbon chain among three basic mutants. This suggests that carbon number(s) in the side chain might affect molecular interactions that might be a critical factor for the penetrating activity. Therefore, we synthesized more mutant peptides that have more or less carbon number(s) with the same amide, carboxylate, or amine functionality at the side chain of 8th residue of LK peptide. Homo glutamine (hQ) was inserted to make analogues of L8N and L8Q for amide functionality. Aminoadipic acid (Aad) was introduced for more carbon numbered carboxylate functionality than Glu. Diaminobutylic acid (Dab), ornithine (Orn), and homo Lys (hK) were used for amine functionality which has less and more carbon numbers than Lys, respectively (Table 2 and Figure 5). We, then, carried out hemolytic activity with acetyl-capped mutants and flow cytometry experiments with TAMRA labeled versions to elucidate the membrane demolishing and cell penetrating activities (Table 2 and Figure 6).

For amide functionality, L8hQ mutant gave negligible membrane demolishing ability ($\text{MHC}_{10} = 640 \, \mu\text{M}$) and a bit improved cell penetrating ability at 1 $\mu\text{M}$ concentration in comparison with N- and Q-mutant. Carboxyl functionality showed no hemolytic activity ($\text{MHC}_{10} > 1280 \, \mu\text{M}$) and no cell penetrating activity with Asp and Glu. In contrast, cell penetrating ability of L8Aad was finally detected at 5 $\mu\text{M}$ concentration, while no membrane-demolishing ability was detected. For amine functionality, L8Dab and L8Orn showed total loss of membrane demolishing ability ($\text{MHC}_{10} > 1280 \, \mu\text{M}$) and cell penetrating ability as well, while L8hK showed similar and negligible membrane demolishing ($\text{MHC}_{10} = 640 \, \mu\text{M}$) and very similar cell penetrating abilities relative to L8K mutant.
Results of data with three different functionalities and different carbon numbered amino acids could be summarized into several factors about two abilities. First of all, the membrane disruption can be significantly reduced by introducing short hydrophilic moiety, especially charged carboxylate or protonated amines, to efficiently break up hydrophobic interactions of hydrophobic face. In this condition, the cell penetrating ability is also erased thoroughly. Secondly, even though these charged functionalities with short side chain ruin both abilities thoroughly, increases of carbon numbers helps to revoke the cell penetrating ability. Finally, a proper breaking up of hydrophobic interactions by neutral amide moiety could be maximized at hQ-mutant, affording negligible membrane demolishing ($MHC_{10} = 640 \mu M$) with the best cell penetrating ability both at 1 and 5 $\mu M$ of concentrations. Although conformational change owing to those partial broken hydrophobic interactions by amide, carboxyl, or amine group was not reflected in $\alpha$-helical propensities (Figure 3A and 3B), some changes were reflected in hydrophobicity measured by retention times of the mutants in HPLC chromatogram (See r.t. in Table 2). Mutants with the more carbon-numbered amino acid in side chain, having the later retention time, afford remained cell penetrating ability and abolished the membrane disrupting ability. This trend was observed in all three functionalities, for example, L8Q and L8hQ for amide functionality, L8Aad for carboxyl functionality, and L8K and L8hK for amine functionality (Figure 6).
**Table 2.** Sequences, structures and hemolytic activities of mutant peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MHC [μM][a]</th>
<th>α-helicity [%][b]</th>
<th>( t_R ) [min][c]</th>
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<td>19.7</td>
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<tr>
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<td>640</td>
<td>18.3/39.4</td>
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<td><strong>Carboxylate functionality</strong></td>
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<td>&gt;1280</td>
<td>16.7/33.9</td>
<td>19.5</td>
</tr>
<tr>
<td>L8E</td>
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<td>&gt;1280</td>
<td>16.7/39.6</td>
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<tr>
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<td>17.1/37.7</td>
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<td>640</td>
<td>18.3/44.1</td>
<td>19.7</td>
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</table>

[a] Minimum hemolytic concentration (MHC) is evaluated as the peptide concentration that produces 10% hemolysis. [b] α-helicity of peptides was calculated in 10 mM potassium phosphate buffer (first value) or in the same buffer with 50% 2,2,2-trifluoroethanol (TFE) (second value). [c] Retention time of peptides. Conditions of HPLC elute: buffer A (0.1% v/v TFA in water) and buffer B (0.1% v/v TFA in acetonitrile); 0-5% (B%) over 5 min, 5-70% (B%) over 25 min; detected at UV, 220nm; column, Zorbax C18 column (3.5 μm, 4.6 x 150 mm)
Figure 5. Structures of amino acids have amide (A), carboxylate (B) and amine (C) functionality.
Figure 6. Cellular uptake of peptides in MDA-MB-231 cell at 1 μM (grey) and 5 μM (black). All of the peptides were labeled with TAMRA dye and used to evaluate cell penetrating abilities in the experiments.
3. Cell penetrating mechanism study on Q and K mutants

Next, we tried to verify that hQ- and K-mutant possess the common internalization mechanisms since L8hQ and L8K possess the greatest cell penetrating activity among their categorized functionalities (Figure 7). The original LK peptide was used as a reference. In the presence of each peptide, cells were incubated at 4 °C or incubated at 37 °C with three different endocytosis inhibitors. Incubation of cells at 4 °C induces energy depletion condition, so it prevents energy dependent penetrating pathway as receptor mediated endocytosis. Those endocytosis inhibitors were 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), nystatin, and wortmannin known to inhibit of macropinocytosis, caveolae-mediated, and both clathrin-mediated endocytosis and macropinocytosis, respectively.[11][12][13][14] All peptides got into cells by energy-dependent mechanism because their penetrating activity were decreased in 4 °C condition. However, in energy-depleted condition, LK peptide possess almost 50 % percent of activity, but L8hQ and L8K peptides lose their cell penetrating activity completely. It showed that both Q and K-mutants got into cells by energy-dependent mechanism(s). Especially, L8hQ and L8K entered the cells through macropinocytosis since their cell penetrating activity was inhibited by EIPA. It represented that cell penetrating pathway could be changed by Q and K mutation from the original LK peptide, which showed partial energy-independent cell penetrating pathway.
**Figure 7.** The effect of inhibitors on cell penetrating pathways using MDA-MB-231 cell treated with LK, L8hQ and L8K peptide. Each bar represents the relative TAMRA positive cell number to control cells treated with peptide only.
4. Biological application using cell penetrating mutant peptides

We next tried to confirm the delivery activity of peptides and used methotrexate (MTX) as a drug. MTX is an antitumor agent that has been used extensively in cancer therapy. As it is an analogue of folic acid, it performs as a metabolic drug for inhibition of dihydrofolate reductase. However, some adverse effects like resistance to MTX of certain cancer cells through several mechanisms and low selectivity restrict the application of the drug.[15][16] Therefore, coupling MTX to the cell penetrating peptide has been used as one of the strategies to overcome these side effects.[15][17]

We conjugated MTX to the N-terminal amide backbone.[18][19] MDA-MB-231 cells were used for this purpose, because the cell line has relatively low toxicity against MTX alone.[20] All non-conjugated peptides showed no cytotoxicity at less than 3 μM concentrations. However, some selected MTX-conjugated peptides showed cytotoxicity at the same concentrations (Figure 8). For example, MTX-conjugated LK peptide without mutation gave the most severe cytotoxicity, followed by MTX-L8G. MTX-L8Q and MTX-L8K showed somewhat weak cytotoxicity, but MTX-L8E showed no toxicity at all. The cytotoxicity of MTX-conjugated peptides have a good correlation with the cell penetrating ability of the as shown in Figure 2. Data suggest that toxicity is not derived from the membrane damage but only proportional to the cell penetrating ability of peptides in these low concentrations. From these results, we concluded that cytotoxicity of the peptides could be brought by the internalized drug by the cell penetrating ability of them.
Figure 8. Cell viability of MDA-MB-231 cells treated with MTX- and non-conjugated peptides at 0.3 μM (grey), 1 μM (dark grey) and 3 μM (black).
5. Effect of Q or K mutation in a natural AMP, LL-37 peptide

Bases on the results coming from Q and K mutations from the LK peptide, we tried the same Q and K mutations on LL-37, a natural AMP, also known as a human cathelicidin.[21] This AMP is ideal for the trial because it has a global amphipathic character when it forms α-helicity (Figure 1B). We did use a truncated form of LL-37, abbreviated as Lt and sequenced as FKRIVQRIKDFLR for this study.[22] Even though it has already Q and K residues, it is in the hydrophilic face that does not perturb hydrophobic residues. Then, we tried to synthesize positional Q- and K-scanned peptides in hydrophobic face. (Table 3) Once again, hemolytic activity of wt Lt (250 µM) was reduced in every Q and K mutant (1280 µM), suggesting that the membrane disrupting ability of the wild type peptide is erased, but at most 5-fold. This is probably owing to the limited amount of peptides used for measuring hemolytic activity, but it also derived from lower membrane disrupting ability of Lt peptide (250 µM) relative to the model LK peptide (5 µM). With LK and its mutant peptides, we observed more than 250-fold reduced hemolytic activity. Then we did try to conjugate TAMRA to every mutant peptide directly and measured cell penetrating ability by flow cytometry experiments (Figure 9). Only one mutant of each series (Lt-F1Q and Lt-F1K) showed a compatible or the same cell penetrating ability relative to wt Lt peptide, suggesting that the position specific Q- and K-mutation only transform its membrane disrupting to penetrating ability, while general Q- and K-mutations just erase membrane disrupting ability.
Table 3. Sequences, structures and hemolytic activity of Lt and Lt mutant peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MHC [μM][a]</th>
<th>α-helicity [%][b]</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; [min][c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lt peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lt</td>
<td>FKRIVQRIKDFLR</td>
<td>250</td>
<td>18.6/41.2</td>
<td>22.0</td>
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<tr>
<td>Q mutant peptides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lt-F1Q</td>
<td>QKRIVQRIKDFLR</td>
<td>&gt;1280</td>
<td>17.5/44.1</td>
<td>18.7</td>
</tr>
<tr>
<td>Lt-I4Q</td>
<td>FKRQVQRIKDFLR</td>
<td>&gt;1280</td>
<td>17.9/51.6</td>
<td>18.6</td>
</tr>
<tr>
<td>Lt-V5Q</td>
<td>FKRIQVRIKDFLR</td>
<td>&gt;1280</td>
<td>17.9/41.3</td>
<td>20.0</td>
</tr>
<tr>
<td>Lt-I8Q</td>
<td>FKRIVQRQKDFLR</td>
<td>&gt;1280</td>
<td>17.6/43.1</td>
<td>16.9</td>
</tr>
<tr>
<td>Lt-F11Q</td>
<td>FKRIVQRIKDQLR</td>
<td>&gt;1280</td>
<td>17.3/42.8</td>
<td>19.4</td>
</tr>
<tr>
<td>Lt-L12Q</td>
<td>FKRIVQRIKDFQR</td>
<td>&gt;1280</td>
<td>17.3/52.8</td>
<td>18.7</td>
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<tr>
<td>K mutant peptides</td>
<td></td>
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<td></td>
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<tr>
<td>Lt-F1K</td>
<td>KKRIVQRIKDFLR</td>
<td>&gt;1280</td>
<td>16.9/27.1</td>
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<tr>
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<td>17.0/38.5</td>
<td>17.3</td>
</tr>
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<tr>
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<td>16.9</td>
</tr>
<tr>
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<td>&gt;1280</td>
<td>16.7/36.5</td>
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</tr>
<tr>
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<td>&gt;1280</td>
<td>16.7/34.9</td>
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</table>

[a] Minimum hemolytic concentration (MHC) is evaluated as the peptide concentration that produces 10 % hemolysis. [b] α-helicity of peptides was calculated in 10 mM potassium phosphate buffer (first value) or in the same buffer with 50 % 2,2,2-trifluoroethanol (TFE) (second value). [c] Retention time of peptides. Conditions of HPLC elute: buffer A (0.1 % v/v TFA in water) and buffer B (0.1 % v/v TFA in acetonitrile); 0-5 % (B %) over 5 min, 5-70 % (B %) over 25 min; detected at UV, 220nm; column, Zorbax C<sub>18</sub> column (3.5 μm, 4.6 x 150 mm)
Figure 9. Cellular uptake of Q and K mutant Lt peptides in MDA-MB-231 cell at 5 μM (grey) and 10 μM (black). All of the peptides were labeled with TAMRA dye and used to evaluate cell penetrating abilities in the experiments.
Conclusion

The 8th position of Leu to Gln or Lys mutation of the model LK peptide deletes membrane disrupting ability but remains membrane translocating ability, which apparently resulted in converting function of the lytic peptide to membrane penetrating peptide that do not give any more lytic toxicity against eukaryotic cell. Even though, these mutations disrupt hydrophobic interactions of the amphipathic peptides, they still possess reasonably high $\alpha$-helical propensity than other mutations and maintain cell penetrating ability. High helical propensity in amphipathic peptide is the common character for both cell disrupting and penetrating abilities, breaking-up hydrophobicity by polar residues theoretically disrupt both membrane disrupting and penetrating ability. In this study, however, we found out that breaking-up hydrophobicity by short charged residues totally erase both abilities, the same breaking-up by longer charged residues (such as K- or hK-mutant) or reasonable sized neutral polar residues (Q- or hQ-mutant) could erase the membrane demolishing ability but remain the cell penetrating ability. In contrast to the original LK peptide, these two types of mutants are translocated into cells by an energy-dependent and caveolae-mediated endocytosis pathway using MDA-MB-231 cells. As a “proof-of-concept” experiment, we made methotrexate conjugation of mutants. Owing to the cell penetrating ability of these mutants, the MTX-conjugated peptides gave increased cytotoxicity in comparison with the corresponding MTX-free mutants in MDA-MB-231 cell. This suggests that the internalized MTX might be a culprit of the extra cytotoxicity. The same Q and K mutational strategy was applied to a truncated version of a natural antimicrobial peptide, LL-37, showing that specific positional Q- and K-mutation maintains their cell penetrating ability, while the mutants totally erase membrane demolishing ability. Taken together, Q- and K-mutation is a key residual change to keep cell penetrating ability, while other polar residue in nonpolar face of the amphipathic peptides just removes both the membrane disrupting ability and cell penetrating ability. These findings can be applied for transforming natural AMPs to cell penetrating peptides or designing new CPP against eukaryotic cells.
References


국문초록

김서연

과학교육과 화학전공

서울대학교

항균 펩타이드는 생명체가 외부 병원체에 대해 갖는 선천적인 면역 반응에 의한 결과로 만들어낸 생성물이다. 이때, 이 항균 펩타이드가 세포막을 파괴하는 것이 병원체를 죽이는 주요 메커니즘이기는 하지만, 세포막을 투과하여 내부의 특정한 표적을 조절하는 것 또한 다른 하나의 메커니즘이 될 수 있다. 그러나 이러한 항균 펩타이드는 세포막을 투과하는 것 외에 전핵세포의 세포막 파괴로 인한 독성을 지닐 가능성 또한 가지고 있다. 이렇게 항균 펩타이드는 세포 파괴성과 세포 투과성을 모두 가지고 있으므로, 우리는 알파나선 형태의 모델 펩타이드(LK peptide)를 체계적으로 변화 시킴으로써 펩타이드의 세포막 파괴성은 없었고, 투과성은 남아있 는 펩타이드를 찾아내고자 노력하였다. 실험 결과, 합성한 대부분의 변종 펩타이드는 막 파괴성과 세포 투과성이 비례하는 모습을 보였다. 그러나 소수성 부분의 상호작용을 진수성 잔기로 적당히 붕괴시킨 L8Q와 L8K 펩타이드의 경우에는 낮은 마이크로 볼 정도의 농도에서 막 파괴성 없이 세포 투과성을 유지하고 있음을 발견하였다. 이후 Q와 K와 같은 작용기를 가지면서 잔기의 길이 가 다른 아미노산을 지닌 펩타이드를 합성하여, 막 파괴성과 세포 투과성을 측정하였다. Q와 K보다 더 긴 잔기를 가진 아미노산을 도입하였을 때에는 기존과 비슷한 세포 투과성을 가졌지만, 더 짧은 잔기를 가진 아미노산의 경우에는 막 파괴성과 함께 세포 투과성이 감소하는 것을 볼 수 있었다. 이후 Methotrexate (MTX)라는 항암제를 펩타이드에 연결시켜, MTX가 달린 펩타이
드와 그렇지 않은 캡타이드간의 암세포에 대한 독성을 비교해보았다. 그 결과, MTX가 달린 캡타이드가 훨씬 독성이 크다는 것을 발견하였고, 이는 캡타이드에 의해 세포 안으로 MTX가 효율적으로 전달되었기 때문임을 알 수 있었다. 마지막으로 이러한 Q와 K의 도입으로 인한 변화가 자연에 존재하는 항균캡타이드에도 적용될 수 있는지 알아보기 위해 Lt 캡타이드의 소수성 부분에 있는 아미노산에 Q와 K로 치환시킨 캡타이드를 합성하였다. 앞선 결과와 마찬가지로 Lt 캡타이드에 Q와 K를 도입하였을 때, 막 파괴성이 사라지고, 세포 투과성이 유지되는 것을 발견하였다.

모든 실험 결과를 종합하여보았을 때, 이러한 Q와 K의 도입은, 항균 캡타이드의 막 파괴성은 줄이고 세포 투과성은 유지시켜, 이를 세포 투과 캡타이드로 만드는 하나의 전략이 될 수 있을 것이다.

주요어 : 항균 캡타이드, 세포 투과 캡타이드, 용혈 현상, 세포 투과 메커니즘, Methotrexate

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