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Short communication

Cell Selectivity of an Antimicrobial Peptide Melittin Diastereomer with D-amino Acid in the Leucine Zipper Sequence

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Melittin (ME), a linear 26-residue non-cell-selective antimicrobial peptide, displays strong lytic activity against bacterial and human red blood cells. To design ME analogue with improved cell selectivity, we synthesized a melittin diastereomer (ME-D) with D-amino acid in the leucine zipper sequence (Leu-6, Lue-13 and Ile-20). Compared to ME, ME-D exhibited the same or 2-fold higher antibacterial activity but 8-fold less hemolytic activity. Circular dichroism analysis revealed that ME-D has much less α-helical content in α-helical content in the presence of zwitterionic EYPC/cholesterol (10 : 1, w/w) liposomes compared to negatively charged EYPE/EYPG (7 : 3, w/w) liposomes. The blue shift of the fluorescence emission maximum of ME-D in zwitterionic EYPC/ cholesterol (10 : 1, w/w) liposomes was much smaller than in negatively charged EYPE/EYPG (7 : 3, w/w) liposomes. These results suggested that the improvement in therapeutic index/cell selectivity of ME-D is correlated with its less permeability to zwitterionic membranes.

Keywords: Antimicrobial peptide, Cell selectivity, Leucine zipper sequence, Melittin

Introduction

Antimicrobial peptides (AMPs) are important and effective components in innate host defenses against infectious pathogens (Boman, 1995; Zasloff *et al*., 2002). Recently, AMPs have been tapped as a new source of potential antibiotics to combat the increasing emergence of drug-resistant bacteria (Andreu and Rivas, 1998; Cudic *et al*., 2002; Park *et al*., 2007). AMPs are classified into two types on the basis of their cell selectivity: those that are toxic for bacterial cells but not mammalian cells (*e.g.* magainins and cecropins), and those that are toxic to both bacterial and mammalian cells (*e.g.* melittin and pardaxin) (Shai, 1999; Glukhov *et al*., 2005).

Melittin (ME) is isolated from the venom of European honey bee, *Apis mellifera* (Habermann and Jentsch, 1967). It is toxic not only to a variety of microorganisms, but also to mammalian cells, *e.g.* causing hemolysis against human erythrocytes, which has prevented its usage as therapeutic agents (Blondelle *et al*., 1993). Several attempts have been done to design analogues of ME with improved selectivity toward bacterial cells (reducing toxicity against mammalian cells and increasing antibacterial activity). For example, a CA-ME hybrid peptide consisting of cecropin A (CA) and ME sequences, and an analogue designed from the C-terminal 15-residue fragment (residues 12-26) exhibited similar antibacterial activity to ME but markedly less hemolytic activity (Oren and Shai, 1997; Yan *et al*., 2003).

Recently, Asthana *et al.* reported that the presence of the leucine zipper sequence in ME, in which every seventh amino acid is leucine or isolucine (Leu-6, Leu-13, and Ile-20 at the 'a' position and Ile-2, Leu-9 and Leu-16 at the 'd' position) (Asthana *et al*., 2004). The leucine zipper sequence in ME was known to play a prominent role in conferring toxicity to mammalian cells but not in its antibacterial activity (Asthana *et al*., 2004).

In this study, to develop a novel ME analogue with improved cell selectivity (reducing hemolytic activity while increasing antibacterial activity), we synthesized a ME diastereomer, in which Leu-6, Lue-13 and Ile-20, which lie at the 'a' position in the leucine zipper sequence, were replaced with D-amino acid. The lytic activity of ME-D against several bacterial strains and human red blood cells were investigated. We examined the secondary structures of the peptides in bacterial or mammalian membrane-mimicking environments by CD spectroscopy. Furthermore, we examined their permeability

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with model phospholipid membranes by tryptophan fluorescence.

Materials and Methods

Materials. Rink amide 4-methylbenzhydrylamine resin, fluoren-9-yl-methoxycarbonyl (Fmoc)-amino acids and other reagents for the peptide synthesis were purchased from Calbiochem-Novabiochem. Egg yolk L-α-phosphatidylcholine (EYPC), egg yolk L-α-phosphatidylethanolamine (EYPE) and egg yolk L-α-phosphatidyl-DL-glycerol (EYPG) were supplied from Sigma Chemical Co. All other reagents were of analytical grade. The buffers were prepared in double glass-distilled water.

Peptide synthesis. The peptides used in this study were prepared by Fmoc-based solid-phase method on rink amide MBHA resin (0.54 mmole/g). DCC (dicyclohexylcarbodiimide) and HOBt (N-hydroxybenzotriazole) were used as coupling reagent, and ten-fold excess Fmoc-amino acids were added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/water/thioanisole/ethanedithiol/triisopropylsilane (81.5 : 5 : 5 : 5 : 2.5 : 1, v/v) for 2 h at room temperature, the crude peptides were repeatedly extracted with diethyl ether and purified by reverse phase HPLC on a preparative Vydac C_{18} column (15 mm, 20 × 250 mm) using an appropriate 0-80% water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid. The final purity of the peptides (>98%) was assessed by reverse phase HPLC on an analytical Vydac C_{18} column (5 mm, 4.6 × 250 mm) and their identities were confirmed by MALDI-TOF MS (matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry) (Shimadzu).

Antimicrobial activity (MIC). The antibacterial activities of the peptides against three Gram-positive bacterial strains and three Gram-negative bacterial strains were examined in sterile 96-well plates using the broth microdilution method as our previous reports (Shin *et al*., 1996; 1999; 2000; Yang *et al*., 2003) Aliquots (100 ml) of a bacterial suspension at 2×10^6 colony-forming units (CFU)/ml in 1% peptone were added to 100 ml of peptide solution (serial 2-fold dilutions in 1% peptone). After incubation for 18-20 h at 37°C, the inhibition of bacterial growth was determined by measuring the absorbance at 620 nm with a Microplate autoreader EL 800 (Bio-Tek Instruments). The minimal inhibitory concentration (MIC) is defined as the minimal peptide concentration that inhibits bacteria growth. Three types of Gram-positive bacteria (*Bacillus subtilis* [KCTC 3068], *Staphylococcus epidermidis* [KCTC 1917] and *Staphylococcus aureus* [KCTC 1621]) and three types of Gram-negative bacteria (*Escherichia coli* [KCTC 1682], *Pseudomonas aeruginosa* [KCTC 1637] and *Salmonella typhimurium* [KCTC 1926]) were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology.

Hemolytic activity (MHC). Fresh human red blood cells (hRBCs) were washed 3 times with PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.4) by centrifugation for 7 min at 1000 × *g* and resuspended in PBS. The peptide solutions (serial 2-fold dilutions in PBS) were added to 100 ml suspension of hRBCs [4% (v/v) in final] in PBS to the final volume of 200 μl and were incubated for 1h at 37°C. The samples were then centrifuged at 1000 × *g* for 5 min, and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at 405 nm by Microplate ELISA Reader. The MHC is defined as the peptide concentration that produces 10% hemolysis against human red blood cells. For negative and positive controls, hRBCs in PBS (A_{blank}) and in 0.1% Triton X-100 (A_{triton}) were used, respectively. The percentage of hemolysis was calculated according to the equation (Oh *et al*., 2000; Song *et al*., 2005; Zhu *et al*., 2006).

$$\% \text{ hemolysis} = 100 \times [(A_{sample} - A_{blank})/(A_{triton} - A_{blank})]$$

Circular dichroism (CD) analysis. The CD spectra of peptides were recorded in the presence of vesicles composed of either 1 mM EYPE/EYPG (7 : 3, w/w) SUVs or 1 mM EYPC/cholesterol (10 : 1, w/w) SUVs by utilizing a Jasco J-715 CD spectrophotometer (Tokyo, Japan). The samples were scanned in a capped quartz cuvette of 1-mm path length in the wavelength range of 250-190 nm at 20°C under nitrogen flush. The fractional helicities were calculated with the help of mean residue ellipticity values at 222 nm by the following equation: % α-helical content = $([\theta]_{222} - [\theta]^0_{222})/([\theta]^{100}_{222} - [\theta]^0_{222}) \times 100$. As reported previously, where $[\theta]_{222}$ was the experimentally observed mean residue ellipticity at 222 nm and values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, which correspond to 0% and 100% helix content at 222 nm, were estimated to be -2000 and -32 000, respectively (Wu *et al*., 1981; Shin *et al*., 2001; Song *et al*., 2005).

Preparation of small unilamellar vesicles (SUVs). Small unilamellar vesicles were prepared by a standard procedure with required amounts of either EYPE/EYPG (7 : 3, w/w) or EYPC/cholesterol (10 : 1, w/w) for tryptophan fluorescence. Dry lipids were dissolved in chloroform in small glass vessel. Solvents were removed by rotary evaporation to form a thin film on the wall of a glass vessel and then lyophilized overnight. Dried thin films were resuspended in Tris-HCl buffer by vortex mixing. The lipid dispersions were then sonicated in ice water for 10-20 min with a titanium-tip ultrasonicator until the solution became transparent.

Tryptophan fluorescence blue shift. The fluorescence emission spectrum of tryptophan of peptides was monitored in aqueous Tris-HCl buffer (10 mM Tris, 0.1 mM EDTA, 150 mM NaCl, pH 7.4), and in the presence of vesicles composed of either EYPE/EYPG (7 : 3, w/w) SUVs or EYPC/cholesterol (10 : 1, w/w) SUVs. In these fluorometric studies, small unilamellar vesicles (SUVs) were used to minimize differential light scattering effects (Mao and Wallace, 1984; Shai *et al*., 1990). The tryptophan fluorescence measurements were taken with a model RF-5301PC spectrophotometer (Shimadzu, Japan). Each peptide was added to 3 ml of Tris-HCl buffer (10 mM Tris, 0.1 mM EDTA, 150 mM NaCl, pH 7.4) containing 0.3 mM liposomes (pH 7.4), and the peptide/liposome mixture (a molar ratio of 1 : 200) was allowed to interact at 20°C for 10 min. The fluorescence was excited at 280 nm, and the emission was scanned from 300 to 400 nm.

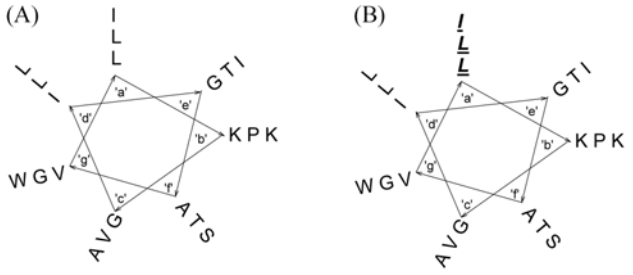

Fig. 1. Helical wheel projections of ME (A) and ME-D (B). ***L*** and ***I*** indicate D-Leu and D-Ile, respectively.

Results and Discussion

Melittin (ME) contains the leucine zipper sequence, in which every seventh amino acid is leucine or isolucine (Leu-6, Leu-13, and Ile-20 at the 'a' position and Ile-2, Leu-9 and Leu-16 at the 'd' position) (Fig. 1 and Table 1). To develop a novel cell-selective ME analogue, we synthesized a ME diastereomer, in which Leu-6, Lue-13, and Ile-20, which lie at the 'a' position in the leucine zipper sequence, were replaced by D-Leu, D-Leu and D-Ile, respectively.

To provide an overall measure of antibacterial activity of the peptides, we calculated the geometric mean (GM) of the minimal concentration that inhibits bacteria growth (MIC) in six selected bacterial strains. As the cytotoxicity of the peptides against mammalian cells, the MHC defined as the peptide concentration that produces 10% hemolysis was used. ME-D exhibited the same or 2-fold higher antibacterial activity and 8-fold less hemolytic activity, compared to ME (Table 2).

To assess the cell selectivity of the peptides, we calculated their therapeutic index, which is a widely accepted measure of the cell selectivity of antimicrobial agents. A larger therapeutic index indicates greater cell selectivity (Chen *et al*., 2005). The therapeutic index of ME was 0.6, which is consistent with its poor cell selectivity (Table 2). In contrast, ME-D had therapeutic index of 8.9 (15-fold increase compared to ME).

CD spectroscopy was used to examine the α-helical contents in the secondary structure of the peptides in negatively charged EYPE/EYPG (7 : 3, w/w) phospholipids (bacterial membrane mimicking environments) or zwitterionic EYPC/cholesterol (10 : 1, w/w) phospholipids (mammalian outer membrane mimicking environments). Although the replacement of the leucine zipper sequence in ME with D-amino acid residue (ME-D) caused reduction in α-helical content in both negatively charged EYPE/EYPG (7 : 3, w/w) and zwitterionic EYPC/cholesterol (10 : 1, w/w) phospholipids, ME-D caused much more significant reduction in α-helical content in the presence of zwitterionic EYPC/cholesterol (10 : 1, w/w) phospholipids compared to negatively charged EYPE/EYPG (7 : 3, w/w) phospholipids. The percent α-helical contents of the peptides in the presence of zwitterionic EYPC/cholesterol (10 : 1, w/w) phospholipids was just 11.5% (Fig. 2 and Table 3).

The present of a tryptophan at position 19 of ME and ME-D allowed us to monitor the binding of the peptides to liposome by the fluorescence emission of the tryptophan (Table 4). ME and ME-D had a fluorescence emission maximum at 350 nm in buffer, which is typical for Trp in a water-polar environment. In negatively charged EYPE/EYPG (7 : 3, w/w) liposomes, the fluorescence emission maxima of ME and ME-D exhibited a significant blue shift of 17 nm and 14 nm, respectively, indicating ME and ME-D penetrate into the hydrocarbon region of anionic lipid bilayer. In zwitterionic

Table 2. Antimicrobial and hemolytic activities of peptides

Cell Types	ME	ME-D
Bacterial cells (MIC: μM)[a]		
Escherichia coli (KCTC 1682)	2	1
Pseudomonas aeruginosa (KCTC 1637)	2	1
Salmonella typhimurium (KCTC 1926)	2	1
Bacillus subtilis (KCTC 3068)	1	0.5
Staphylococcus epidermidis (KCTC 1917)	1	0.5
Staphylococcus aureus (KCTC 1621)	0.5	0.5
GM (μM)[b]	1.4	0.7
MHC (μM)[c]	0.78	6.25
Therapeutic index (MHC/GM)[d]	0.6	8.9

[a]Each MIC is the mean determined from three independent experiments performed in triplicate with a standard deviation of 23.0%.
[b]The geometric mean of MIC values from all bacterial strains in this Table.
[c]MHC is defined as the peptide concentration that produces 10% hemolysis against human red blood cells.
[d]The ratio of the MHC (μM) over the geometric mean MIC (μM).

Table 1. Amino acid sequences, calculated and observed molecular masses of the peptides

Peptide	Amino acid sequence[a]	Molecular mass (Da)	
		Calculated	Observed[a]
ME	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2846.5	2846.4
ME-D	GIGAV***L***KVLTTG***L***PALISW***I***KRKRQQ-NH_2	2846.5	2847.0

L and ***I*** indicate D-Leu and D-Ile, respectively.
[a]Molecular mass were determined by MALDI-TOF-MS.

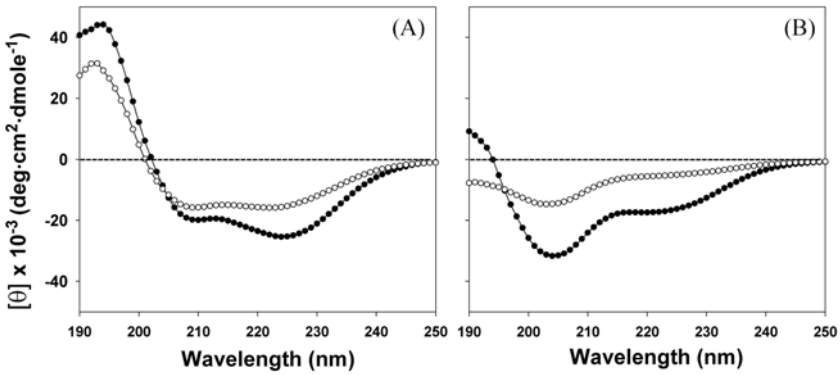

Fig. 2. The CD spectra of the peptides in 1 mM EYPE/EYPG (7 : 3, w/w) SUVs (A) or 1 mM EYPC/cholesterol (10 : 1, w/w) SUVs (B). The concentration of the peptide was 50 μM. Peptides are indicated as follows: ME (●-●-●); ME-D (○-○-○).

Table 3. Mean residual ellipticity at 222 nm ($[\theta]_{222}$) and percent α-helical contents of the peptides in the presence of 1 mM EYPE/EYPG (7 : 3, w/w) liposomes or 1 mM EYPC/cholesterol (10 : 1, w/w) liposomes

Peptides	EYPE/EYPG (7 : 3, w/w)		EYPC/cholesterol (10 : 1, w/w)	
	$[\theta]_{222}$	α-helix (%)	$[\theta]_{222}$	α-helix (%)
ME	-24724.7	75.7	-15896.7	46.3
ME-D	-17232.7	50.8	-5443.0	11.5

Table 4. Tryptophan emission maxima of 1.5 μM peptides in Tris-buffer (pH 7.4) or in the presence of 0.3 mM EYPE/EYPG (7 : 3, w/w) SUVs and 0.3 mM EYPC/cholesterol (10 : 1, w/w) SUVs

Peptides	Tris-HCl buffer (nm)	EYPE/EYPG (7 : 3, w/w) (nm)	EYPC/cholesterol (10 : 1, w/w) (nm)
ME	350	333 (17)[a]	336 (14)
ME-D	350	336 (14)	345 (5)

[a]Blue shift of Trp emission maxima compared to Tris-HCl buffer.

EYPC/cholesterol (10 : 1, w/w) phospholipids, ME still exhibited a marked blue shift of 14 nm, but ME-D showed the smallest blue shift of 5 nm in the fluorescence emission maximum. These results suggest that ME-D did not deeply insert into the hydrophobic lipid phase of the zwitterionic lipid bilayer unlike ME. The less permeability of ME-D than ME to zwitterionic membranes was well in agreement with the less hemolysis of ME-D than ME.

Taken together, CD spectroscopy and tryptophan fluorescence spectroscopy suggested that the improvement in therapeutic index/cell selectivity of ME-D is correlated with its less permeability to zwitterionic membranes.

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