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Cell selectivity-membrane phospholipids relationship of the antimicrobial effects shown by pleurocidin enantiomeric peptides‡

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Previously, we investigated the antimicrobial properties of pleurocidin (Ple) enantiomers. Our studies showed that the L-enantiomer exhibited about a 2–16 fold more potent activity against bacterial strains as compared to that of the D–enantiomer. However, fungal strains were about two–fold more susceptible to the D–enantiomer than to the L-enantiomer. In this study, confocal laser scanning microscopy indicates that the Ple enantiomers internalize into the cell surface. The present results also suggest that they could be characterized by a membrane–active mechanism. To further elucidate their selective membranolytic activities, we conducted a fluorescence analysis. A study with 1,6–diphenyl–1,3,5–hexatriene, a hydrophobic molecule, showed that the L–and the D–enantiomer exert more potent antibacterial or antifungal activity than their opposite enantiomer, respectively. Furthermore, we synthesized liposomes by using representative phospholipids consisting of bacterial or fungal membranes. Our results show that the L-enantiomer causes significant dye leakage from negatively charged liposomes (PG/CL; 58 : 42, PC/PG; 1 : 1, w/w) which mimic bacterial membranes such as *Staphylococcus aureus*. Conversely, the D–enantiomer has more potent leakage effects against fungal liposomes (PC/PE/PI/ergosterol; 5 : 4 : 1 : 2, w/w/w/w, PC/ergosterol; 10 : 1, w/w). In summary, these results suggest that the selective antimicrobial effects of the Ple enantiomers against bacterial and fungal cells may be due to the different lipid compositions of prokaryotes and eukaryotes. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: pleurocidin; enantiomer; antimicrobial effect; cell selectivity; lipid composition

Introduction

Naturally occurring, antimicrobial peptides are usually from 12- to 50-amino acid long and are folded into a variety of structures, including α-helices, β-sheets, extended helices, and loops [1]. Despite this structural variation as well as an extensive sequence variability, most antimicrobial peptides contain two unique features, in that they are polycationic, with a net positive charge of more than +2, and fold into amphipathic structures, characterized by hydrophobic and hydrophilic domains [1]. These properties allow them to interact with the negatively charged surface molecules of gram-negative bacteria, such as lipopolysaccharides (LPS), and to insert into the negatively charged cytoplasmic membranes of most bacteria [2].

Among the cationic and amphipathic peptides, cecropins [3,4] and other antibiotic peptides of the animal kingdom, including defensins [5], magainins [6], and melittin [7], are thought to exert their antimicrobial activity by forming ion channels in lipid bilayer membranes [8]. This idea has been based on the studies of electrical conductivity in artificial lipid bilayers [5,9–11], in which the activity is a function of the peptide structure and composition of the membrane lipids [8]. Additionally, it is known that the formation of transmembrane pores or ion channels on the cellular membranes may cause leakage of metabolites and, eventually, disruption of the microbial cell structures which are the major killing mechanism for these peptides [12]. However, although many studies have been published addressing this issue, the exact molecular mechanism underlying antimicrobial peptide-mediated cell death is still a subject of debate [2].

Pleurocidin (Ple) (GWGSFFKKAAHVGKHVGKAALTHYL-NH_2), one of the most well-known cationic antimicrobial and a membrane-active peptide as well, was isolated from the skin mucous of the winter flounder *Pleuronectes americanus* [13,14]. This peptide has been reported to exhibit a broad range of antimicrobial activities, to display a primary sequence homology with dermaseptins from the skin of the arboreal frog *Phyllomedusa bicolor* [15] and ceratatoxins [16], and also to possess an amphipathic α-helical structure [17]. Previously, we proposed not only that the antifungal effects of Ple are due to its membrane-disruption mechanism [18] but also that the discrepancy of the secondary structure between the L- and the D-enantiomer makes their antibacterial activities

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distinct [14]. Furthermore, interestingly, according to our previous studies, the L- and the D-enantiomers exert more potent activity against bacterial and fungal cells, respectively.

Therefore, in this study we investigated in detail the specific biological activities of the two enantiomeric Ple peptides. We also suggest that the lipid composition of bacteria and fungi could be the primary factor for the cell selectivity of the Ple enantiomers.

Materials and Methods

Materials

Fmoc-L-amino acids, Fmoc-D-amino acids, and reagents (DIC, HOBt) used during peptide chain assembly were purchased from Novabiochem as synthesis grade. Dichloromethane (DCM), N,N-dimethylformamide (DMF), N-methylpyrrolidinone (NMP), and N,N-diisopropylethylamine (DIEA) were purchased from Deajung Chemicals Co., Ltd.

Solid-phase Peptide Synthesis of Ple and FITC-labeled Ple

We entrusted peptide synthesis to Anygen Co., a peptide company. The following procedures of peptide synthesis are offered by Anygen. The assembly of peptides consisted of a 60-min cycle for each residue at ambient temperature as follows: (i) the 2-chlorotrityl (or 4-methylbenzhydrylamine (MBHA) amide) resin was charged to a reactor and then washed with DCM and DMF respectively; (ii) a coupling step with vigorous shaking using a 0.14 mM solution of a Fmoc-L-amino acid (or a Fmoc-D-amino acid) and a Fmoc-L-amino acid (or a Fmoc-D-amino acid) preactivated for approximately 60 min with a 0.1 mM solution of 0.5 M HOBt/DIC in DMF; (iii) after the final assembly of the amino acids, FITC was coupled at the *N*-terminus of the peptide resin [FITC labeling procedures: (i) FITC was dissolved in NMP; (ii) this FITC solution was poured into the reactor which contained the peptide resin; (iii) a 2 M DIEA solution was slowly added into the reactor and then shaken for 3 h at ambient temperature]. Finally, the peptide was cleaved from the resin using a trifluoroacetic acid (TFA) cocktail solution at ambient temperature [19,20].

Analysis and Purification of the Peptide Using HPLC

Analytical and preparative reverse-phase high performance liquid chromatography (HPLC) runs were performed with a Shimadzu 20A or 6A gradient system. Data were collected by using an SPD-20A detector at 230 nm. Chromatographic separations were achieved with a 1%/min linear gradient of buffer B in A (A = 0.1% TFA in H_2O; B = 0.1% TFA in CH_3CN) over 40 min at flow rates of 1 ml/min and 8 ml/min using Shimadzu C_{18} analytical (5 μm, 0.46 cm × 25 cm) and preparative C_{18} (10 μm, 2.5 cm × 25 cm) columns, respectively (Table 1).

Confocal Laser Scanning Microscopy (CLSM)

The intracellular distribution of the peptides was analyzed by means of a confocal laser scanning microscopy (CLSM). *Candida albicans* (ATCC 90 028) cells were treated with the FITC-labeled peptides and incubated for 2 min at 28 °C. After incubation, cells were harvested by means of centrifugation at 10 000 rpm for 5 min and washed with ice-cold phosphate buffered saline (PBS [pH 7.4]). Visualization and localization of the labeled peptides were conducted using the Laser Scanning Spectral Confocal Microscope (Leica TCS SP2, Leica, Swiss) [21].

Measurements of Plasma Membrane Fluorescence Intensity

Fluorescent intensities from the plasma membrane of bacterial or fungal cells, labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Eugene, Oregon), were used to compare the activities of the Ple enantiomers. Specifically, 0.6 mM DPH, dissolved in DMF, was added to a final concentration of 120 μM in PBS. *Pseudomonas aeruginosa* (ATCC 27 853) cells [2×10^8 cells in Müller–Hinton (MH) media], containing the peptides in the concentration range between 0 and 10 μM, were incubated at 37 °C, the physiological temperature, in a rotary shaker at 140 rpm for 2 h. The control cells were incubated without any peptide. For DPH labeling, the cells were resuspended in PBS and incubated at 37 °C for 45 min in DPH solution, followed by several washings in PBS [22]. *C. albicans* (ATCC 90 028) cells (2×10^6 cells/ml) were treated with the peptides in the concentration range between 0 and 10 μM and incubated for 2 h at 28 °C. Samples of the fungal cultures were fixed by 0.37% formaldehyde, collected and washed with PBS. The cells were frozen using liquid nitrogen. For labeling purposes, cells were thawed with PBS and resuspended in PBS. The suspended mixture was incubated with DPH solution for 45 min at 28 °C, followed by washing with PBS. The fluorescent intensities were measured by spectrofluorimetry (Shimadzu RF-5301PC, Shimadzu, Japan) at $\lambda_{ex} = 350$ nm and $\lambda_{em} = 425$ nm [18,23].

Dye Leakage from Liposomes

For dye leakage measurement purposes, L-α-phosphatidyl-DL-glycerol (PG), L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylinositol (PI) and ergosterol were purchased from Sigma. Cardiolipin (CL) was purchased from Avanti. Calcein-encapsulated large unilamellar vesicles (LUVs), composed of PG/CL (58 : 42, w/w) and PC/PG (1 : 1, w/w) for bacteria, PC/PE/PI/ergosterol (5 : 4 : 1 : 2, w/w/w/w) and PC/ergosterol (10 : 1, w/w) for fungi were prepared by vortexing the dried lipids in a dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4). The suspension was subjected to frozen–thawed cycles in liquid nitrogen for ten cycles and extruded through polycarbonate filters (two stacked 100 nm pore size filters) by means of a LiposoFast extruder (Avestin Inc., Canada). Untrapped calcein was removed by means of gel filtration on a Sephadex G50 column. The calcein leakage from the LUVs was monitored by measuring the fluorescent intensity at $\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm by spectrofluorimetry. The measurements were conducted at 25 °C. For determination of 100% dye leakage, 20 μl of 10% Triton X-100 was added to the vesicles. The percentage of dye leakage caused by the peptides was calculated as follows: dye leakage (%) = $100 \times (F - F_0)/(F_t - F_0)$, where F represents the fluorescent intensity achieved by the peptides, and F_0 and F_t represent the fluorescent intensities without the peptides and after addition of Triton X-100, respectively [24,25].

Results and Discussion

Intracellular Localization of Ple Enantiomers in Microbial Cells

Due to its property as a cationic peptide, the mode of action of Ple has been actively studied. More recently, dye-leakage tests showed that Ple, like magainin, is able to form pores in the lipid membranes [17]. However, the Ple mechanism of action has not been fully understood.

Journal of PeptideScience

Table 1. Amino acid sequence, molecular weight, HPLC retention time, and purity of the Ple enantiomers

Peptide	Amino acid sequence	Calculated value (MW)	Observed value (MW)	Retention time (min)	Purity (%)
L-enantiomer	GWGSFFKKAAHVGKHVGKAALTHYL-NH_2	2710.1	2710.5	18.227	96.9
D-enantiomer	GWGSFFKKAAHVGKHVGKAALTHYL-NH_2	2710.1	2710.6	18.140	97.4

Therefore, in order to provide further information regarding the target sites of Ple, we investigated the cellular distribution of the Ple enantiomers in fungal cells, especially *C. albicans*, by using CLSM. *C. albicans* is a significant pathogen with regard to opportunistic fungal infections and is becoming of increasing importance in patients who are immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation, or human immunodeficiency virus infections [26]. CLSM permits the user to depict and obviously identify all fluorescently labeled proteins or compounds at microscopical resolution and allows for the visualization and characterization of structures not only at the surface, but also inside the particles, provided the material is sufficiently transparent and can be fluorescently labeled [27].

Our results show that the FITC-labeled Ple enantiomers interact and accumulate rapidly within the cell surface of *C. albicans* after peptide treatment (Figure 1). This finding indicates that the major target site of both the L-enantiomer and the D-enantiomer is the cell surface, which is comprised of the cell plasma membranes and the cell walls. Furthermore, our data demonstrate that the Ple enantiomers could affect the membranes, thereby exerting their activity by a membrane-active mechanism.

Comparison of Activity of Ple Enantiomers on Intact Cells

We compared the antimicrobial activities of the Ple enantiomers against intact cells of bacteria and fungi. In our previous study, the L-enantiomer showed more potent antibacterial activities, particularly against antibiotic-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and antibiotic-resistant *P. aeruginosa* [14]. On the other hand, human pathogenic fungal strains, such as *C. albicans* and *Malassezia furfur*, are more susceptible to the D-enantiomer than to the L-enantiomer. Therefore, we performed a fluorescence analysis against intact cells by employing DPH, a fluorescent membrane probe, to confirm our previous results. DPH is a hydrophobic molecule. This property enables it to associate with the hydrocarbon tail region of phospholipids within the cytoplasmic membrane without disturbing the structure of the lipid bilayer [18,28,29]. This fluorescent probe is commonly applied to investigate not only membrane structure but also membrane biophysical characteristics [30]. Also, membrane fluidity can be measured by spectrofluorometric analysis by using the DPH molecule. If the antimicrobial activities of the Ple enantiomers cause membrane damage induced by depolarization, then the DPH molecules could not be incorporated into the lipophilic tails of phospholipids in the bilayer [18]. Based on these concepts, we could compare the potency of the antimicrobial activities of the Ple enantiomers on the cell plasma membrane. Our results demonstrate that bacterial and fungal membranes are structurally perturbed and possibly disrupted when exposed to the Ple enantiomers (Figure 2). Additionally, our findings are compatible with those of the antimicrobial susceptibility tests that have been conducted in the previous study. In other words, bacterial membranes are more sensitive to the Ple L-enantiomer, while the D-enantiomer exhibits a more potent activity on the fungal membranes. This result indicates that the two Ple enantiomeric peptides show cell selectivity between eukaryotes and prokaryotes. It is reasonable to assume that the cell selectivity would be due to the different lipid compositions of bacterial and fungal membranes.

Comparison of Activity of Ple Enantiomers on Model Membranes

Liposomes are vesicle-like structures basically constituted of phospholipids organized as concentrical bilayers containing an aqueous compartment in their interior [31]. Due to their amphipathic characteristics, they can incorporate substances in the aqueous compartment, the lipidic bilayer, or even distributed in both compartments [32]. Also, liposomes, such as LUVs or small unilamellar vesicles (SUVs), are considered regarding powerful tools in membrane studies. In this investigation, dye-entrapped

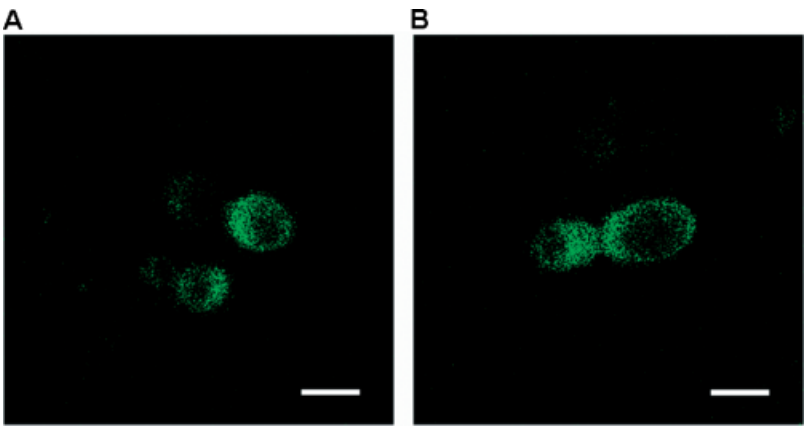

Figure 1. Confocal laser scanning microscopy of *C. albicans* cells treated with FITC-labeled Ple enantiomers. (A) Cells treated with the L-enantiomer, (B) Cells treated with the D-enantiomer. The bar corresponds to 5 μm.

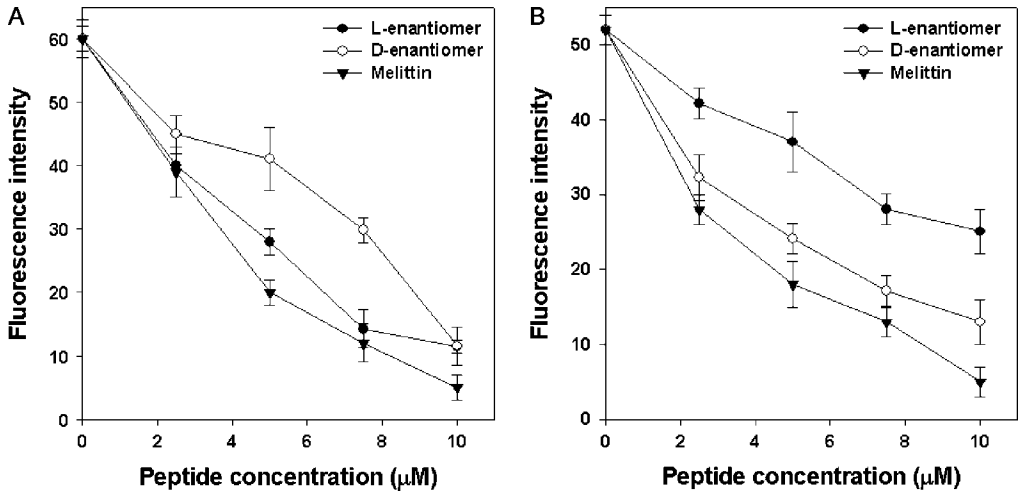

Figure 2. DPH fluorescence intensity after peptide treatment on bacteria and fungi. (A) Sub-cultured *P. aeruginosa* cells containing peptides of various concentration levels (2.5, 5, 7.5, 10 μM) were incubated at 37 °C for 2 h. (B) Sub-cultured *C. albicans* cells containing peptides of various concentration levels (2.5, 5, 7.5, 10 μM) were incubated at 28 °C for 2 h. The error bars represent the standard deviation values for three independent experiments, each performed in triplicate.

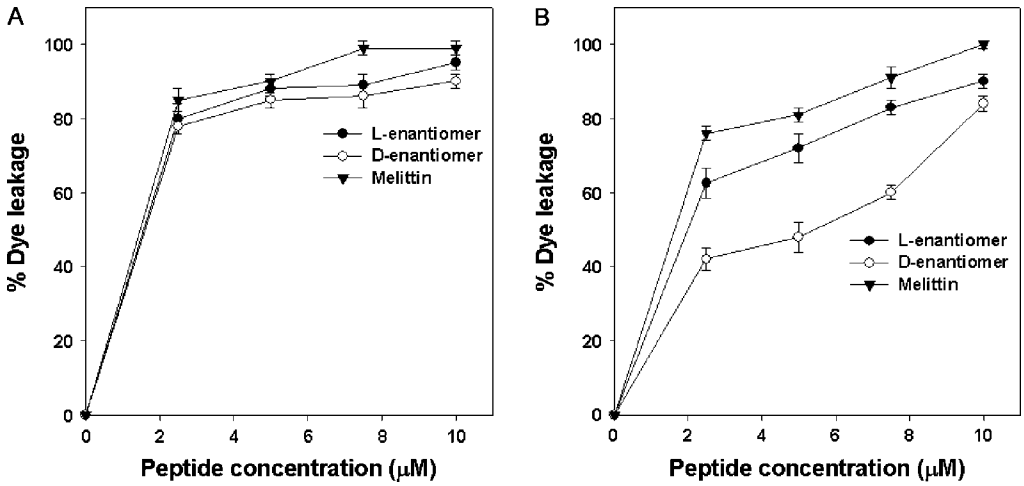

Figure 3. Peptide-induced dye leakage from calcein-entrapped liposomes, mimicking bacterial membranes, measured 2 min after peptide treatment. (A) PG/CL (58 : 42, w/w) LUVs; (B) PC/PG (1 : 1, w/w) LUVs. The error bars represent the standard deviation values for three independent experiments, each performed in triplicate.

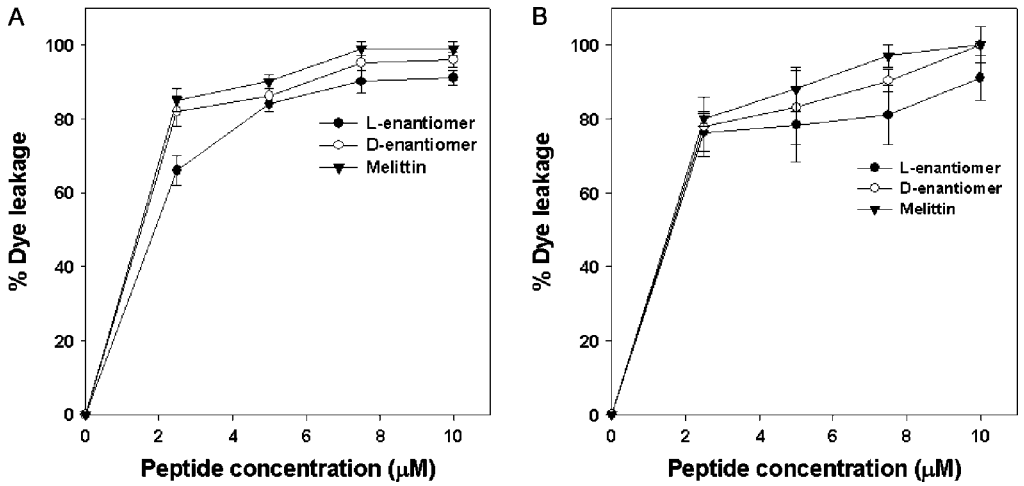

Figure 4. Peptide-induced dye leakage from calcein-entrapped liposomes, mimicking fungal membranes, measured 2 min after peptide treatment. (A) PC/PE/PI/ergosterol (5 : 4 : 1 : 2, w/w/w/w) LUVs; (B) PC/ergosterol (10 : 1, w/w) LUVs. The error bars represent the standard deviation values for three independent experiments, each performed in triplicate.

Journal of PeptideScience

liposomes were used for comparing the ability of two enantiomeric peptides to disrupt model membranes.

To confirm the results of the DPH analysis discussed above, we used calcein-entrapped liposomes based on the following lipid mixtures which mimic the membrane compositions of several different bacteria and fungi: *S. aureus* [PG : CL = 58 : 42] [33]; representative of a typical bacterial membrane [PC : PG = 1 : 1] [34]; and *C. albicans* [PC : PE : PI : ergosterol = 5 : 4 : 1 : 2] [35]. We also utilized a liposome preparation mixture, consisting of a 10 : 1 mixture of PC and ergosterol, mimicking the fungal membranes. Our data show ambiguous patterns, with the exception of PC/PG membranes (Figure 3B). However, in general the results are similar to those extracted from the DPH analysis (Figures 3 and 4). Specifically, the Ple L-enantiomer is more active on the bacterial PG/CL or PC/PG membranes, whereas the D-enantiomer exhibits a more potent disrupting ability with respect to the fungal PC/PE/PI/ergosterol or PC/ergosterol membranes. These findings indicate that the discrepant membranolytic effects of the two Ple enantiomeric peptides originate from the different characteristics of the membrane phospholipids of fungi and bacteria.

Conclusions

In this study, the cell selectivity of the Ple enantiomers against bacteria and fungi was investigated. Actually, various attempts have been made in the literature to shed light on the problem of enantiospecificity of peptides on cellular membranes. Unfortunately, most tested peptides exhibited no specific patterns. However, the Ple enantiomers do show selectivity against bacterial and fungal cells. Although the exact reasons for this selectivity must be further investigated, it is hypothesized that the antimicrobial effects of the Ple enantiomers, including cell selectivity, are due to the different lipid compositions of the membranes. Also, we suggest that enantiomeric peptides could be selectively applied as therapeutic agents with respect to bacterial or fungal diseases in humans.

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