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The Comparison of Characteristics Between Membrane-Active Antifungal Peptide and Its Pseudopeptides

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Abstract—By the introduction of various amide surrogates, novel pseudopeptides corresponding to a membrane active depsipeptide were synthesized and their native characteristics compared with that of the peptide. The pseudopeptides had more resistance to serum proteases than the peptide and similar antimicrobial activities to that of the peptide without hemolytic activity. The pseudopeptides like the peptide were active against current drug resistant fungi and pathogenic fungi isolated from patients, and also had a strong synergism with current antifungal drugs against *Candida albicans*. The leakage assay suggested that the pseudopeptides also acted on the lipid membrane of pathogenic cells. These results indicated that the novel pseudopeptides had advantages over the peptide as a candidate for a novel antifungal drug and backbone modifications can be a tool in the development of a novel antifungal agent from membrane-active peptides isolated from natural sources or chemically synthesized.

Introduction

The incidence of fungal infections has increased dramatically in the past 20 years because of the increase in the number of people whose immune systems are compromised by AIDS, aging, organ transplanation, or cancer therapy.[1,2] Accordingly, the increases in the rates of morbidity and mortality because of fungal infections have been regarded as a major problem.[3] The development of resistant fungal strains in response to the widespread use of current antifungal drugs has been also regarded as a serious problem.[4] The recent emergence of fungal infections and resistant strains has demanded the development of novel antifungal drugs with different mechanisms.[5,6]

Recently, many host defense peptides were isolated from a variety of natural sources and their functions characterized.[7–10] These peptides were suggested to do their actions through the lipid membrane of pathogenic cells. As these membrane active peptides were not required to enter the cytoplasmic level of target cells for biological actions, drug resistance mechanisms, such as enzymatic degradation and export process, must not circumvent the biological action of these peptides. Therefore, they have received attention as novel therapeutic candidates. Some of them had a potent antifungal activity as well as an antibacterial activity and a low level of toxicity against mammalian cells.[11–13]

In a previous study, we identified a novel membrane-active decapeptide named KSL against *Candida albicans* membrane using combinatorial chemistry.[14] This peptide was active against bacteria and fungi without hemolytic activity. We also developed the depsipeptide named MP, which was twofold more active against *C. albicans* than KSL, using amino acid augmentation (data not shown). This peptide irreversibly inhibited the growth of resistant *C. albicans* and specially had a broad range of activities against pathogenic fungi isolated from patients without hemolytic activity. Nevertheless, the membrane-active peptides can not be used as therapeutic agents because of their poor in vivo stability.

Recently, we first developed antimicrobial pseudopeptides more resistant to serum proteases than the peptide by the incorporation of reduced amide bond $\Psi[CH_2NH]$ and carbamate bond $\Psi[CH_2OCONH]$ into the membrane-active decapeptide (KSL).[15] Although this result provides the possibility in the development of pseudopeptides from the membrane-active peptide by backbone modifications, it is necessary for further study about the effect of various backbone modifications on

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the native characteristics of the membrane-active peptides such as structural parameters, biological activity, mechanism, etc.

In the present study, considering the recent dramatic increase of fungal infections, we chose the antifungal depsipeptide (MP) as a model system and evaluated backbone modifications as a novel tool in the development of known membrane-active peptides as therapeutic agents. For this purpose, we incorporated various amide surrogates (reduced amide,[16] carbamate,[17] and peptoid[18]) into the peptide and compared native characteristics between membrane-active peptide and its pseudopeptides such as antifungal activity, antibacterial activity, hemolytic activity, secondary structure, membrane-leakage potency, and synergic effect with current antifungal drugs. These results will provide useful information about the effect of various backbone modifications on the membrane-active peptide and show the possibility of backbone modifications as a novel tool for developing the membrane-active peptides as therapeutic agents.

Results

Design of pseudopeptides corresponding to MP

Since MP, a membrane-active depsipeptide was more active against pathogenic fungi than KSL and non-hemolytic, this peptide was selected as a model peptide. Among many amide surrogates, reduced amide $\Psi[CH_2NH]$,[16] carbamate $\Psi[CH_2OCONH]$,[17] and peptoid $\Psi[CONR]$[18] were incorporated into MP. In the previous study, it was found that the N-terminal amide bond in KSL was the most susceptible to serum proteases and the C-terminal amide bond was a next cleaved site, suggesting that exoprotease in the serum play an important role in the degradation of the peptide.[15] Thus, peptide backbone modifications were applied for the N-terminal amide bond in MP and C-terminal L-amino acid was simultaneously replaced by D-amino acid for easy monitoring the half-life of the pseudopeptides in the presence of serum. The single D-amino acid replacement at N- or C-terminal end of MP had little effect on the half-life in the presence of serum (data not shown).

Characterization of pseudopeptides

As shown in Table 1, three kinds of pseudopeptides were synthesized and their structural parameters, such as α helicity, retention time, and net positive charge, were investigated. The incorporation of reduced amide (MP1-2) resulted in the increase of a net positive charge and the decrease of α helicity in the presence of 50% TFE. It was reported that reduced amide $\Psi[CH_2NH]$ existed in a protonated form $\Psi[CH_2NH_2^+]$ in physiological condition, which interrupted the hydrogen bonding for α helical structure.[19] The incorporation of *N*-substituted glycine (NSG) (peptoid monomer) maintained amide backbone but caused the loss of chirality and the change of the side chain position. As we expected, the incorporation of NSG (MP3-5) resulted in the decrease of α helicity. The incorporation of carbamate $\Psi[CH_2OCONH]$ into the peptide (MP6) maintained α helical structure as well as net positive charge.

We also measured the retention time of the pseudopeptides using C_{18} column because retention time, which reflected hydrophobic interactions between peptide and C_{18} stationary phase, was reported to be parallel to the hydrophobicity of the peptides or to the α helicity of the peptides with same amino acid composition.[20,21] Interestingly, the incorporation of various amide surrogates had little effect on retention times in spite of the decrease of α helicity and/or the increase of net positive charge, suggesting that all pseudopeptides had similar hydrophobic interactions with C_{18} stationary phase.

Stabilities of pseudopeptides in the presence of serum

As shown in Table 1, we compared half-lives of the pseudopeptides in the presence of serum. All pseudopeptides except for MP3 were more stable than the peptide in the presence of serum. Among them, the pseudopeptide with reduced amide bonds (MP2) was the most stable in the pseudopeptides employed in this study. Although MP4 and 5 contained the same kind of amide surrogate (*N*-substituted glycine), MP4 was more stable than MP5, which may be due to the relative bulkiness of side chain in the *N*-substituted glycine.

Antimicrobial and hemolytic activities of the pseudopeptides

As shown in Table 2, the activities of pseudopeptides against bacteria and fungi were evaluated. The peptide (MP) and the pseudopeptides (MP1-6) were more active against test microorganisms than magainin II amide used as a control.[13] All pseudopeptides had similar activities to that of model peptide (MP) against test microorganisms.

To check the cytotoxicity against mammalian cells, MP and pseudopeptides (MP1 and 6) were added to mouse erythrocytes. Amphotericin B and melittin, well-known cytolytic molecule, caused 100% lysis at a concentration greater than 10 μg/mL, whereas MP and pseudopeptides (MP1 and 6) did not show hemolytic activities at a concentration of up to 500 μg/mL.

Leakage of dyes from liposomes caused by MP and its pseudopeptides

To study whether backbone modifications change the primary target of the peptide, we compared the leakage potency of dye from liposomes induced by the peptide and the pseudopeptides (MP1 and 6). As shown in Fig. 1, the increase in concentrations of peptide and its pseudopeptides resulted in the increase in the level of leakage of dye from the liposomes consisting of phosphatidylglycerol with similar potency. Fluconazole as a negative control, which acted on enzyme in cytoplasmic level,[22] did not cause any leakage of dye in the same assay condition.

Table 1. Characterization of MP and its pseudopeptides

Name	Sequence[a]	Net charge	Retention time (min)	Helicity (%)	Half-life (min)[b]
MP	KKVVFKVKFKK-$CONH_2$	+7	27.2	55	7 ± 2
MP1	kψ[CH_2NH]KVVFKVKFKk-$CONH_2$	+8	27.0	18	70 ± 20
MP2	Kψ[CH_2NH]Kψ[CH_2NH]VVFKVKFKk-$CONH_2$	+9	27.0	8	240 ± 110
MP3	R_1NHCH_2CO-KVVFKVKFKk-$CONH_2$	+7	27.3	43	10 ± 4
MP4[c]	R_1NHCH_2ψ[$CONR_1$]CH_2CO-VVFKVKFKk-$CONH_2$	+7	28.1	20	80 ± 30
MP5[d]	R_2NHCH_2ψ[$CONR_2$]CH_2CO-VVFKVKFKk-$CONH_2$	+7	27.3	19	20 ± 7
MP6	Kψ[CH_2OCONH]KVVFKVKFKk-$CONH_2$	+7	27.4	53	30 ± 10

[a] Small letter indicated D-amino acid.
[b] The half life with a standard error.
[c] $R_1 = CH_2CH_2CH_2N(CH_3)_2$.
[d] $R_2 = CH_2CH_2NH_2$.

Table 2. Antimicrobial activity of MP and its pseudopeptides

Name	Minimum inhibitory concentration (MIC) (μg/mL)[a]				
	Staphylococcus aureus ATCC 6538	*Micrococcus luteus* ATCC 9341	*Escherichia coli* ATCC 2592	*Pseudomas aeruginosa* ATCC 9027	*Candida albicans* ATCC 36232
MP	4	5	11	6	5
MP1	6	4	17	10	5
MP2	6	4	17	10	6
MP3	6	3	17	12	9
MP4	6	6	17	17	9
MP5	14	8	42	17	17
MP6	4	3	21	8	5
Magainin II amide	40	50	20	16	40

[a] Average MIC values were calculated from three independent experiments performed in duplicate, which provided a standard deviation below 30%.

Antifungal activities against resistant strains and pathogenic fungi

To evaluate the potential of pseudopeptides as antifungal drugs, we measured the activities of pseudopeptides against current drug resistant fungi. As shown in Table 3, MP and its pseudopeptides (MP1 and 6) retained activities against *C. albicans* ATCC 200955[23] resistant to a potent fungicidal agent, amphotericin B.[24,25] They also retained activities against *C. krusei* ATCC 200917[26] resistant to the well-known fungistatic agent, fluconazole.[22]

As shown in Table 4, we also measured the activities of pseudopeptides (MP1 and 6) against various pathogenic fungi isolated from patients. Minimum inhibitory concentrations (MICs) and minimum lethal concentrations (MLCs) were determined by the fungal testing laboratory at the University of Texas Health Science Center at San Antonio. To lend credence to the activity in this assay, amphotericin B and fluconazole were simultaneously assayed and their activities were compared. Amphotericin B known to be the most effective antifungal agent had the lowest MICs and MLCs for test pathogenic fungi. MP1 and 6 were less active than amphotericin B against test microorganisms, but they were more active than fluconazole. Pseudopeptides, MP1 and 6 inhibited the growth of *Cryptococcus neoformans*, *Candida tropicalis*, *C. albicans*, and *Candida glabrata* at concentrations ranging from 8 to 32 μg/mL. Interestingly, pseudopeptides showed clear MLCs like MP at concentrations ranging from 8 to 32 μg/mL, suggesting that MP1 and six had fungicidal activities.

Synergic effects of the pseudopeptides with current antifungal drugs

The synergic effect of MP1 and MP6 in combination with amphotericin B and fluconazole against *C. albicans* ATCC 36232 was studied. As shown in Table 5, fractional inhibitory concentration (FIC) indices showed that MP and its pseudopeptides (MP1 and MP6) acted synergically with amphotericin B and fluconazole, respectively. The MIC_{80} of fluconazole without the pseudopeptide was 0.312 μg/mL for *C. albicans*, whereas the addition of the pseudopeptide (0.05 μg/mL) resulted in the decrease 300-fold in MIC_{80} of fluconazole (FIC index of 0.02). Interestingly, we also observed a synergic effect between amphotericin B and the pseudopeptides. The MIC of amphotericin B was 0.625 μg/mL for *C. albicans*. Combination of the peptide or pseudopeptide with amphotericin B resulted in 12-fold decrease in the MIC of amphotericin B. (FIC index of 0.1). As amphotericin B,[27] the peptide, and the pseudopeptides acted on the lipid membrane as a primary target, the synergic effect between amphotericin B and MP was an unexpected result. Further study is currently under way to elucidate the mechanism of this synergic effect.

Discussion

The emergence of fungal infections resistant to current therapies underscore for need of new antifungal agents which are effective against resistant strains and non-toxic against mammalian cells. Recently, many membrane-active antifungal peptides were naturally isolated or chemically synthesized. As most of them seemed to induce resistant strains unlikely and were non-hemolytic, they were expected to have a considerable potential as a candidate of a novel antifungal agent.[9] However, peptides themselves have limitations as potential therapeutic agents; rapid breakdown by proteolytic enzymes and rapid clearance from circulation.

In the present study, we incorporated several kinds of amide surrogates into the peptide and compared native characteristics between peptide and pseudopeptides. We studied whether the incorporation of backbone modifications altered the primary target of the membrane-active peptide. The assay of leakage of dyes from the liposomes caused by the peptide and pseudopeptides revealed that the pseudopeptides acted on the lipid membrane as a primary target, suggesting that backbone modification did not alter the primary target of the membrane-active peptide.

Many SAR studies about antimicrobial membrane-active peptides indicated that α helical structure, net positive charge, and hydrophobicity were regarded as major factors for antimicrobial activity.[28–30] Generally, a positively charged membrane-active peptide initially binds with negatively charged lipid membranes of microorganisms mainly by electrostatic interactions. In this step, the net positive charge of the peptide facilitated the binding of the peptide with lipid membranes. Next, the peptide was immersed into lipid membranes inside mainly by hydrophobic interactions and caused the perturbation of lipid membrane, resulting in antimicrobial action. In this step, the hydrophobicity of the peptide itself and the secondary structure, which was induced in lipid membranes, played an important role in hydrophobic interactions between the peptide and lipid membranes. In the present study, the pseudopeptides had lower α helicities than the peptide, but retained antimicrobial activities. This result can be explained by following suggestion; the amide surrogate itself causing the decrease of α helicity is more hydrophobic than amide bond of the peptide, which compensate for the decrease of hydrophobic interactions by the decrease of α helicity. Therefore, the incorporation of amide surrogate can maintain hydrophobic interactions required for membrane-perturbation. This suggestion can be supported by the fact that all pseudopeptides employed in this study have similar retention times, which reflects hydrophobic interactions between the molecule and C_{18} stationary phase, to that of the peptide. Thus, we expect

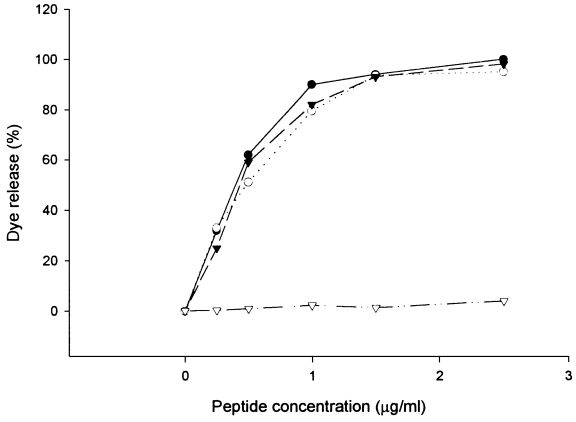

Figure 1. Leakage of dye from liposomes: MP (●), MP1 (○), MP6 (▼), fluconazole (▽). The peptide and its pseudopeptides were incubated with liposomes consisted of phosphatidylglycerol in 10 mM Tris buffer (pH 7.4) at 37°C.

Table 4. MIC and MLC values of MP and its pseudopeptides against pathogenic fungi isolated from patients[a]

Name	MIC/MLC[b] (μg/mL)			
	C. tropicalis 96-804[c]	*C. glabrata* 96-827	*C. albicans* 96-802	*C. neoformans* 96-806
MP	4/4	32/>32	8/8	4/16
MP1	8/8	32/32	16/32	8/8
MP6	8/8	32/32	16/32	8/8
Amphotericin B	0.25/1	0.5/2	1/2	0.25/1
Fluconazole	2/—[d]	>64/—	>64/—	16/—

[a] MIC and MLC values were measured by the fungal testing laboratory at the University of Texas Health Science Center at San Antonio according to the recommendation of the National Committee for Clinical Laboratory Standards. MIC and MLC values were measured for 48 h. In the case of fluconazole, MIC indicated MIC_{80}.
[b] Minimum lethal concentration.
[c] Laboratory number.
[d] The MLC of fluconazole was not determined because fluconazole was known as fungistatic agent.

Table 3. MIC values of MP and its pseudopeptides against current drug resistant fungi

Name	Minimum inhibitory concentration (μg/mL)			
	Candida albicans ATCC 36232	*Candida albicans*[a] ATCC 200955	*Candida krusei* ATCC 90878	*Candida kruesi*[b] ATCC 200917
MP	3.12–6.25	3.12–6.25	1.56–3.12	1.56–3.12
MP1	3.12–6.25	3.12–6.25	1.56–3.12	1.56–3.12
MP6	3.12–6.25	3.12–6.25	1.56–3.12	1.56–3.12
Ampotericin B	0.312–0.625	1.25–2.5	1.25–2.5	0.625–1.25
Fluconazole	0.312–0.625	0.625–1.25	>10	>10

[a] Resistant to amphotetricin B.
[b] Resistant to fluconazole.

Table 5. FIC indices for the synergism of the pseudopeptides with current antifungal drugs, as measured by using *Candida albicans* 36232 as the target organism

Name	FIC index with	
	Fluconazole	Amphotericin B
MP	0.02	0.1
MP1	0.02	0.1
MP6	0.02	0.1

that even though the incorporation of amide surrogate changed α helical structure induced in lipid membrane, the incorporation of amide surrogate could maintain hydrophobic interactions under enough net positive charges, which resulted in the retention of the antimicrobial activity.

We also found that MP and its pseudopeptides (MP1 and 6) were non-hemolytic. However, we could not observe the real effect of the introduction of backbone modifications on hemolytic activity, because MP itself had no hemolytic activity in the assay condition. This result suggested that the incorporation of various amide surrogates did not increase the hemolytic activity of the peptide.

Pseudopeptides, MP1 and 6 like the peptide, retained activities against *C. albicans* and *C. krusei* resistant to current antifungal drugs. MP1 and 6 were more active than fluconazole against various pathogenic fungi. In addition, they showed clear cutoff value against pathogenic fungi, while fluconazole did not. Interestingly, MP1 and 6 had a synergic effect with current antifungal agents such as amphotericin B and fluconazole, which enlarge the extent of the use of peptide or pseudopeptide as an antifungal agent.

Our investigations about pseudopeptides indicated that the incorporation of backbone modifications into the peptide had little effect on the native characteristics of the peptide. Considering the development of fungal resistant strains and the toxicity of the current antifungal drug, antifungal pseudopeptides with an improved stability developed in this study have potential as novel antifungal agents.

Conclusions

Backbone modifications, which were easily incorporated into membrane-active peptides in solid phase synthesis, retained biological properties of the membrane-active peptides and simultaneously improved the stability toward enzymatic degradation. Therefore, backbone modifications can be a tool in the development of novel antifungal agents from membrane-active peptides which were isolated from natural sources or chemically synthesized. Our investigations also indicated that the pseudopeptide could be a more potent candidate as a therapeutic agent than the peptide itself.

Experimental

Materials

N-α-9-Fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives for solid phase peptide synthesis were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). 5-(4-Aminomethyl-3,5-dihydroxyphenoxy)valeric acid (PAL) resin was purchased from PerSeptive Biosystems GmbH (Hamburg, Germany). Piperidine, acetic anhydride, methyl alcohol, dicyclohexylcarbodiimide (DCC), *N*-methylpyrrolidone (NMP), and *N*-hydroxybenzotriazole (HOBt) were obtained from Applied biosystems, Inc. (Foster City, CA, USA). Phospholipid, NaCl, KCl, ethylenediaminetetraacetic acid (EDTA), calcein, amphotericin B, and tris(hydroxymethyl)aminomethane (Tris) were purchased from sigma (St Louis, MO, USA). RPMI 1640 was purchased from Gibco BRL (Gaithersburg, MD, USA). Other chemicals were purchased from Aldrich (Milwaukee, WI, USA). All chemicals were used without further purification.

Methods

Synthesis of peptide and pseudopeptides. Peptides were prepared by stepwise solid-phase synthesis on an 431A automatic peptide synthesizer. The peptide chain was assembled on PAL resin with a Fmoc/*tert*-butyl strategy.[31] The reduced amide bonds $\Psi(CH_2NH)$ were formed by the reductive alkylation of the preformed protected amino aldehyde with the free amino terminal of the resin bound peptide, in the presence of an excess $NaBH_3CN$ (1.0 M solution in tetrahydrofuran) in 1% acetic acid in *N*,*N*-dimethylformamide (DMF).[16,32] The completion of reaction was monitored by ninhydrin test.[33] Carbamate bonds were prepared by the coupling of Fmoc-carbonate monomer with the free amino terminal of the resin bound peptide, in the presence of *N*,*N*-diisopropylethylamine (DIEA) and HOBt in NMP for 2–4 h at 25°C.[17] Peptoid was incorporated into the peptide by two steps; free amino group of the resin bound peptide was coupled with bromoacetic acid in DMF in the presence of 1,3-diisopropylcarbodiimide (DIC) and DIEA for 30 min at 25°C. The reaction was repeated once more. Next, resin-bound bromoacetamide was replaced by addition of the amine in DMSO (2 h, at 25°C).[18]

Deprotection was achieved by treatment with a mixture of trifluoroacetic acid (TFA):water:thioanisole (9:0.5:0.5, v/v/v) at room temperature for 2–4 h. After filtration of the resin and a washing with TFA, a gentle stream of nitrogen was used to remove the excess TFA. The crude peptide was triturated with diethyl ether chilled at −20°C and was centrifuged at 3000×*g* for 10 min. Diethyl ether was decanted and the crude peptide was dried under nitrogen. The peptide was purified by high performance liquid chromatography with a Phenomenex C_{18} column (21.2×250 mm; Phenomenex, Torrance, CA, USA). The homogeneity of the peptide and pseudopeptides (>95%) was checked by analytical HPLC with a Waters Delta Pak C_{18} column (3.9×150 mm; Waters, Milford, MA, USA). The peptide was eluted using solvent A consisting of 0.1% TFA in water and solvent B

consisting of 0.1% TFA in acetonitrile and monitored by absorbance at 214 nm. Mass spectrometry on a Platform II (Fisons Instruments, Manchester, UK) was used to measure the mass of the purified peptide and pseudopeptides. MP (ES–MS: 1376.43 $[M+1]^+$, calculated mass 1377.92), MP1 (ES–MS: 1363.14 $[M+1]^+$, calculated mass 1363.92), MP2 (ES–MS: 1348.73 $[M+1]^+$, calculated mass 1349.92), MP3 (ES–MS: 1390.86 $[M+1]^+$, calculated mass 1391.92), MP4 (ES–MS: 1405.3 $[M+1]^+$, calculated mass 1405.92), MP5 (ES–MS: 1320.15 $[M+1]^+$, calculated mass 1321.92), MP6 (ES–MS: 1406.76 $[M+1]^+$, calculated mass 1407.92).

Antifungal and antibacterial assay. In vitro antifungal assays were performed by the broth microdilution method according to the recommendation of the National Committee for Clinical Laboratory Standards.[34] RPMI 1640 was used as the assay medium. *C. albicans* ATCC 36232 and *C. krusei* ATCC 90878 were used in bioassays. *C. albicans* ATCC 200955 and *C. krusei* ATCC 200917 were used in bioassays as resistant strains against amphotericin B and fluconazole. *Candida* cells freshly grown on slopes of Sabouraud dextrose agar were suspended in physiological saline, and the cell concentration was adjusted to 10^4 cells per 1 mL of 2×-concentrated medium for use as the inoculum. Peptide solution was added to the wells of a 96-well plate (100 μL per well) and serially diluted twofold. The final concentration of peptide mixture ranged from 0.2 to 500 μg/mL. After inoculation (100 μL per well, 5×10^3 cells per mL), the 96-well plate was incubated at 30°C for 48 h and the absorbance was measured at 620 nm by using an enzyme-linked immunosorbent assay reader (SLT, Salzburg, Austria) to assess cell growth. Minimal inhibition concentration (MIC) was defined as the lowest concentration exhibiting no visible growth of the test organism. All MICs were measured from three independent experiments performed in duplicate. Fractional inhibition concentration (FIC) indices were calculated as follows;[35] $[(A)/MIC_A]+[(B)/MIC_B]=FIC_A+FIC_B=$ FIC index where MIC_A and MIC_B are the MICs of drug A and B, defined separately, and (A) and (B) are the MICs of drug A and B when determined in combination. Drugs were interpreted to be synergistic when the FIC index was ≤ 0.5.

Hemolytic assay. The detailed method for hemolytic activity was described elsewhere.[27] Packed mouse erythrocytes were washed three times with buffer (150 mM KCl, 5 mM Tris–HCl, pH 7.4), and then packed erythrocytes were suspended in 10 volumes of the same buffer (stock cell suspension). For antibiotic treatment, the cell stock suspension was diluted 25-fold with the same buffer and preincubated in the water bath at 37°C for 15 min and then the test sample was added. After incubation for 1 h, the sample was centrifuged at $4000\times g$ for 5 min and the absorbance of the supernatant was determined at 540 nm. Hemolysis effected by 0.1% Triton X-100 was considered to be 100%.

CD measurement. Circular dichroism (CD) spectra were recorded on a J-715 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell of 1 mm path length, at wavelengths ranging from 190 to 245 nm. CD spectra were obtained with a 0.5 nm bandwidth and a scan speed of 10 nm/min at room temperature. Two scans were averaged to improve the signal to noise ratio. CD spectra were expressed as the mean residue ellipticity and the α helicity was calculated from the mean residue ellipticity [θ] at 222 nm.[36]

Preparation of liposomes. Phospholipid (egg PG) (10 mg) in chloroform was placed in a vial. After evaporation of the solvent, residual film was dried overnight under vacuum. The dried film was hydrated with 2 mL of test buffer (10 mM Tris, pH 7.4, 154 mM NaCl, 0.1 mM EDTA) containing 70 mM calcein for leakage measurement. The suspension was vortexed for 10 min. This turbid liposome solution was sonicated (under nitrogen, in ice-water) for 10 min (×5) by using a titanium tip sonicator. The solution was freeze-thawed for six cycles. Untrapped calcein was removed from the vesicles by gel filtration on a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column that was equilibrated with 10 mM Tris buffer (pH 7.4) containing 154 mM NaCl and 0.1 mM EDTA. The concentration of liposome (0.8 mM) was determined on the basis of the method described by Vaskovsky et al.[37]

Leakage measurement. A liposome solution (5 μL) was added to 2 ml of 10 mM Tris buffer (154 mM NaCl, 0.1 mM EDTA) in the cuvette. To the mixture was added an appropriate concentration of peptides. When the leakage occurred, the calcein was released from the liposomes and emitted the fluorescence. Therefore, the leakage was directly measured by determining the relative change in fluorescence. Fluorescence, excited at 490 nm and emitted at 520 nm, was measured with a Jasco J-777 spectrofluorometer (Jasco, Tokyo, Japan). For determination of 100% dye release, 20 μL of 10% Triton X-100 solution was added to liposome solution. The percent of dye-release caused by sample was evaluated by the equation, dye-release $(\%)=100\times(F-F_0)/(F_t-F_0)$, where F was the fluorescence intensity achieved by the peptides, F_0 and F_t are intensities of the fluorescence without the peptides and with Triton X-100, respectively.

Half-lives measurement in the presence of serum. 1 mL of 25% mouse serum/RPMI media (v/v) in 1.5 mL Eppendorf tube was temperature-equilibrated at 37°C for 15 min before adding 10 μL of peptide stock solution (10 mg/mL) to make the final peptide concentration 100 μg/mL. The initial time was recorded and 100 μL of reaction solution was removed at known time intervals and added into 100 μL of 10% aq TCA solution. The cloudy reaction sample was cooled at 4°C for 15 min and spun at 13,000 g for 15 min to precipitate serum protein.[38] Peptide analysis was carried out by reverse phase HPLC with Waters C_{18} column. Each half-life was determined by non-linear least square fitting using following equations. $\%P=(\%P_0-\%P_{inf})e^{-kt}+\%P_{inf}$; half life $=\ln 2/k$. In the equation, %P, $\%P_0$, $\%P_{inf}$ are percent peak area at time t, 0, and infinite, respectively, and k is the first order rate constant.

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