Biochemical and Biophysical Research Communications 443 (2014) 483–488

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Synthesis and antimicrobial activity of cysteine-free coprisin nonapeptides

Jaeho Lee [a], Daeun Lee [a], Hyemin Choi [b], Ha Hyung Kim [c], Ho Kim [d], Jae Sam Hwang [e], Dong Gun Lee [b], Jae Il Kim [a,*]

[a] School of Life Sciences, Gwangju Institute of Science and Technology, Oryong-dong, Buk-gu, Gwangju 500-712, Republic of Korea
[b] School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Daehak-ro 80, Buk-gu, Daegu 702-701, Republic of Korea
[c] College of Pharmacy, Chung-Ang University, Seoul, Republic of Korea
[d] School of Life Sciences and Biotechnology, College of Natural Science, Daejin University, Pocheon, Gyeonggido, Republic of Korea
[e] Department of Agricultural Biology, Natural Academy of Agricultural Science, RDA, Suwon 441-853, Republic of Korea

ARTICLE INFO

Article history:
Received 19 November 2013
Available online 7 December 2013

Keywords:
Antimicrobial activity
Antimicrobial peptide
Cysteine-free coprisin
Nonapeptide
Synergic effect

ABSTRACT

Coprisin is a 43-mer defensin-like peptide from the dung beetle, *Copris tripartitus*. CopA3 (LLCIALRKK-NH_2), a 9-mer peptide containing a single free cysteine residue at position 3 of its sequence, was derived from the α-helical region of coprisin and exhibits potent antibacterial and anti-inflammatory activities. The single cysteine implies a tendency for dimerization; however, it remains unknown whether this cysteine residue is indispensible for CopA3's antimicrobial activity. To address this issue, in the present study we synthesized eight cysteine-substituted monomeric CopA3 analogs and two dimeric analogs, CopA3 (Dimer) and CopIK (Dimer), and evaluated their antimicrobial effects against bacteria and fungi, as well as their hemolytic activity toward human erythrocytes. Under physiological conditions, CopA3 (Mono) exhibits a 6/4 (monomer/dimer) molar ratio in HPLC area percent, indicating that its effects on bacterial strains likely reflect a CopA3 (Mono)/CopA3 (Dimer) mixture. We also report the identification of CopW, a new cysteine-free nonapeptide derived from CopA3 that has potent antimicrobial activity with virtually no hemolytic activity. Apparently, the cysteine residue in CopA3 is not essential for its antimicrobial function. Notably, CopW also exhibited significant synergistic activity with ampicillin and showed more potent antifungal activity than either wild-type coprisin or melittin.

1. Introduction

The increasing prevalence of pathogenic bacteria resistant to conventional antibiotics has prompted an intensive search for new antibacterial agents [1,2]. Antimicrobial peptides (AMPs) are attractive in this regard because they mainly kill through global actions at bacterial membranes and do not interact with specific intercellular components, which reduces the likelihood that resistance will develop [3,4]. AMPs are found in a wide variety of organisms including plants, insects, invertebrates and mammals, and are classified into four categories based on their sequence and structure: amphipathic α-helical molecules, extended disordered molecules, cyclic peptide with a single disulfide bond, and cysteine-rich β-sheet molecules [5]. One representative AMP group is the defensin family, members of which contain three or four intramolecular disulfide bonds. Defensins identified to date include sapecin A/B, drosomycin, heliomicin, defensin A and lucifensin [6–10]. Widely distributed in diverse insect species, these molecules exhibit a broad antimicrobial spectrum against bacteria, fungi and enveloped viruses [11].

Another insect defensin, coprisin, was recently isolated from *Copris tripartites* [12]. Its NMR solution structure reveals the presence of the conserved cysteine-stabilized α-helix/*β-sheet* motif frequently seen in the three-dimensional structures of insect defensins [13]; it has an amphipathic α-helical structure from Ala^{19} to Arg^{28} and β-sheets from Gly31 to Gln35 and Val38 to Arg42. Our research group examined the structure–activity relationship of coprisin in an effort to understand its antimicrobial action and to provide insight into the mechanism of action of an insect defense system [12–17]. In addition, we also designed CopA3 (LLCIALRKK-NH_2), a nonapeptide derived from the α-helical region of coprisin, and showed that CopA3 has an effective spectrum of biological properties, including antimicrobial, antifungal, anticancer and anti-inflammatory activities [14–17]. Notably,

Abbreviations: AMP, antimicrobial peptide; ATCC, American Type Culture Collection; CLSI, Clinical and Laboratory Standards Institute; HPLC, high performance liquid chromatography; KCTC, Korean Collection for Type Cultures; LPS, lipopolysaccharide; PBS, phosphate buffered saline.

* Corresponding author.
E-mail address: jikim@gist.ac.kr (J.I. Kim).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.11.125

CopA3 possesses just a single cysteine residue in its sequence, which prompted us to wonder whether this cysteine is indispensable for the biological function of CopA3. To address that question, in the present study we chemically synthesized a set of cysteine-related CopA3 analogs that included eight monomeric analogs and two dimeric analogs and tested their antibacterial activity against Gram-negative, Gram-positive and drug-resistant bacteria, as well as their hemolytic activity toward human red blood cells (hRBCs). Our findings indicate that CopW, a cysteine-free nonapeptide derived from CopA3, possesses potent antibacterial and antifungal activities without hemolytic activity, indicating the cysteine residue in CopA3 is not essential for its antimicrobial function.

2. Materials and methods

2.1. Chemical synthesis and oxidative refolding of 9-mer peptides

All peptides were chemically synthesized by Anygen (Gwangju, Republic of Korea) using the solid-phase synthesis, and were then purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on C_{18} columns (20×250 mm; Shim-pack). The purity and molecular masses of the peptides were determined using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Japan).

2.2. Bacterial strains and antibacterial test

Escherichia coli (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637), *Salmonella typhimurium* (KCTC 1926), *Bacillus subtilis* (KCTC 3068), *Staphylococcus epidermidis* (KCTC 1917), and *Staphylococcus aureus* (KCTC 1621) were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (Daejeon, Republic of Korea). *Enterococcus faecium* (KCCM 12118), *Enterococcus faecalis* (KCCM 29212), and methicillin-resistant *S. aureus* (MRSA) (KCCM 40510) were purchased from the Korean Culture Center of Microorganisms (Seoul, Republic of Korea). Vancomycin-resistant *E. faecalis* (ATCC 51575) and *E. faecium* (ATCC 51559) were purchased from the American Type Culture Collection (Manassas, VA, USA).

Minimal inhibitory concentrations (MICs) were determined using broth microdilution assays. Bacteria were grown to mid-log phase in LB broth and then diluted to 10^6 CFU/ml. Synthetic peptides diluted to final concentrations of 64, 32, 16, 8, 4, 2 and 1 μg/ml were added to the diluted bacteria, after which the mixture was incubated for 20 h at 37 °C. The MIC was defined as the lowest concentration of antibiotic causing complete inhibition of visible growth, as compared to the growth in an antibiotic-free control well. MICs were determined by three independent assays.

2.3. Fungal strains and antifungal susceptibility test

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Malassezia furfur* (KCTC 7744) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Most fungal strains were cultured in YPD broth (Difco) at 28 °C with aeration; *M. furfur* was cultured at 32 °C in modified YM broth (Difco) containing 1% olive oil. Fungal cells at log phase (2×10^6/ml) were inoculated into YPD or YM broth and then dispensed into microtiter plates at 100 μl/well. The MIC values were determined by three independent assays.

2.4. Hemolytic activity

Fresh hRBCs were washed through three cycles of rinsing in phosphate-buffered saline (PBS; 35 mM phosphate containing 10 mM NaCl, pH 7.4). Aliquots (100 μl) of 8% (v/v) hRBCs in PBS were dispensed into 96-well plates, after which 100 μl of the peptide solution were added to give a final hRBC concentration of 4% (v/v). The plates were then incubated for 1 h at 37 °C before centrifugation at 1000*g* for 10 min. Aliquots (100 μl) of the resultant supernatant were then transferred to new 96-well plates, and hemolysis was monitored at 414 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Controls for 0% and 100% hemolysis were determined in PBS and 0.1% Triton X-100 solution, respectively. The percent hemolysis was calculated using the following formula: $\%\ \text{hemolysis} = [(\text{Abs}_{414\text{nm}}\ \text{in the peptide solution} - \text{Abs}_{414\text{nm}}\ \text{in PBS})/(\text{Abs}_{414\text{nm}}\ \text{in}\ 0.1\%\ \text{Triton X-100} - \text{Abs}_{414\text{nm}}\ \text{in PBS})] \times 100$.

2.5. Peptide-induced permeabilization for E. coli ML35

E. coli strain ML35, a lactose permease-deficient strain with cytoplasmic β-galactosidase activity, was used to monitor permeation of two chromogenic substrates. The lactose analogue 2-nitrophenyl β-D-pyranoside (ONPG) and nitrocefin are chromogenic reporter molecules that cannot cross the inner or outer membranes under normal conditions. Upon membrane disruption, however, nitrocefin and ONPG diffuse into the bacterial periplasmic and cytoplasmic regions, where they are hydrolyzed by β-lactamase and β-galactosidase, respectively, causing a color change. Thus cleavage of nitrocefin and ONPG can be monitored by measuring the absorbance at 450 and 490 nm, respectively, using a SpectraMax plate spectrophotometer (Molecular Devices, Sunnyvale, CA). For the assay, a single colony of ML35 was inoculated into 5 ml of LB media. After growth overnight at 37 °C, the bacteria were washed in PBS containing 3% LB and diluted to an OD_{600} of 0.2 (1×10^8 CFU/ml). Aliquots of this suspension (100 μl) were added to all the wells of sterilized 96-well plates. In duplicate wells, bacteria were exposed to the test peptides in the presence of 2.5 mM ONPG and 30 μM nitrocefin for 1 h at 37 °C, during which hydrolysis of ONPG and nitrocefin was monitored as described above. Readings were taken every 2 min.

2.6. Killing kinetics

Exponential phase *S. aureus* and *E. coli* were washed three times with PBS and resuspended to 10^6 CFU/ml. CopW was then added to the cells to final concentrations of 16 or 32 μg/ml and 0.5×10^6 CFU/ml. This mixture was incubated for 2 h at 37 °C, after which 100-μl aliquots were collected at timed intervals. Each aliquot was diluted 1000-fold with PBS, and 100 μl of each diluent was spread on an LB agar plate and incubated for 16 h. Cell viability was then assessed by counting the resultant colonies on the plates.

2.7. Measurement of NO in conditioned culture medium

Nitric oxide (NO) levels in the culture supernatants were measured using the Griess reaction. Cells from the RAW264.7 mouse macrophage line were pre-incubated overnight and then plated at a density of 5×10^5 cells/well in 12-well plates. To stimulate the cells, LPS (1 μg/ml) from *E. coli* O111:B4 (Sigma) in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) was added to each well with or without CopW. After 24 h, the culture media were collected and mixed with the same volume of Griess reagent [1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, 2% phosphoric acid]. NO production was then quantified by comparing

the measured absorbance at 540 nm to standard curve generated using sodium nitrite. Three independent experiments were performed, and the data are presented as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. LPS alone.

2.8. Synergy assays

The synergistic effects of CopW in combination with each of conventional antibiotics were evaluated using a checkerboard microtiter format. All experiments were performed according to NCCLS approved standard M7-A6 [18]. For the combined treatments, a two-dimensional checkerboard with twofold dilutions of each agent was set up. After incubation for 18 h at 37 °C, MICs were determined as described above. Each test was performed in triplicate. The fractional inhibitory concentration index (FICI) was calculated as the sum of each FIC. FICI = (MIC_{DrugA} in combination/MIC_{DrugA} alone) + (MIC_{DrugB} in combination/MIC_{DrugB} alone). The FICIs were then interpreted as follows: FICI ⩽ 0.5 synergy, 0.5 < FICI < 1 partial synergy, FICI = 1 additive, 1 < FICI ⩽ 4 no interaction, 4 < FICI antagonism [19].

3. Results and discussion

3.1. Design and antibacterial activity of coprisin 9-mer analogs

To investigate the structural/functional role of the cysteine residue on the antibacterial activity of CopA3 (LLCIALRKK-NH_2), we designed and synthesized a set of two homodimers, CopA3 (Dimer) and CopIK (Dimer); two monomers, CopA3 (Mono) and CopIK (Mono); and six cysteine-substituted CopA3 analogs with differing chemical and physical properties (Table 1). Two analogs, CopIK and CopLV, were designed to enhance the amphipathicity of CopA3. The relative hydrophobicity of the CopA3 analogs was determined by measuring their retention times on a reverse-phase HPLC C_{18} column. Because both CopA3 (Mono) and CopIK (Mono) have just one free cysteine residue in their sequence, it would be expected that the majority of the reduced linear molecules would spontaneously dimerize in neutral solution. Interestingly, however, dimerization of CopA3 (Mono) was quite different from that of CopIK (Mono) in 0.1 M NH_4OAc solution (pH 7.0). As shown in Fig. 1, dimerization of CopA3 (Mono) yielded a 6/4 monomer/dimer ratio in HPLC area percent, whereas CopIK (Mono) remained a monomer, even under neutral conditions. This means the biological effects of CopA3 (Mono) on bacterial strains reflect the CopA3 (Mono)/CopA3 (Dimer) mixture.

Table 1
Amino acid sequences and properties of the tested 9-mer coprisin analogs.

Name	Sequence[a]	Net charge	Retention time (min)[b]
CopSe	LL-SelenoCys-IALRKK-NH_2	+4	19.2
CopMe	LL-MethylCys-IALRKK-NH_2	+4	17.1
CopR	LLRIALRKK-NH_2	+5	15.2
CopL	LLLIALRKK-NH_2	+4	18.0
CopW	LLWIALRKK-NH_2	+4	17.7
CopLV	LLLIVLRKK-NH_2	+4	18.9
CopA3 (Mono)	LLCIALRKK-NH_2	+4	18.6
CopA3 (Dimer)	(LLCIALRKK-NH_2)$_2$	+8	20.7
CopIK (Mono)	LLCKALRKI-NH_2	+4	18.6
CopIK (Dimer)	(LLCKALRKI-NH_2)$_2$	+8	20.7

[a] Substituted sites are indicated using underlines.
[b] Retention times are indicative of the relative hydrophobicity of the peptides and were determined by measuring the elution time with a reverse phase HPLC C_{18} column.

We next examined the antibacterial activities of the coprisin 9-mer analogs against a representative set of Gram-negative (*E. coli*, *Salmonella typhimurium*, *P. aeruginosa*), Gram-positive (*S. aureus*, *S. epidermidis*, *B. subtilis*, *E. faecium* and *E. faecalis*), and drug-resistant (vancomysin-resistant *E. faecium*, vancomysin-resistant *E. faecalis* and MRSA) strains. The activities of the test peptides were compared with those of melittin, which has prominent antibacterial activity against a wide range of bacterial strains. As summarized in Table 2, the tested peptides exhibited a relatively broad spectrum of antibacterial activity, which is consistent with the general characteristics of AMPs targeting the lipid membrane. Among the 10 analogs, CopL, CopW, CopLV and CopA3 (Dimer) showed strong antibacterial activity against Gram-negative, Gram-positive and drug-resistant strains, with about 2- to 8-fold greater activity than CopA3 (Mono). It is particularly interesting that CopA3 (Dimer) greatly inhibited vancomycin-resistant *E. faecium and E. faecalis* at low concentrations (MIC = 1–2 μg/ml). Their activities were thus similar to those of melittin (MIC = 2–4 μg/ml). Two cysteine-free derivatives, CopMe and CopSe, in which cysteine was replaced by methylcysteine and selenocysteine, respectively, were more potent against Gram-negative (MIC = 4–8 μg/ml) than Gram-positive (MIC = 8–16 μg/ml) bacteria and drug-resistant strains (MIC = 8–64 μg/ml). Taken together, these results indicate that the antibacterial activity of monomeric CopA3 can be maintained or even enhanced in the absence of a cysteine residue in its primary structure. Thus the cysteine residue in the CopA3 sequence does not appear to be essential for its antibacterial activity.

It was previously reported that wild-type coprisin induced substantial leakage of entrapped fluorescent dye from negatively

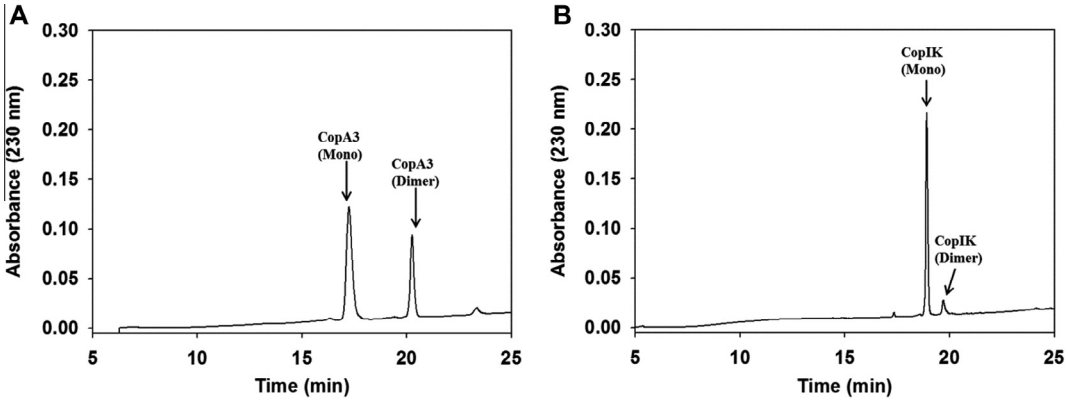

Fig. 1. Reverse phase HPLC profile showing spontaneous dimerization of CopA3(Mono) and CopIK(Mono). The peptide concentration was 64 μg/ml, which is the highest concentration used in the antimicrobial susceptibility test. Peptides were solubilized in 100 mM NH_4OAc (pH7.0) for 1 day at room temperature. (A) The CopA3(Mono)/CopA3(Dimer) ratio is 68.5:31.5. (B) The CopIK(Mono)/CopIK(Dimer) ratio is 90.5:9.5.

Table 2
MICs (μg/ml) and hemolytic activities of the peptides. Results are the averages of three independent experiments. The hemolytic activity was determined using 200 μg/ml peptide.

Microorganism	Minimal inhibitory concentration (μg/ml)										
	CopSe	CopMe	CopR	CopL	CopW	CopLV	CopA3 (Mono)	CopA3 (Dimer)	Cop IK (Mono)	Cop IK (Dimer)	Melittin
Gram-negative bacteria											
E. coli	8	4	4	4	4	4	8	4	16	8	4
S. typhimurium	8	4	8	2	4	4	16	4	16	8	4
P. aeruginosa	8	8	8	4	4	8	16	8	16	8	4
Gram-positive bacteria											
S. aureus	16	16	32	4	8	4	16	8	8	8	2
S. epidermidis	8	4	4	2	4	4	8	4	16	8	4
B. subtilis	8	16	16	4	4	2	16	4	8	4	2
E. faecium	16	16	32	4	8	2	8	8	16	4	4
E. faecalis	16	8	32	4	8	2	2	1	16	8	2
Drug-resistant bacteria											
VRE (*E. faecium*)	8	4	8	2	2	1	4	2	8	2	2
VRE (*E. faecalis*)	64	32	64	8	16	4	2	1	32	32	2
MRSA	8	16	16	4	8	4	16	8	8	8	2
% Hemolysis	1.6	1	1	30	1.5	13.6	2	20	2	2.6	100

charged bacterial membrane-mimetic phospholipid vesicles, but caused much less leakage from neutral mammalian cell membrane-mimetic vesicles [13]. We next assessed the peptides' mammalian cytotoxicity by testing their ability to lyse hRBCs. The bottom row in Table 2 shows the % hemolysis determined based on the evoked release hemoglobin from hRBCs. Melittin and 0.1% Triton X-100 were used as a positive control and standard for comparison, respectively. Melittin induced 100% hemolysis at a concentration of 12.5 μg/ml, whereas most of the CopA3-derived peptides exhibited little cytotoxicity, even at concentrations as high as 200 μg/ml. In particular, CopW exhibited potent antibacterial activity with virtually no hemolytic activity, even at a concentration of 200 μg/ml. Among the peptides tested, we consider CopW to best discriminate between bacterial and mammalian cells.

3.2. Killing kinetics and membrane permeabilization

To further investigate the mechanism of action of CopW, we tested its killing kinetics with Gram-negative *E. coli* and Gram-positive *S. aureus* as well as the kinetics of its effect on membrane permeability using *E. coli* ML35 strain. It took CopW 2 h to completely kill the Gram-negative *E. coli* and 4 h to kill the Gram-positive *S. aureus*, which suggests CopW is more effective against Gram-negative than Gram-positive bacteria (Fig. 2). This killing time for Gram-negative bacteria is a little slower than other insect defensins; for example, sapecin and defensin A exhibit killing times of 30 and 60 min, respectively, against Gram-negative bacterial. On the other hand, CopW acts more quickly than conventional antibiotics, which require more than 6 h [20–22].

Because CopW appeared to be more effective against Gram-negative bacteria, we next examined its effect on the membrane permeability of *E. coli* ML35 cells. This strain expresses two enzymes, β-lactamase and β-galactosidase, which localize in the periplasmic and cytoplasmic regions, respectively. In this assay, damage to the bacterial outer or inner membrane enables hydrolysis of two chromogenic reporter molecules, nitrocefin and ONPG, which cannot otherwise cross the bacterial membranes. Melittin, which is a powerful membrane lytic AMP that efficiently interacts with both the inner and outer membranes of Gram-negative bacteria, was used as a positive control. When log-phase *E. coli* ML35 were exposed to melittin and CopW in the presence of 30 μM nitrocefin or 2.5 mM ONPG, both peptides induced rapid hydrolysis of nitrocefin (Fig. 3, left panel). By contrast, CopW was unable to induce inner membrane permeability. These results suggest that the antibacterial activity of CopW reflects its ability to permeabilize the bacterial outer membrane.

3.3. Anti-inflammatory effect of CopW

LPS is a major component of the outer membrane of Gram-negative bacteria. When mammals are infected by a Gram-negative bacterium, LPS is released, which induces systematic production of pro-inflammatory cytokines and NO. It was previously reported

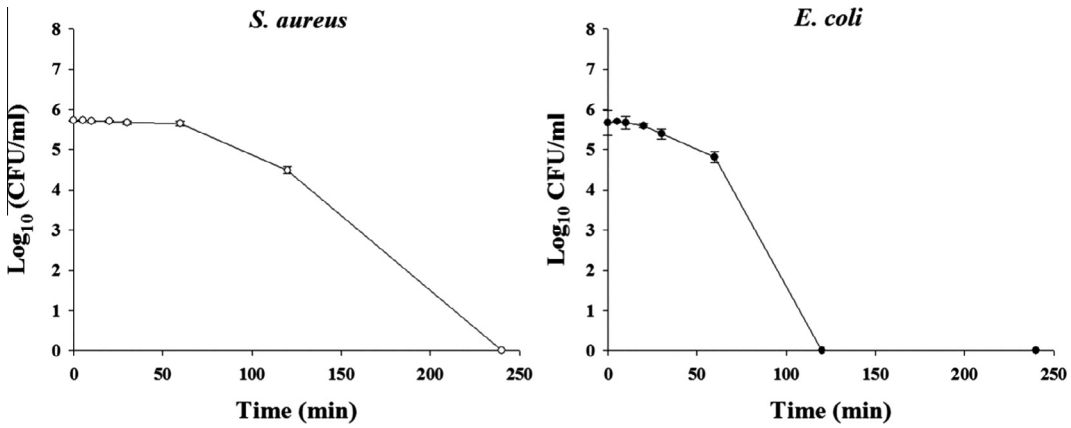

Fig. 2. In vitro killing-time curves for CopW against *S. aureus* KCTC 1621 and *E. coli* KCTC 1682. The CopW concentration was twice the MIC in PBS.

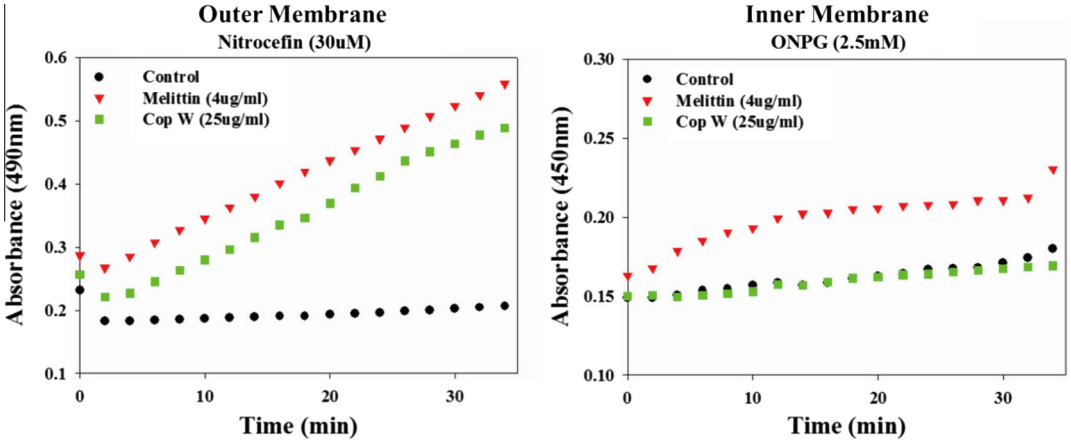

Fig. 3. Membrane permeabilization of *E. coli* ML35 by CopW. Melittin was used as a positive control. Nitrocefin absorbance was measured at 490 nm, while ONPG absorbance was measured at 450 nm.

that CopA3 has anti-inflammatory properties reflecting its ability to neutralize LPS, and that LPS interacts electrostatically with the positively charged residues of CopA3 [13]. Since the positive residues present in CopA3 are conserved in CopW, we investigated the anti-inflammatory properties of CopW. We found that in RAW264.7 mouse macrophages, LPS (1 μg/ml)-induced NO production was dose-dependently inhibited by 1.0–200 μg/ml CopW (Fig. 4), indicating that CopW retains the anti-inflammatory properties of CopA3.

3.4. Synergistic effects of CopW with conventional antibiotics

The synergistic effects of CopW in combination with chloramphenicol, vancomycin, ampicillin and kanamycin against drug-resistant bacteria were evaluated based on their FICIs using the checkerboard method [18]. As outlined in Table 3, half of the combinations showed synergistic or partially synergistic effects. In particular, the combination of CopW and ampicillin was especially synergistic, with a FICI of 0.258, and showed a highly synergistic effect against MRSA. Similarly, Hwang et al. reported that wild-type coprisin exhibits a broad antibacterial spectrum against a variety of bacterial strains and significant synergistic activity when combined with conventional antibiotics [23]. They suggested that combination therapies using an AMP with a conventional antibiotic could increase the susceptibility of bacterial strains to lower concentrations of the agents.

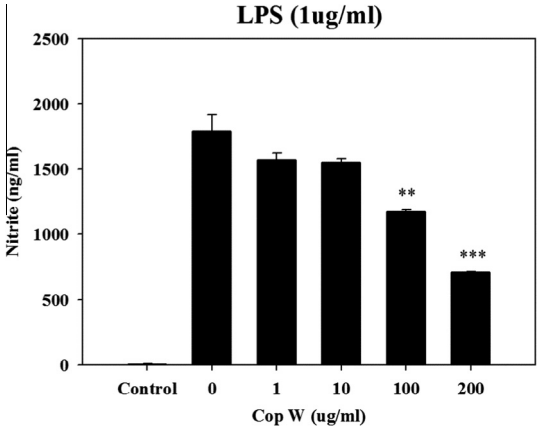

Fig. 4. Inhibition of NO production by CopW in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated for 24 h with CopW (1, 10, 100 or 200 μg/ml) in the presence of LPS (1 μg/ml). Three independent experiments were performed, and the bars depict means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. LPS alone.

3.5. Anti-fungal activity of CopW

It was also previously reported that although its activity is weaker than that of melittin, wild-type coprisin exhibits meaningful antifungal and fungicidal activity (MICs = 5–20 μM) against many strains of fungal cells [24]. We assessed the antifungal activity of CopW using four human pathogens, *C. albicans*, *C. parapsilosis*, *M. furfur* and *T. beigelii*, and compared it that of melittin, which exhibits strong antifungal activity (Table 4). Notably, CopW exhibited more potent antifungal activity than either wild-type coprisin or melittin. The MICs for CopW ranged from 0.7 to 1.5 μg/ml,

Table 4
Antifungal activities of CopW and melittin.

Fungal strains	MIC (μg/ml)	
	CopW	Melittin
C. albicans	0.7	7.1
C. parapsilosis	0.7	14.3
M. furfur	0.7	14.3
T. beigelii	1.5	14.3

Table 3
FICI[a] index of CopW with conventional antibiotics against drug-resistant bacteria.

Strains	Cop W-Chloramphenicol	Cop W-Vancomycin	Cop W-Ampicillin	Cop W-Kanamycin
MRSA	1.0 (A)	1.5 (NI)	0.258 (S)	0.75 (PS)
VRE (*E. faecium*)	0.625 (PS)	1.0 (A)	0.75 (PS)	ND
VRE (*E. faecalis*)	1.5 (NI)	0.75 (PS)	0.75 (PS)	ND

[a] The fractional inhibitory concentration index (FICI) was calculated using the formula: FICI = (MIC_{DrugA} in combination/ MIC_{DrugA} alone) + (MIC_{DrugB} in combination/ MIC_{DrugB} alone). S, synergy; PS, partial synergy; A, additive; NI, no interaction; ND, not determined.

whereas the MICs for melittin ranged from 7.1 to 14.3 μg/ml, indicating CopW has 10- to 20-fold greater antifungal activity than melittin. Collectively then, CopW appears to exert potent antibacterial and antifungal effects with no hemolytic activity.

In conclusion, the therapeutic potential of peptide antibiotics lies in their ability to effectively kill bacterial cells without exhibiting significant cytotoxicity toward mammalian cells. Based on its structure–activity relationships, we have identified CopW, a new nonapeptide derived from CopA3, which shows potent antibacterial and antifungal activity with virtually no hemolytic activity. This makes CopW an attractive potential alternative for use in the treatment the drug-resistant pathogens. In addition, the strong efficacy of CopW makes it clear that the cysteine residue in sequence of CopA3 is not essential for its antimicrobial function.

Acknowledgments

This research was supported by Grants from the Next-Generation BioGreen 21 Program (No. PJ008158), Rural Development Administration, Republic of Korea; a National Research Foundation of Korea grant funded by the Korean Government (MEST) (NRF-C1ABA001-2011-0018559); and the BioImaging Research Center at the Gwangju Institute of Science and Technology.

References

[1] A. Coates, Y. Hu, R. Bax, C. Page, The future challenges facing the development of new antimicrobial drugs, Nat. Rev. Drug Discov. 1 (2002) 895–910.
[2] A. Makovitzki, D. Avrahami, Y. Shai, Ultrashort antibacterial and antifungal lipopeptides, Proc. Natl. Acad. Sci. USA 103 (2006) 15997–16002.
[3] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[4] T. Ganz, Defensins: antimicrobial peptides of innate immunity, Nat. Rev. Immunol. 3 (2003) 710–720.
[5] R.E. Hancock, Cationic peptides: effectors in innate immunity and novel antimicrobials, Lancet Infect. Dis. 1 (2001) 156–164.
[6] K. Yamada, S. Natori, Characterization of the antimicrobial peptide derived from sapecin B, an antibacterial protein of *Sarcophaga peregrina* (flesh fly), Biochem. J. 3 (1994) 623–628.
[7] C. Landon, P. Sodano, C. Hetru, J. Hoffmann, M. Ptak, Solution structure of drosomycin, the first inducible antifungal protein from insects, Protein Sci. 6 (1997) 1878–1884.
[8] M. Lamberty, A. Caille, C. Landon, S. Tassin-Moindrot, C. Hetru, P. Bulet, F. Vovelle, Solution structures of the antifungal heliomicin and a selected variant with both antibacterial and antifungal activities, Biochemistry 40 (2001) 11995–12003.
[9] B. Cornet, J.M. Bonmatin, C. Hetru, J.A. Hoffmann, M. Ptak, F. Vovelle, Refined three-dimensional solution structure of insect defensin A, Structure 3 (1995) 435–448.
[10] M.K. Nygaard, A.S. Andersen, H.H. Kristensen, K.A. Krogfelt, P. Fojan, R. Wimmer, The insect defensin lucifensin from *Lucilia sericata*, J. Biomol. NMR 52 (2012) 277–282.
[11] M. Pasupuleti, A. Schmidtchen, M. Malmsten, Antimicrobial peptides: key components of the innate immune system, Crit. Rev. Biotechnol. 32 (2012) 143–171.
[12] J.S. Hwang, J. Lee, Y.J. Kim, H.S. Bang, E.Y. Yun, S.R. Kim, H.J. Suh, B.R. Kang, S.H. Nam, J.P. Jeon, I. Kim, D.G. Lee, Isolation and characterization of a defensin-like peptide (Coprisin) from the Dung Beetle, *Copris tripartitus*, Int. J. Pept. (2009), http://dx.doi.org/10.1155/2009/136284.
[13] E. Lee, J.K. Kim, S. Shin, K.W. Jeong, A. Shin, J. Lee, D.G. Lee, J.S. Hwang, Y. Kim, Insight into the antimicrobial activities of coprisin isolated from the dung beetle, *Copris tripartitus*, revealed by structure–activity relationships, Biochim. Biophys. Acta 1828 (2012) 271–283.
[14] B.R. Kang, H. Kim, S.H. Nam, E.Y. Yun, S.R. Kim, M.Y. Ahn, J.S. Chang, J.S. Hwang, CopA3 peptide from *Copris tripartitus* induces apoptosis in human leukemia cells via a caspase-independent pathway, BMB Rep. 45 (2012) 85–90.
[15] H.J. Nam, A.R. Oh, S.T. Nam, J.K. Kang, J.S. Chang, D.H. Kim, J.H. Lee, J.S. Hwang, K.E. Shong, M.J. Park, H. Seok, H. Kim, The insect peptide CopA3 inhibits lipopolysaccharide-induced macrophage activation, J. Pept. Sci. 18 (2012) 650–656.
[16] I.W. Kim, S.J. Kim, Y.N. Kwon, E.Y. Yun, M.Y. Ahn, D.C. Kang, J.S. Hwang, Effects of the synthetic coprisin analog peptide, CopA3 in pathogenic microorganisms and mammalian cancer cells, J. Microbiol. Biotechnol. 22 (2012) 156–158.
[17] S.J. Kim, I.W. Kim, Y.N. Kwon, E.Y. Yun, J.S. Hwang, Synthetic Coprisin analog peptide, D-CopA3 has antimicrobial activity and pro-apoptotic effects in human leukemia cells, J. Microbiol. Biotechnol. 22 (2012) 264–269.
[18] Wayne PA. Performance standards for antimicrobial susceptibility testing, fifteenth information supplement. Clinical and Laboratory Standards Institute MS100-S15, 2005.
[19] K. Mazumdar, N.K. Dutta, K.A. Kumar, S.G. Dastidar, In vitro and in vivo synergism between tetracycline and the cardiovascular agent oxyfedrine HCl against common bacterial strains, Biol. Pharm. Bull. 28 (2005) 713–717.
[20] K. Matsuyama, S. Natori, Mode of action of sapecin, a novel antibacterial protein of *Sarcophaga peregrina* (flesh fly), J. Biochem. 108 (1990) 128–132.
[21] J. Lambert, E. Keppi, J.L. Dimarcq, C. Wicker, J.M. Reichhart, B. Dunbar, P. Lepage, A. Van Dorsselaer, J. Hoffmann, J. Fothergill, Insect immunity: isolation from immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides, Proc. Natl. Acad. Sci. USA 86 (1989) 262–266.
[22] S.J. Jung, I.N. Yun, H.S. Park, H.H. Lee, J.W. Jeong, Y.Z. Kim, Y.L. Cho, J.H. Kwak, Antibacterial activity of LCB01-0062, a novel oxazolidinone, Int. J. Antimicrob. Agents 40 (2012) 539–543.
[23] I. Hwang, J. Lee, J. Hwang, J.H. Hwang, H. Choi, E. Lee, Y. Kim, D.G. Lee, Synergistic effect and antibiofilm activity between the antimicrobial peptide coprisin and conventional antibiotics against opportunistic bacteria, Curr. Microbiol. 66 (2013) 56–60.
[24] J. Lee, J. Hwang, I. Hwang, J. Cho, E. Lee, Y. Kim, D.G. Lee, Coprisin-induced antifungal effects in *Candida albicans* correlate with apoptotic mechanisms, Free Radic. Biol. Med. 52 (2012) 2302–2311.