Synthetic Coprisin Analog Peptide, D-CopA3 has Antimicrobial Activity and Pro-Apoptotic Effects in Human Leukemia Cells

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Recently, we reported that the synthetic Coprisin analog peptide 9-mer dimer CopA3 (consisted of all-L amino acid sequence) was designed based on a defensin-like peptide, Coprisin isolated from *Copris tripartitus*. The 9-mer dimer CopA3 (L-CopA3) had antibacterial activity and induced apoptosis in human leukemia cells *via* a caspase-independent pathway. In this study, all of amino acid sequences of L-CopA3 were modified to all D-form amino acids (D-CopA3) to develop a more effective antimicrobial peptide. We investigated whether D-CopA3 had antimicrobial activities against pathogenic microorganisms and pro-apoptotic effects in human leukemia cells (U937, Jurkat, and AML-2). The synthetic peptide D-CopA3 had antimicrobial activities against various pathogenic bacteria and yeast fungus with MIC values in the 4~64 μM range. Moreover, D-CopA3 caused cell growth inhibition, and increased the chromosomal DNA fragmentation and the expression of inflammatory cytokines, TNF-α and IL1-β, transcripts in human leukemia cells. The all-D amino acid peptide D-CopA3 proved as effective as the L-CopA3 reported previously. These results provide the basis for developing D-CopA3 as a new antibiotic peptide.

Keywords: Coprisin analog peptide D-CopA3, antimicrobial peptide, defensin, antimicrobial activity, apoptosis

Antimicrobial peptides (AMPs) are important components of the innate immune defense against microbial pathogens in a broad range of most living organisms [3, 10, 12, 21, 26]. Of these, insect AMPs are cationic and amphipathic with variable length, sequence, and structure, but most have relatively small (below 5 kDa) molecular masses [6, 7]. Coprisin belongs to the defensin family of insect AMPs which were first isolated from cell cultures of the fleshfly *Sarcophaga peregrina* [14]. Insect defensins are members of the widely distributed family of AMPs that contain six cysteine/three disulfide bonds [6, 7] and have antimicrobial activity against Gram-positive bacteria and fungi [5, 7]. Moreover, it has been reported that several insect AMPs show cytotoxic effects against broad ranges of cancer cell lines, such as mouse myeloma, melanoma, lymphomas, leukemia, breast cancer, and lung cancer [1, 12, 15, 20, 25]. Recently, we isolated Coprisin from the bacteria immunized Dung beetle, *Copris tripartitus*. Coprisin consisted of 80 amino acids with a predicted molecular mass of 8.6 kDa and was found to be 79.1% and 67.4% identical to those of defensin peptides of *Anomala cuprea* and *Allomyrina dichotoma*, respectively [11]. In addition, we reported that the synthetic 9-mer dimer analog peptide CopA3 (LLCIALRKK-NH$_2$; L-type amino acid structure), derived from the Leu22 to Lys30 in 43 mature peptides of Coprisin, had antimicrobial activities against various pathogenic microorganisms, inhibited the cell growth of pancreatic and hepatocelluar cancer cells, and induced apoptosis in human leukemia cells *via* a caspase-independent pathway (submitted). Furthermore, CopA3 prevented *Clostridum difficile*-mediated acute inflammation and mucosal damage through selective antimicrobial activity [13].

Many AMPs with preferentially disrupt prokaryotic and cancer cell membranes might be highly potent and effective novel therapeutic agents, but the use of native all-L amino acid AMPs *in vivo* is mainly limited owing to the loss of their function in serum, partially because of enzymatic degradation and binding to the serum components [17]. To overcome this limitation, the D-amino acid enantiomers would be expected to be inactive of proteolytic cleavage and inactivation by serum components [1, 2, 17, 23]. Several studies have shown that the enantiomer of all-D amino acids exhibits antibacterial activity nearly equivalent to that of all-L structure and is not sensitive to proteolytic cleavage [2, 23]. These results suggest that D-amino acid peptides would be a very attractive candidate as a therapeutic agent.

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Therefore, in this study, we synthesized the D-enantiomer CopA3 on the basis of the natural all-L amino acids peptide CopA3 and investigated whether D-CopA3 had antimicrobial activities against pathogenic microorganisms and pro-apoptotic effects in human leukemia cell (U937, Jurkat, and AML-2).

MATERIALS AND METHODS

Peptide Synthesis
The synthetic peptide 9-mer dimer D-CopA3 (LLCIALRKK-NH$_2$; all-D amino acids enantiomer) and 26-mer Melittin (GIGAVLKVL TTGLPALISWIKRKRQQ-NH$_2$) were synthesized by Anygen Co. (Gwangju, Korea).

Antimicrobial Activity Assay
For antimicrobial activity assay, the strains *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Entercoccus hirae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, multidrug resistant *E. coli* (MDREC), multidrug-resistant *P. aeruginosa* (MDRPA), methicillin-resistant *S. aureus* (MRSA), and yeast fungus *Candida albicans* were purchased from the CCARM (Culture Collection Antimicrobial Resistant Microbes, Korean) or the KACC (Korean Agricultural Culture Collection). MIC (minimum inhibitory concentration) test of D-CopA3 against these strains was performed as described previously [11].

Cell Culture
Human leukemia cells (AML2, Jurkat, and U937) were obtained from the KCLB (Korean Cell Line Bank). These cancer cells were maintained in a RPMI-1640 with 10% FBS, penicillin G (100 unit/ml), and streptomycin (100 µg/ml) and incubated at 37°C and 5% CO$_2$ in a humidified incubator.

Cell Viability Assay
Human leukemia cells (AML2, Jurkat, and U937) were plated into 96-well tissue culture plates (2×10^3 cells per well), and treated with various concentrations (25, 50, 75, and 100 µM) of D-CopA3 or without D-CopA3 as a control. After incubation for 24 h, the growth inhibition of cancer cells was measured following the vendor's protocols for the Cell Titer 96 AQ$_{ueous}$ One Solution cell proliferation assay (Promega, USA), and then the optical density (OD) was measured with a Microplate Reader (BecKman DTX 8800 multi detector) at 490 nm.

DNA Fragmentation Assay
For the DNA fragmentation assay, 2×10^6 cells were seeded into 6-well plates and treated with D-CopA3 at a concentration of 75 µM or without (as a control) for 24 h. These cells were collected, washed with PBS, lysed in a solution containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0), and 0.5% Triton X-100 on ice for 30 min, and then centrifuged at 15,000 rpm for 5 min. The supernatants were digested with 0.1 mg of RNase A/ml and 1 mg of proteinase K/ml for 55°C for 1 h in the presence of 1% sodium dodecyl sulfate (SDS), extracted with phenol and chloroform, precipitated in cold ethanol, and subjected to electrophoresis on 2% agarose gels containing ethidium bromide. DNA fragments were visualized by UV light

transillumination. Photographs were taken by a computer-assisted image processor (Core-bio i-max Gel Image analysis system).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL Assay)
Human leukemia cells (AML2, Jurkat, and U937) were plated into 6-well plates (2×10^6/ml) and treated with D-CopA3 at a concentration of 75 µM or without (as a control) for 24 h. Apoptotic cells were determined by TUNEL assay according to the manufacturer's instructions (Promega, Madison, WI, USA).

RNA Isolation and Quantitative RT-PCR
Total RNA was isolated from cultured cells with or without D-CopA3 using Trizol reagent (Invitrogen). First-strand complementary DNA was synthesized by using the High Capacity cDNA Reverse Transcription kit (AB Applied Biosystems) according to the manufacturer's protocols. Quantitative RT-PCR was performed using SYBR Green PCR Master mix. (AB Applied biosystems) containing 1µl of cDNA as a template and the specific primers. The specific primers used were (1) human TNF-α (NM_000594) sense 5'-AGC ACT GAA AGC ATG ATC CG-3' and antisense 5'-GGC CAG AGG GCT GAT TAG AG-3'; (2) human IL-1β (NM_000576) sense 5'-GTA CCT GAG CTC GCC AGT GA-3' and antisense 5'-TGA AGC CCT TGC TGT AGT GG-3'; and (3) human β-actin (NM_001101) sense 5'-CGA CAG GAT GCA GAA GGA GA-3' and antisense 5'-TAG AAG CAT TTG CGG TGG AC-3'. The PCR product was quantitated with the Applied Biosystems 7500 Fast Real Time PCR system (AB Applied Biosystems).

RESULTS AND DISCUSSION

Synthetic Peptide D-CopA3 has Antimicrobial Activity Against Pathogenic Microorganisms.
Recently, we showed that the synthetic Coprisin analog CopA3 (L-form amino acids) had not only antimicrobial activities but also cell growth inhibitions against pancreatic and hepatocellular cancer cells (submitted). The purpose of the present study was to determine the antimicrobial activity of the all-D amino acid enantiomers D-CopA3 derived from the natural, all-L amino acids, CopA3. The antimicrobial activities were tested by MIC (minimum inhibitory concentration) test against pathogenic microorganisms; Gram-negative bacteria, Gram-positive bacteria, and yeast fungus (Table 1). Melittin peptide is known to have powerful antimicrobial and hemolytic activities [7, 8, 22], and was used as a positive control in the MIC test.

D-CopA3 showed growth inhibitory properties against pathogenic bacteria. The MIC values of Gram-positive and -negative bacteria and antibiotic-resistant bacteria tested in this study were 16~64 µM, 4~16 µM, and 4~32 µM ranges, respectively (Table 1), whereas melittin also showed antibacterial activities against these bacteria with a 2~32 µM range of MIC values (Table 1). Furthermore, D-CopA3 had antifugal activity against the human pathogenic yeast fungus *Candida albicans* with 16 µM of MIC value, but

Table 1. Antimicrobial activities against pathogenic microorganisms.

| Microorganism	MIC (μM)	
	D-CopA3	Melittin
Gram-negative bacteria		
Escherichia coli	16	8
Klebsiella pneumoniae	64	8
Pseudomonas aeruginosa	32	32
Gram-positive bacteria		
Enterococcus faecalis	4	4
Enterococcus hirae	4	8
Staphylococcus aureus	8	4
Staphylococcus epidermidis	16	16
Antibiotic-resistant bacteria		
MDR *E. coli*	16	4
MDR *P. aeruginosa*	32	32
MR *S. aureus*	4	2
Yeast fungus		
Candida albicans	16	2

MIC values were determined in three independent experiments

these effects were less strong than those of melittin (2 μM of MIC value) (Table 1).

Wade *et al.* [23] synthesized the D-enantiomer peptides of three naturally occurring antimicrobial peptides (cecropinA, magainin 2 amide, melittin) and two of their hybrid analogs. These peptides show potent antibacterial activity against Gram-positive and -negative bacteria and have resistance to enzymatic degradation. They suggest that all D-enantiomer peptides might have a considerable therapeutic importance. Our results indicated that D-CopA3 exhibited antibacterial and antifungal activities nearly identical to that of the all-L amino acid CopA3 reported previously. Thus, we consider that D-CopA3 may be a new candidate as an antibiotic peptide, even though we do not know whether D-CopA3 peptide is inactive to enzyme degradation or not.

Synthetic Peptide D-CopA3 Inhibits Cell Viability and Induces Pro-Apoptosis in Human Leukemia Cells

We examined the cell growth inhibition of the synthetic peptide D-CopA3 in human leukemia cells (AML2, Jurkat, and U937). These cancer cells were treated with various concentrations (25, 50, 75, and 100 μM) of D-CopA3 for 24 h, and the growth inhibition of these cells was measured by MTT assay. As shown in Fig. 1A, D-CopA3 inhibited the cell growth of human leukemia cells in a dose-dependent manner. Specifically, Jurkat and U937 showed a
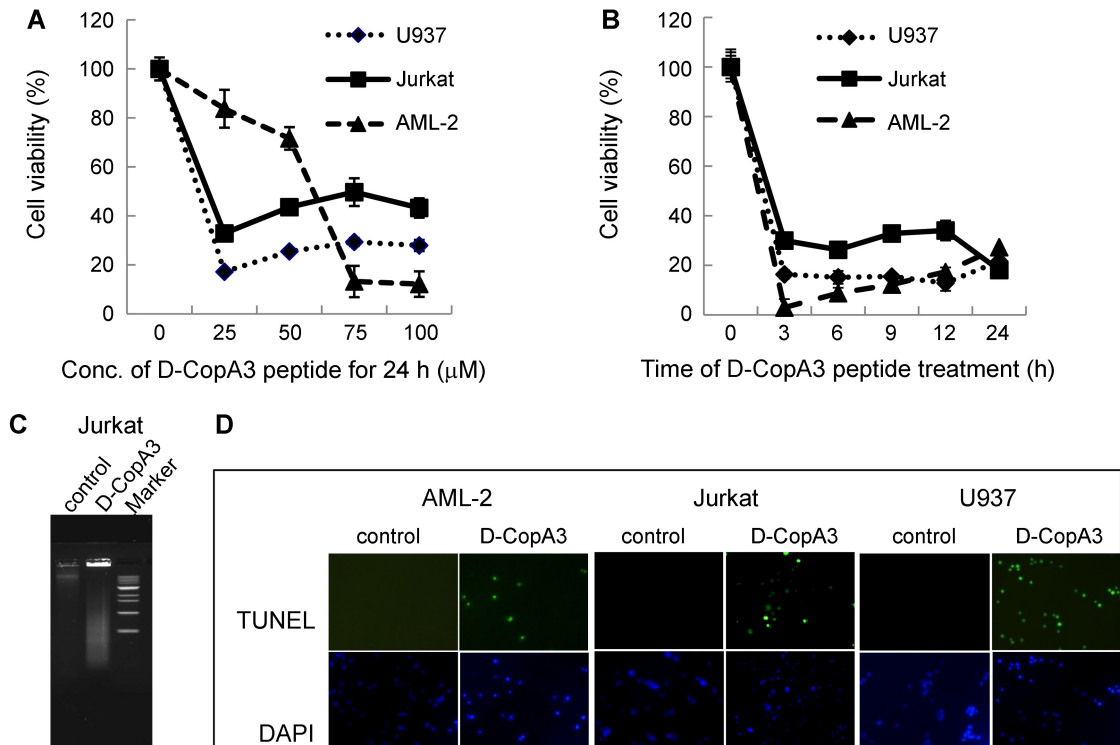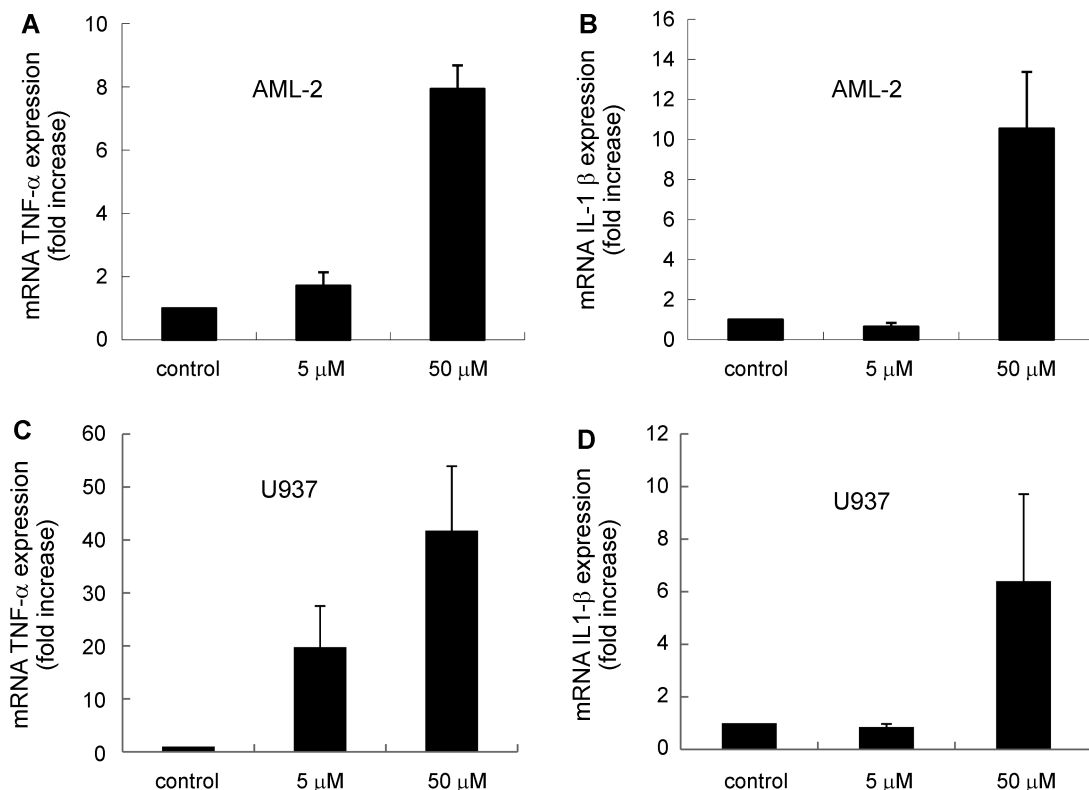

Fig. 1. Synthetic peptide D-CopA3 decreases the cell viability and induces pro-apoptosis in human leukemia cells.
These cancer cells (AML2, Jurkat, and U937) were treated with the different concentrations (0, 25, 50, 75, and 100 μM) of D-CopA3 for 24 h (**A**), or 75 μM of D-CopA3 for different times (0, 3, 6, 9, 12, and 24 h) (**B**), and then cell viabilities were measured by the MTT assay. An error bar was used to show the standard deviation derived from three independent experiments. DNA fragmentation (**C**) and TUNEL (**D**) assays were carried out to demonstrate apoptotic cells following treatment of the cancer cells at a concentration of 75 μM D-CopA3 for 24 h. The microphotographs were taken at ×400 magnification (**D**).

strong inhibition of cell growth at a concentration of 25 μM D-CopA3. Additionally, these cell growth inhibitions were dependent on the incubation time treated with 75 μM of D-CopA3 in these cancer cells, which resulted in a significant inhibition of cell growth even at 3 h. After that time, we observed the growth inhibitions of these cancer cells treated with D-CopA3 (Fig. 1B). Therefore, our results conclude that D-CopA3 may have a potent anticancer activity in the human leukemia cells.

Next, to determine whether or not apoptosis is involved in the inhibition of cell growth of the human leukemia cells, we performed the chromosomal DNA fragmentation and TUNEL assays in these cells after treating with 75 μM of D-CopA3 for 24 h. Apoptosis, or programmed cell death, is a pivotal physiological process that is required for the normal development and maintenance of tissue homeostasis in multicellular organisms [24]. During apoptosis, certain morphological characteristics are involved including membrane blebbing, nuclear and cytoplasmic shrinkages, chromatic condensation, and DNA fragmentation [18]. As shown in Fig. 1C, the chromosomal DNA of Jurkat cells

was fragmented by D-CopA3. In agreement with results in Fig. 1C, the number of positive TUNEL apoptotic cells increased in AML2, Jurkat, and U937 cells treated with D-CopA3 as compared with the untreated cells (control) (Fig. 1D). Based on these results, we consider that the pro-apoptosis effects may lead to the cell viability inhibition of the human leukemia cells treated with D-CopA3.

A few of native all-L amino acid AMPs show anticancer activity *in vitro* but could be not be used *in vivo* because of their inactivation by blood components [4, 16]. These studies showed that intravenous injection of a diasteromer (containing 33% D amino acid) of antimicrobial peptide, but not the all-L amino acid parental peptide, cures neutropenic mice infected with gentamicin-sensitive *Pseudomonas aeruginosa* and gentamicin-resistant *Acinetobacter baumannii* bacteria. Moreover, this research group designed a 15-amino acid peptide, composed of L and D amino acids (diastereomer), which targets both androgen-independent and androgen-dependent human prostate carcinoma cells. Most importantly, the diasteromer (amphipathic D) markedly reduced tumor weights in the CL1 and 22RV1 prostate

Fig. 2. Synthetic peptide D-CopA3 increases the expression of inflammatory cytokines TNF-α and IL1-β in the AML2 (**A** and **B**) and U937 (**C** and **D**) cells.
The cancer cells were treated with 5 μM or 50 μM of D-CopA3 or without D-CopA3 for 24 h. The expressions of TNF-α (**A** and **C**) and IL1-β (**B** and **D**) mRNA transcripts were analyzed by quantitative RT-PCR and normalized to the β-actin housekeeping gene. The average normalized expression level is presented as a fold value relative to that of control, which is given the value of 1. An error bar is used to show the SD derived from more than three independent experiments.

carcinoma xenogragfts and the tumor completely disappeared in 40% of the mice. In contrast, the parental all-ʟ amino acids peptide is highly active only *in vitro* and could not discriminate between tumor and non-tumor cells. Thus, the synthetic peptide ᴅ-CopA3 would have certain practical advantages as an antibiotic peptide, although ᴅ-CopA3 peptide has a potent anticancer activity in human leukemia cells *in vitro*.

Synthetic Peptide ᴅ-CopA3 Increases the Expression of TNF-α and IL-1 Cytokines in AML2 and U937 Cells

Numerous studies have shown that cytokines such as IL-1β, TNF-α, and IFN-γ in relation to their signaling pathway induce apoptosis in many cells. The IL-1β signaling pathway, which is mediated through MAPK activation of JNK and p38, can induce apoptosis in pancreatic RINm5F cells [19]. It is reported that TNF-α induces apoptosis in endothelial cells *via* phosphorylation and down-regulation of Bcl-x_L [9].

Thus, we determined whether the tumor apoptosis mechanism induced by ᴅ-CopA3 was due to the production of inflammatory cytokines, and so the mRNA expressions of TNF-α and IL1-β in AML2 and U937 cancer cells were measured by quantitative RT-PCR. The results revealed that the expression of TNF-α transcript in AML2 and U937 cancer cells after treating with 50 μM of ᴅ-CopA3 for 24 h was increased by 7.9-fold (Fig. 2A) and 41.7-fold (Fig. 2C), respectively, as compared with the control. Even with treatment of only 5 μM of ᴅ-CopA3, the TNF-α transcript in AML2 and U937 cells was increased by 1.7-fold and 19.7-fold, respectively (Fig. 2A and 2C). Similarly, at 50 μM of ᴅ-CopA3, IL1-β transcripts were also increased by 10-fold (in AML2) and 6.4-fold (in U937) (Fig. 2B and 2D). These data suggest that the expressions of TNF-α and IL1-β mRNA transcripts after treating with ᴅ-CopA3 could induce apoptosis in the human leukemia cells.

In conclusion, we showed that the synthetic peptide ᴅ-CopA3 has antimicrobial and anticancer activities *in vitro*. In addition, the synthetic peptide ᴅ-CopA3 has pro-apoptotic effects in the human leukemia cells, which increased the chromosomal DNA fragmentation and the expression of cytokines TNF-α and IL1-β mRNA transcripts. From these results, we conclude that the synthetic peptide ᴅ-CopA3 has potential for development as a new type of antibiotic peptide.

Acknowledgments

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