Addition of L-cysteine to the N- or C-terminus of the all-d-enantiomer [$_D$(KLAKLAK)$_2$] increases antimicrobial activities against multidrug-resistant *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Escherichia coli*

Maki K Ohno [Corresp., Equal first author, 1], **Teruo Kirikae** [2], **Eisaku Yoshihara** [3], **Fumiko Kirikae** [2], **Isao Ishida** [Equal first author, 1]

[1] Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Tokyo, Japan

[2] Department of Microbiology, Juntendo University School of Medicine, Tokyo, Japan

[3] Tokai University School of Medicine, Kanagawa, Japan

Corresponding Author: Maki K Ohno
Email address: m.ohno@thu.ac.jp

Background. Antimicrobial peptides have a broad spectrum of antimicrobial activities and are attracting attention as promising next-generation antibiotics against multidrug-resistant (MDR) bacteria. The all-d-enantiomer [$_D$(KLAKLAK)$_2$] has been reported to have antimicrobial activity against *Escherichia coli* and *Pseudomonas aeruginosa*, and to be resistant to protein degradation in bacteria because it is composed of D-enantiomer compounds. In this study, we demonstrated that modification of [$_D$(KLAKLAK)$_2$] by the addition of an L-cysteine residue to its N- or C- terminus markedly enhanced its antimicrobial activities against Gram-negative bacteria such as MDR *Acinetobacter baumannii*, *E. coli*, and *P. aeruginosa*.

Methods. The peptides [$_D$(KLAKLAK)$_2$] (DP), DP to which L-cysteine was added at the N-terminus C-DP, and DP to which L-cysteine was added at the C-terminus DP-C, were synthesized at >95% purity. The minimum inhibitory concentrations of peptides and antibiotics were determined by the broth microdilution method. The synergistic effects of the peptides and the antibiotics against MDR *P. aeruginosa* were evaluated using the checkerboard dilution method. In order to assess how these peptides affect the survival of human cells, cell viability was determined using a Cell Counting Kit-8.

Results. C-DP and DP-C enhanced the antimicrobial activities of the peptide against MDR Gram-negative bacteria, including *A. baumannii*, *E. coli,* and *P. aeruginosa*. The antimicrobial activity of DP-C was greater than that of C-DP, with these peptides also having antimicrobial activity against drug-susceptible *P. aeruginosa* and drug-resistant *P. aeruginosa* overexpressing the efflux pump components. C-DP and DP-C also showed antimicrobial activity against colistin-resistant *E. coli* harboring *mcr-1*, which encodes a lipid A modifying enzyme. DP-C showed synergistic antimicrobial activity against MDR *P. aeruginosa* when combined with colistin. The LD_{50} of DP-C against a human cell line HepG2 was six times higher than the MIC of DP-C against MDR *P. aeruginosa.* The LD_{50} of DP-C was not altered by incubation with low-dose colistin.

Conclusion. Attachment of an L-cysteine residue to the N- or C-terminus of [$_D$(KLAKLAK)$_2$] enhanced its antimicrobial activity against *A. baumannii*, *E. coli*, and *P. aeruginosa*. The combination of C-DP or DP-C and colistin had synergistic effects against multi-drug resistant *P. aeruginosa*. In addition, DP-C and C-DP showed much stronger antimicrobial activity against MDR *A. baumannii* and *E. coli* than against *P. aeruginosa.*

Addition of L-cysteine to the N- or C-terminus of the all-d-enantiomer [$_D$(KLAKLAK)$_2$] increases antimicrobial activities against multidrug-resistant *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Escherichia coli*

Maki Kamiya Ohno [1*], Teruo Kirikae [2], Eisaku Yoshihara [3], Fumiko Kirikae [2], Isao Ishida [1*]

[1] Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Tokyo Japan
[2] Department of Microbiology, Juntendo University School of Medicine, Tokyo, Japan
[3] Tokai University School of Medicine, Kanagawa, Japan
[*]These authors contributed equally to this work.

Corresponding Author:
Maki Ohno[1]
4-21-2 Nakano, Nakano-ku, Tokyo, 164-8530, Japan
Email address: m.ohno@thu.ac.jp

Abstract

Background. Antimicrobial peptides have a broad spectrum of antimicrobial activities and are attracting attention as promising next-generation antibiotics against multidrug-resistant (MDR) bacteria. The all-d-enantiomer [$_D$(KLAKLAK)$_2$] has been reported to have antimicrobial activity against *Escherichia coli* and *Pseudomonas aeruginosa*, and to be resistant to protein degradation in bacteria because it is composed of D-enantiomer compounds. In this study, we demonstrated that modification of [$_D$(KLAKLAK)$_2$] by the addition of an L-cysteine residue to its N- or C-terminus markedly enhanced its antimicrobial activities against Gram-negative bacteria such as MDR *Acinetobacter baumannii*, *E. coli*, and *P. aeruginosa*.

Methods. The peptides [$_D$(KLAKLAK)$_2$] (DP), DP to which L-cysteine was added at the N-terminus C-DP, and DP to which L-cysteine was added at the C-terminus DP-C, were synthesized at >95% purity. The minimum inhibitory concentrations of peptides and antibiotics were determined by the broth microdilution method. The synergistic effects of the peptides and the antibiotics against MDR *P. aeruginosa* were evaluated using the checkerboard dilution method. In order to assess how these peptides affect the survival of human cells, cell viability was determined using a Cell Counting Kit-8.

Results. C-DP and DP-C enhanced the antimicrobial activities of the peptide against MDR Gram-negative bacteria, including *A. baumannii*, *E. coli,* and *P. aeruginosa*. The antimicrobial activity of DP-C was greater than that of C-DP, with these peptides also having antimicrobial activity against drug-susceptible *P. aeruginosa* and drug-resistant *P. aeruginosa* overexpressing efflux pump components. C-DP and DP-C also showed antimicrobial activity against colistin-resistant *E. coli* harboring *mcr-1*, which encodes a lipid A modifying enzyme. DP-C showed synergistic antimicrobial activity against MDR *P. aeruginosa* when combined with colistin. The LD_{50} of DP-C against a human cell line HepG2 was six times higher than the MIC of DP-C against MDR *P. aeruginosa.* The LD_{50} of DP-C was not altered by incubation with low-dose colistin.

Conclusion. Attachment of an L-cysteine residue to the N- or C-terminus of $[_D(KLAKLAK)_2]$ enhanced its antimicrobial activity against *P. aeruginosa, A. baumannii* and *E. coli*. The combination of C-DP or DP-C and colistin had synergistic effects against multi-drug resistant *P. aeruginosa*. In addition, DP-C and C-DP showed much stronger antimicrobial activity against MDR *A. baumannii* and *E. coli* than against *P. aeruginosa.*

Introduction

The emergence and spread of multidrug-resistant (MDR) Gram-negative pathogens has become a serious public health problem worldwide. A global priority list of antibiotic-resistant bacteria published by the World Health Organization (WHO) to guide research, discovery, and development of new antibiotics listed carbapenem-resistant *Acinetobacter baumannii, Pseudomonas aeruginosa* and carbapenem-resistant and third-generation cephalosporin-resistant *Enterobacteriaceae* as first priority pathogens (Shai, 2002).

Antimicrobial peptides (AMPs) are produced by various host organisms and partially contribute to the host's innate immunity (Plesniak et al., 2004; Onuchic, Jennings & Ben-Jacob, 2013). These peptides exhibit potent antimicrobial activities against a wide range of microorganisms, including viruses, bacteria, protozoa, and fungi (Shai, 2002). AMPs are chemically amphiphilic polycationic peptides, generally comprising 6–50 amino acid residues, and they constitute a unique and diverse group of molecules (Peters, Shirtliff & Jabra-Rizk, 2010). AMPs are classified according to their secondary structures including mixtures of α-helices, β-sheets, loops, and extended peptides. Most of these peptides are thought to bind to the cytoplasmic membrane, forming micelle-like aggregates that destroy the membrane (Peters, Shirtliff & Jabra-Rizk, 2010). These peptides orient parallel to the interface, and associate with the membrane surface. After reaching a threshold concentration on the bilayer surface, they aggregate promoting channel formation through the bilayer and disrupt the membrane (Matsuzaki et al., 1995; Plesniak et al., 2004; Onuchic, Jennings & Ben-Jacob, 2013). Because of their mechanisms of action, AMPs show antimicrobial activities against MDR as well as drug-susceptible Gram-negative bacteria. Thus, AMPs are attracting attention as promising next-generation antibiotics for the treatment of MDR bacterial infections (Hancock & Lehrer, 1998; Marr, Gooderham & Hancock, 2006).

The all-D-enantiomer, [$_D$(KLAKLAK)$_2$] is an amphipathic lysine-leucine-rich α-helical peptide with high antimicrobial activity against *Escherichia coli* and *P. aeruginosa*, but with low toxicity against mouse 3T3 cells (Javadpour et al., 1996; McGrath et al., 2013). [$_D$(KLAKLAK)$_2$] orients parallel to the interface and associates with the outer membrane surface. After reaching a threshold concentration on the outer membrane surface, the peptides aggregate to promote channel formation through the bilayer (Matsuzaki et al., 1995; Plesniak et al., 2004; Onuchic, Jennings & Ben-Jacob, 2013). [$_D$(KLAKLAK)$_2$] was reported to selectively interfere with the bilayer of the outer membranes of *E. coli* and *P. aeruginosa*, leading to cell death by membrane disruption and loss of membrane potential. [$_D$(KLAKLAK)$_2$] has shown antimicrobial activity against Gram-negative bacteria but not Gram-positive bacteria, as this peptide was unlikely to disrupt the thick peptidoglycan layer of the latter (McGrath et al., 2013). One of the strategies used to protect AMPs from protease degradation was sequence modification of D-amino acids to replace L-amino acids (Choi et al., 1993; Braunstein, Papo & Shai, 2004; Lee & Lee, 2008). Because this peptide is an all-D-enantiomer, it is highly resistant to proteolysis in bacteria and has low immunogenicity. This stability and low immunogenicity may prolong its half-life and enhance its efficacy at low doses *in vivo*. Initially we added L-cysteine to the N- or C-terminus of [$_D$(KLAKLAK)$_2$] in order to conjugate with protein such as a single chain antibody. The side chain of cysteine contains sulfhydryl group, which can make a covalent coupling with an amino group of the protein via a cross-linker molecule such as sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate). The cysteine-rich AMPs were isolated from leguminous plants and the granular hemocytes of mangrove crabs (Sivakamavalli, Nirosha & Vaseeharan, 2015; Maróti, Downie & Kondorosi, 2015). The functionalized textiles and nasal prongs modified with L-cysteine exhibited antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* (Gouveia, Sá & Henriques, 2012; Caldeira et al., 2013; Xu et al., 2017; Odeberg et al., 2018).

Modification of [$_D$(KLAKLAK)$_2$] by the addition of an L-cysteine residue to its N- or C-terminus markedly enhanced its antimicrobial activities against Gram-negative bacteria such as *P. aeruginosa*, *E. coli*, and *A. baumannii*. The present study describes the antimicrobial activities of the modified peptides against clinical isolates of MDR *P. aeruginosa*, *A. baumannii* and *E. coli*, and its synergistic effects with low dose colistin.

Materials & Methods

Peptide design and synthesis

The peptides [(D-Lys-D-Leu-D-Ala-D-Lys-D-Leu-D-Ala-D-Lys)$_2$] (DP) (9), DP to which L-cysteine was added at the N-terminus, [L-Cys-(D-Lys-D-Leu-D-Ala-D-Lys-D-Leu-D-Ala-D-Lys)$_2$] (C-DP) and DP to which L-cysteine was added at the C-terminus [(D-Lys-D-Leu-D-Ala-D-Lys-D-Leu-D-Ala-D-Lys)$_2$-L-Cys] (DP-C), were synthesized at >95% purity (Scrum Inc., Tokyo, Japan). DP-C was dimerized by heating at 60 ° C for 30 min (DP-C dimer) to convert cysteine to cystine. Purity of synthetic peptides was checked on a SunFire C18 column (100Å, 5 µm, 4.6 mm inner diameter × 250 mm, Waters). Each peptides were gradiently eluted with

solution A (water containing 0.1% trifluoroacetic acid) and solution B (acetonitrile containing 0.1% trifluoroacetic acid) at a flow rate of 1.0 mL/min. The elution program for DP and C-DP was as follows: at 0 min, 10% of B; at 20 min, 60% of B. The elution program for DP-C was as follows: at 0 min, 0% of B; at 20 min, 100% of B. The separated components were detected at 220 nm. The DP-C dimer was separated on a COSMOSIL 5C18-AR-300 reversed phase column (4.6 mm inner diameter × 250 mm, Nacalai Tesque, Kyoto, Japan), using an automated HPLC system (LC-2010AHT; Shimadzu, Kyoto, Japan). The reaction products were gradiently eluted with solution A (water containing 0.086% trifluoroacetic acid) and solution B (acetonitrile containing 0.086% trifluoroacetic acid) at a flow rate of 1.0 mL/min. The elution program for DP-C dimer was as follows: at 0 min, 20% of B; at 20 min, 50% of B. The peptide masses were determined by MALDI-TOF MS on a microflex (Bruker, Billerica MA).

Bacterial strains

MDR *P. aeruginosa* NCGM2.S1 (Miyoshi-Akiyama et al., 2011); drug-susceptible *P. aeruginosa* PAO1 (Weinstein, 2018); *P. aeruginosa* OCR1 (Poole et al., 1996); and *P. aeruginosa* PAO4290 (Yoneyama et al., 1997), were grown in Luria Bertani broth (LB Broth; BD Japan, Tokyo, Japan) or on LB plates containing 15 g/L agar, at 37° C. Drug-susceptible *E. coli* ATCC 25922, a clinical isolate of *E.coli* NCCHD1261-5 (Uchida et al., 2018), and *S. aureus* ATCC 25923 were grown at 37℃ in tryptic soy broth (TSB; BD Japan). Drug-susceptible *A. baumannii* ATCC 15308; a clinical isolate of MDR *A. baumannii* IOMTU433 (Tada et al., 2015) (GenBank accession no. AP014649); a clinical isolate of MDR *A. baumannii* NCGM237 (Tada et al., 2015) (GenBank accession no. AP013357); a clinical isolate of MDR *A. baumannii* NCGM253 (Tada et al., 2015) (GenBank accession no. AB823544); *K. pneumoniae* ATCC-BAA-2146; *K. pneumoniae* ATCC15380 (Reading & Cole, 1977) and *Serratia marcescens* NBRC102204^T, were grown at 37° C in Difco™ Nutrient broth (BD).

Drug susceptibility testing

The minimum inhibitory concentrations (MICs) of peptides and antibiotics, including meropenem, amikacin, ofloxacin, and colistin, were determined by the broth microdilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines (Weinstein, 2018). Bacterial strains were inoculated at 5 x 10^5 CFU/ml per well into 96-well round-bottom microtiter plates (Watson Bio Lab, Kobe, Japan) containing an equal volume of serially diluted peptide or antibiotic. Three independent experiments were performed to confirm reproducibility.

The synergistic effects of the peptides and the antibiotics amikacin, colistin, meropenem, ofloxacin, and rifampicin against *P. aeruginosa* NCGM2.S1 were evaluated using the checkerboard dilution method. The peptides were two-fold serially diluted to final concentrations ranging from 0.125- to 2-times the MIC longitudinally in 96-well round-bottom microtiter plates (Watson Bio Lab). Subsequently, antibiotic was two-fold serially diluted to final concentrations ranging from 0.125- to 2-times the MIC transversely into the plates. NCGM2.S1 was inoculated at 5 x 10^5 CFU/ml per well at a volume equal to that of the diluted peptide and antibiotic. Three

independent experiments were performed to confirm reproducibility. The synergistic effect of the peptides and antibiotics was assessed by determining the fractional inhibitory concentration (FIC) index (Berenbaum, 1978), using the formula:

$$\text{FIC} = \frac{\text{MIC of peptide in combination}}{\text{MIC of peptide alone}} + \frac{\text{MIC of antimicrobial agent in combination}}{\text{MIC of antimicrobial agent alone}}$$

An FIC index ≤0.5 was defined as synergistic, an FIC index >0.5 to 4.0 was defined as additive or unrelated, and an FIC index > 4.0 was defined as antagonistic.

Cytotoxicity tests

The human hepatoblastoma cell line, HepG2 (ATCC HB-8065), was obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). HepG2 cells were seeded at 3000 cells/well in 96-well cell culture-treated flat bottom microtiter plates (Falcon, Corning NY). The cells were incubated at 37 °C for 48 h in an atmosphere containing 5% CO_2, followed by the addition of peptides, at a final concentration of 0–256 μg/ml, or colistin, at a final concentration of 0–3000 μg/ml, by serial dilution. The plates were incubated with 0.2% FBS in 5% CO_2 at 37 °C for 48 h; under these conditions, HepG2 cells were alive but did not grow. Cell viability was determined using a Cell Counting Kit-8 (Dojin, Tokyo, Japan), and colorimetric changes were determined at $OD_{450\ nm}$ with a microplate reader (Corona Electric Co Ltd., Ibaraki, Japan). The LD_{50} was defined as the concentration of peptides or colistin that resulted in 50% cell viability. Three independent experiments were performed to confirm reproducibility.

Statistical analysis

The Mann-Whitney U test was used to compare the MIC values of C-DP, DP-C, and DP-C dimer with DP in *P. aeruginosa* and *A. baumannii*. P-values less than 0.05 were considered statistically significant. Cell survival was expressed as a percentage of the control were obtained as mean ± Standard Deviation (SD) of three independent experiments done in three replicates for each treatment. Significant differences of cell survival rate between each concentration and the control were statistically evaluated by Student's t-test.

Results

Addition of L-cysteine to the N- or C- terminus enhanced the antimicrobial activity of the original peptide

The HPLC analysis indicated that the purities of synthetic DP, C-DP, and DP-C were 100, 95.52 and 98.68%, respectively (*Fig. S1-3*). Additionally the masses of DP, C-DP, and DP-C by MALDI-TOF MS analysis were 1525.444, 1626.595, and 1627.151, respectively that matched well with the theoretical molecular weights (1524.0, 1627.1, and 1627.1) (*Fig. S1-3*). Similarly the formation of DP-C dimer was assessed by HPLC and MALDI-TOF MS. The HPLC analysis of heat treated DP-C showed one large peak estimated as DP-C dimer and one

small peak estimated as DP-C monomer, the peak area ratio was 6.3 : 1 (*Fig. S4*). The MALDI-TOF MS result of heat treated DP-C was 3252.7, which was in consistent with the estimated molecular weight of DP-C dimer (*Fig. S4*). The grand average hydropathy (GRAVY) values were -0.07 for DP and 0.1 each for C-DP and DP-C, indicating that the addition of L-cysteine affected the hydrophobicity of DP.

Assessment of antibiotic susceptibility showed that drug-susceptible *P. aeruginosa* PAO-1 (Weinstein, 2018) and PAO4290 expressing a normal level of MexAB-OprM (Yoneyama *et al*., 1997), were susceptible to all antibiotics tested; MDR *P. aeruginosa* NCGM2.S1 (Miyoshi-Akiyama *et al*., 2011) was susceptible to colistin, but resistant to amikacin, meropenem and ofloxacin; and OCR1, a *nalB* multidrug-resistant mutant that overproduces the outer membrane protein OprM (Poole *et al*., 1996) was susceptible to amikacin and colistin, intermediately susceptible to ofloxacin, but resistant to meropenem (*Table 1*). DP showed antimicrobial activity against PAO-1, with an MIC of 300 μg/mL, consistent with previous findings (McGrath et al., 2013). DP also had antimicrobial activity against NCGM2.S1 and PAO4290, with MICs of 300 μg/mL, but DP showed no antimicrobial activity against OCR1 within the tested concentration range. C-DP had greater antimicrobial activities than DP against all of these strains (The Mann-Whitney U test, $p < 0.05$), with MICs of 64–128 μg/mL, and DP-C had greater antimicrobial activities than C-DP and DP (The Mann-Whitney U test, $p < 0.05$), with MICs 16–32 μg/mL (*Table 1*). The DP-C dimer also had antimicrobial activities and showed MICs identical to DP-C against all the strains tested. These results indicate that the addition of L-cysteine to the N- or C-terminus of [$_{D}$(KLAKLAK)$_2$] increased its antimicrobial activity.

DP showed antimicrobial activity against four *A. baumannii* strains, with MICs of 64 to 300 μg/mL, and against a clinical isolate of colistin- and carbapenem-resistant *E. coli* NCCHD1261-5 co-harboring *mcr-1* and *bla*$_{\text{NDM-5}}$ genes (Uchida *et al*., 2018), with an MIC of 64 μg/mL. In contrast, DP was inactive against drug-susceptible *E. coli* ATCC 25922, two *K. pneumoniae* strains, *S marcescens* NBRC102204 and *S. aureus* ATCC 25923 (*Table 2*). C-DP and DP-C showed higher antimicrobial activities than DP (The Mann-Whitney U test, $p < 0.05$), with MICs of 4 to 8 μg/mL against the four *A. baumannii* strains and MICs of 4 to 16 μg/mL against the two *E. coli* strains. C-DP and DP-C showed antimicrobial activity against carbapenem-resistant *K. pneumonia*e ATCC BAA-2146 harboring *bla*$_{\text{NDM-1}}$, with both having MICs of 16 μg/mL, but not against penicillin-resistant, β-lactamase-producing *K. pneumonia*e ATCC 15380, *S. marcescens* NBRC102204 and *S. aureus* ATCC 25923 (*Table 2*).

Synergistic effects of peptides and antibiotics

The combinations of DP, C-DP and DP-C with colistin had synergistic effects on antimicrobial activity (*Table 3*). For example, the growth of *P. aeruginosa* NCGM2.S1 was inhibited by a combination of one-sixteenth the MIC of DP (19 μg/mL) and one-fourth the MIC of colistin (0.25 μg/mL), by a combination of one-fourth the MIC of C-DP (32 μg/mL) and one-eighth the MIC of colistin (0.125 μg/mL) and by a combination of one-eighth the MIC of DP-C (4.0 μg/mL) and one-fourth the MIC of colistin (0.25 μg/mL). Although AMPs that induced

susceptibility to rifampicin were reported in clinical MDR isolates of *P. aeruginosa* (Baker et al., 2019), DP-C only slightly enhanced the susceptibility to rifampicin. The combinations of C-DP with meropenem or ofloxacin had additive effect on antimicrobial activity. The synergistic effects were not observed when DP-C was combined on amikacin.

Cytotoxicity of antimicrobial peptides to HepG2 cells

The LD_{50} values of each peptide in HepG2 cells were >256, >256, 192, and 2100 μg/ml for DP, C-DP, DP-C, and colistin, respectively (*Fig. S5*, *Fig. S6*). The raw data of *Fig. S5* and *Fig. S6* are available in Supplementary file 2 and Supplementary file 3 respectively. Since the antimicrobial activity of these peptides against *P. aeruginosa* were synergistic with colistin, we examined whether a combination of these peptides and colistin was more toxic than the peptide alone. The combination of DP-C and colistin dose-dependently induced the death of HepG2 cells (*Figure 1*), with more than 50% of the cells dying at 256 μg/ml DP-C and 25.6 μg/ml colistin. The cytotoxicity of the peptide was not enhanced by combination of low doses of colistin. There was no cytotoxicity to HepG2 at 4 μg/ml DP-C and 0.4 μg/ml colistin, which concentrations showed synergistic effects of colistin and DP-C. The raw data of *Fig. 1* is available in Supplementary file 2.

Discussion

We added L-cysteine to the N- or C-terminus of $[_D(KLAKLAK)_2]$ in order to bind it with another protein like a single-chain antibody in the beginning of the experiment. This attachment yielded two compounds, DP-C and C-DP, with significantly greater antimicrobial activities against *A. baumannii*, *E. coli and P. aeruginosa* than the original DP. Similarly, the addition of L-cysteine to AMPs, Andersonin-Y1, HBcARD, buforinII or lysin, enhanced its antimicrobial activity, with higher membrane disruption activity than the original peptide (Chen et al., 2018; Pal et al., 2019). The cysteine-derived cationic dipeptides lysine–cysteine, arginine–cysteine and histidine–cysteine presented antimicrobial activity, SEM analysis suggests that these dipeptides interact with cell walls to disrupt membrane integrity (Tsai et al., 2020). Whereas addition of an L-cysteine to the C-terminus of indolicidin, magainine or epinecidin-1 did not change their antimicrobial activity (Chen et al., 2018). It remains unclear what could be the mechanism by which the addition of cysteine to the N- or C-terminus to AMPs enhances antimicrobial activity. It is unlikely that this effect is simply due to peptide dimerization via cysteine disulfide bond formation because DP-C dimer showed the same MIC value as DP-C monomer against *P. aeruginosa* strains tested. The cysteine-rich region in Factor C receptors in the horseshoe crab specifically binds to bacterial lipopolysaccharides on Gram-negative bacteria (Koshiba, Hashii & Kawabata, 2006). The addition of L-cysteine to the N- or C-terminus of the peptide may have facilitated its binding to the bacterial membrane surface and form structures that disrupt their cell wall. The potential mechanism of efficacy enhancement by the attachment of cysteine residue to AMPs requires further investigation.

Systematic hybridization of two lead peptides from unrelated classes of AMPs showed no associations of net charge, charge density, and antipneumococcal activity among the hybrid peptides, although AMPs with higher hydrophobicity values have been reported to have greater antimicrobial activity against *Streptococcus pneumoniae* (Le et al., 2015). The peptides we tested showed a similar trend, when GRAVY was calculated, DPC showed higher hydrophobicity than DP. L-cysteine exhibited preferred antimicrobial activity against *S. aureus* compared with D-cysteine, whereas D-cysteine showed stronger antimicrobial activity against *E. coli*, *Listeria monocytogenes* and *Salmonella enteritis* (Wang et al., 2019). D-amino acid is highly resistant to proteolysis in bacteria, addition of D-cystein instead of L-cystein may be effective.

DP-C and C-DP had potent activity against multidrug-resistant Gram-negative pathogens. The emergence and spread of these drug-resistant pathogens has become a serious worldwide public health problem (Boucher et al., 2009; Tacconelli et al., 2018). Carbapenem is a last resort β-lactam antibiotic administered to treat infections with drug-resistant Gram-negative pathogens. The development of new antibiotics against carbapenem-resistance pathogens is of top priority (Tacconelli et al., 2018). DP-C and C-DP also had antimicrobial activity against *E. coli* strains harboring the plasmid-mediated colistin resistance *mcr-1* gene. Colistin is a last line polycationic peptide antibiotic which is used to treat infections with carbapenem-resistant Gram-negative pathogens (Paterson & Harris, 2016). However, colistin-resistant *mcr-1* producers have emerged in humans and animals in China (Liu et al., 2016) and have spread worldwide. DP-C and C-DP, like DP, possess a positive charge and hydrophobic regions, suggesting that they target the lipid bilayer of the membrane and destroy it, causing loss of membrane potential and ultimately cell death (McGrath et al., 2013). These peptides had antimicrobial activity against Gram-negative but not Gram-positive pathogens. These peptides were also inactive against intrinsically colistin-resistant *S. marcescens*, indicating that the addition of positively charged 4-amino-4-deoxy-L-arabinopyranose 1 to lipopolysaccharide changes the membrane charge and prevents peptide binding. The combination of DP-C and colistin reduced effective doses of both and may reduce peptide toxicity and colistin clinical nephrotoxicity. Although D-amino acid-based AMPs have been used clinically in the topical treatment of acne (Gordon, Romanowski & McDermott, 2005) but not yet for systemic infectious diseases. It should be rewarding to explore in the systemic treatment whether attachment of cysteine to the N- or C-terminus of AMPs could help broaden the spectrum and enhance the activity of AMPs against various drug-resistant microorganisms.

An algorithm predicting the effectiveness of silico designed stapled AMPs that are stable, active and selective toward bacterial membranes *in vivo*, has enabled the modification of magainin II (Mag2) and other known AMPs (Mourtada et al., 2019). Modified Mag(i+4)1,15(A9K) was found to have MICs <4 μg/ml for MDR *P. aeruginosa*, *A. baumannii* and *E. coli*, with concentrations as high as ~100 μg/ml having almost no red blood cell hemolytic activity. Combinations of colistin with DP-C and C-DP may achieve the same level of anti-microbial activity against these MDR bacteria, as well as widening the safety windows of both drugs. Furthermore, linking of chimeric DP-C and C-DP to macrocycles derived from polymyxin and colistin could have synergistic antimicrobial activity. Chimeric peptidomimetic antibiotics,

in which a β-hairpin peptide macrocycle is linked to the macrocycles found in polymyxin and colistin, have shown potent antimicrobial activity against Gram-negative bacteria (Luther et al., 2019). These polymyxin and colistin-derived macrocycles targeting lipopolysaccharide were found to synergize with the macrocycles targeting β-barrel outer membrane proteins.

Conclusions

Attachment of an L-cysteine residue to the N- or C-terminus of [$_D$(KLAKLAK)$_2$] enhanced its antimicrobial activity against *P. aeruginosa, E. coli*, and *A. baumannii*. The combination of C-DP or DP-C and colistin had synergistic effects against multi-drug resistant *P. aeruginosa*. In addition, DP-C and C-DP showed much stronger effects against MDR *A. baumannii* and *E. coli* than against *P. aeruginosa.*

Acknowledgements

We thank Dr. Atsushi Ohnishi (Faculty of Pharmaceutical Sciences, Teikyo Heisei Univ.) for providing us with the HPLC ana data of the DP-C dimer. We also thank Azumi Inaba for technical assistance and David Price for proofreading the manuscript.

References

Baker KR, Jana B, Hansen AM, Nielsen HM, Franzyk H, Guardabassi L. 2019. Repurposing Azithromycin and Rifampicin Against Gram-Negative Pathogens by Combination With Peptidomimetics. *Frontiers in Cellular and Infection Microbiology* 9:236. DOI: 10.3389/fcimb.2019.00236.

Berenbaum MC. 1978. A Method for Testing for Synergy with Any Number of Agents. *The Journal of Infectious Diseases* 137:122–130. DOI: 10.1093/infdis/137.2.122.

Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. 2009. Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America. *Clinical Infectious Diseases* 48:1–12. DOI: 10.1086/595011.

Braunstein A, Papo N, Shai Y. 2004. In Vitro Activity and Potency of an Intravenously Injected Antimicrobial Peptide and Its DL Amino Acid Analog in Mice Infected with Bacteria. *Antimicrobial Agents and Chemotherapy* 48:3127–3129. DOI: 10.1128/AAC.48.8.3127-3129.2004.

348 Caldeira E, Piskin E, Granadeiro L, Silva F, Gouveia IC. 2013. Biofunctionalization of cellulosic fibres
349 with l-cysteine: Assessment of antibacterial properties and mechanism of action against
350 Staphylococcus aureus and Klebsiella pneumoniae. *Journal of Biotechnology* 168:426–435. DOI:
351 10.1016/j.jbiotec.2013.10.021.
352 Chen H-L, Su P-Y, Kuo S-C, Lauderdale T-LY, Shih C. 2018. Adding a C-terminal Cysteine (CTC) Can
353 Enhance the Bactericidal Activity of Three Different Antimicrobial Peptides. *Frontiers in*
354 *Microbiology* 9:1440. DOI: 10.3389/fmicb.2018.01440.
355 Choi H, Hwang J-S, Kim H, Lee DG. 1993. Antifungal effect of CopA3 monomer peptide via membrane-
356 active mechanism and stability to proteolysis of enantiomeric d-CopA3. *Biochemical and*
357 *Biophysical Research Communications* 440:94–98. DOI: 10.1016/j.bbrc.2013.09.021.
358 Gordon YJ, Romanowski EG, McDermott AM. 2005. A Review of Antimicrobial Peptides and Their
359 Therapeutic Potential as Anti-Infective Drugs. *Current Eye Research* 30:505–515. DOI:
360 10.1080/02713680590968637.
361 Gouveia IC, Sá D, Henriques M. 2012. Functionalization of wool with L-cysteine: Process
362 characterization and assessment of antimicrobial activity and cytotoxicity. *Journal of Applied*
363 *Polymer Science* 124:1352–1358. DOI: 10.1002/app.34587.
364 Hancock REW, Lehrer R. 1998. Cationic peptides: a new source of antibiotics. *Trends in Biotechnology*
365 16:82–88. DOI: 10.1016/S0167-7799(97)01156-6.
366 Javadpour MM, Juban MM, Lo W-CJ, Bishop SM, Alberty JB, Cowell SM, Becker CL, McLaughlin ML.
367 1996. De Novo Antimicrobial Peptides with Low Mammalian Cell Toxicity. *Journal of*
368 *Medicinal Chemistry* 39:3107–3113. DOI: 10.1021/jm9509410.
369 Koshiba T, Hashii T, Kawabata S -i. 2006. A Structural Perspective on the Interaction between
370 Lipopolysaccharide and Factor C, a Receptor Involved in Recognition of Gram-negative Bacteria.
371 *Journal of Biological Chemistry* 282:3962–3967. DOI: 10.1074/jbc.M609198200.

372 Le C-F, Yusof MYM, Hassan H, Sekaran SD. 2015. In vitro properties of designed antimicrobial peptides
373 that exhibit potent antipneumococcal activity and produces synergism in combination with
374 penicillin. *Scientific Reports* 5:9761. DOI: 10.1038/srep09761.
375 Lee J, Lee DG. 2008. Structure-antimicrobial activity relationship between pleurocidin and its
376 enantiomer. *Experimental and Molecular Medicine* 40:370. DOI: 10.3858/emm.2008.40.4.370.
377 Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F,
378 Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, Shen J. 2016. Emergence of
379 plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a
380 microbiological and molecular biological study. *The Lancet Infectious Diseases* 16:161–168.
381 DOI: 10.1016/S1473-3099(15)00424-7.
382 Luther A, Urfer M, Zahn M, Müller M, Wang S-Y, Mondal M, Vitale A, Hartmann J-B, Sharpe T, Monte
383 FL, Kocherla H, Cline E, Pessi G, Rath P, Modaresi SM, Chiquet P, Stiegeler S, Verbree C,
384 Remus T, Schmitt M, Kolopp C, Westwood M-A, Desjonquères N, Brabet E, Hell S, LePoupon
385 K, Vermeulen A, Jaisson R, Rithié V, Upert G, Lederer A, Zbinden P, Wach A, Moehle K, Zerbe
386 K, Locher HH, Bernardini F, Dale GE, Eberl L, Wollscheid B, Hiller S, Robinson JA, Obrecht D.
387 2019. Chimeric peptidomimetic antibiotics against Gram-negative bacteria. *Nature* 576:452–458.
388 DOI: 10.1038/s41586-019-1665-6.
389 Maróti G, Downie JA, Kondorosi É. 2015. Plant cysteine-rich peptides that inhibit pathogen growth and
390 control rhizobial differentiation in legume nodules. *Current Opinion in Plant Biology* 26:57–63.
391 DOI: 10.1016/j.pbi.2015.05.031.
392 Marr A, Gooderham W, Hancock R. 2006. Antibacterial peptides for therapeutic use: obstacles and
393 realistic outlook. *Current Opinion in Pharmacology* 6:468–472. DOI:
394 10.1016/j.coph.2006.04.006.
395 Matsuzaki K, Murase O, Fujii N, Miyajima K. 1995. Translocation of a channel-forming antimicrobial
396 peptide, magainin 2, across lipid bilayers by forming a pore. *Biochemistry* 34:6521–6526. DOI:
397 10.1021/bi00019a033.

McGrath DM, Barbu EM, Driessen WHP, Lasco TM, Tarrand JJ, Okhuysen PC, Kontoyiannis DP, Sidman RL, Pasqualini R, Arap W. 2013. Mechanism of action and initial evaluation of a membrane active all-D-enantiomer antimicrobial peptidomimetic. *Proceedings of the National Academy of Sciences* 110:3477–3482. DOI: 10.1073/pnas.1221924110.

Miyoshi-Akiyama T, Kuwahara T, Tada T, Kitao T, Kirikae T. 2011. Complete Genome Sequence of Highly Multidrug-Resistant Pseudomonas aeruginosa NCGM2.S1, a Representative Strain of a Cluster Endemic to Japan. *Journal of Bacteriology* 193:7010–7010. DOI: 10.1128/JB.06312-11.

Mourtada R, Herce HD, Yin DJ, Moroco JA, Wales TE, Engen JR, Walensky LD. 2019. Design of stapled antimicrobial peptides that are stable, nontoxic and kill antibiotic-resistant bacteria in mice. *Nature Biotechnology* 37:1186–1197. DOI: 10.1038/s41587-019-0222-z.

Odeberg J, Wirsén A, Norberg Å, Frie J, Printz G, Lagercrantz H, Gudmundsson GH, Agerberth B, Jonsson B. 2018. A novel cysteine-linked antibacterial surface coating significantly inhibits bacterial colonization of nasal silicone prongs in a phase one pre-clinical trial. *Materials Science and Engineering: C* 93:782–789. DOI: 10.1016/j.msec.2018.08.040.

Onuchic JN, Jennings PA, Ben-Jacob E. 2013. Learning from cancer how to defeat bacteria. *Proceedings of the National Academy of Sciences* 110:3212–3213. DOI: 10.1073/pnas.1222882110.

Pal I, Bhattacharyya D, Kar RK, Zarena D, Bhunia A, Atreya HS. 2019. A Peptide-Nanoparticle System with Improved Efficacy against Multidrug Resistant Bacteria. *Scientific Reports* 9:4485. DOI: 10.1038/s41598-019-41005-7.

Paterson DL, Harris PNA. 2016. Colistin resistance: a major breach in our last line of defence. *The Lancet Infectious Diseases* 16:132–133. DOI: 10.1016/S1473-3099(15)00463-6.

Peters BM, Shirtliff ME, Jabra-Rizk MA. 2010. Antimicrobial Peptides: Primeval Molecules or Future Drugs? *PLoS Pathogens* 6:e1001067. DOI: 10.1371/journal.ppat.1001067.

Plesniak LA, Parducho JI, Ziebart A, Geierstanger BH, Whiles JA, Melacini G, Jennings PA. 2004. Orientation and helical conformation of a tissue-specific hunter-killer peptide in micelles. *Protein Science* 13:1988–1996. DOI: 10.1110/ps.04853204.

Poole K, Tetro K, Zhao Q, Neshat S, Heinrichs DE, Bianco N. 1996. Expression of the multidrug resistance operon mexA-mexB-oprM in Pseudomonas aeruginosa: mexR encodes a regulator of operon expression. *Antimicrobial Agents and Chemotherapy* 40:2021–2028. DOI: 10.1128/AAC.40.9.2021.

Reading C, Cole M. 1977. Clavulanic Acid: a Beta-Lactamase-Inhibiting Beta-Lactam from Streptomyces clavuligerus. *Antimicrobial Agents and Chemotherapy* 11:852–857. DOI: 10.1128/AAC.11.5.852.

Shai Y. 2002.From Innate Immunity to de-Novo Designed Antimicrobial Peptides. *Available at https://www.ingentaconnect.com/content/ben/cpd/2002/00000008/00000009/art00005* (accessed June 24, 2019). DOI: info:doi/10.2174/1381612023395367.

Sivakamavalli J, Nirosha R, Vaseeharan B. 2015. Purification and Characterization of a Cysteine-Rich 14-kDa Antibacterial Peptide from the Granular Hemocytes of Mangrove Crab Episesarma tetragonum and Its Antibiofilm Activity. *Applied Biochemistry and Biotechnology* 176:1084–1101. DOI: 10.1007/s12010-015-1631-1.

Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, Pulcini C, Kahlmeter G, Kluytmans J, Carmeli Y, Ouellette M, Outterson K, Patel J, Cavaleri M, Cox EM, Houchens CR, Grayson ML, Hansen P, Singh N, Theuretzbacher U, Magrini N, Aboderin AO, Al-Abri SS, Awang Jalil N, Benzonana N, Bhattacharya S, Brink AJ, Burkert FR, Cars O, Cornaglia G, Dyar OJ, Friedrich AW, Gales AC, Gandra S, Giske CG, Goff DA, Goossens H, Gottlieb T, Guzman Blanco M, Hryniewicz W, Kattula D, Jinks T, Kanj SS, Kerr L, Kieny M-P, Kim YS, Kozlov RS, Labarca J, Laxminarayan R, Leder K, Leibovici L, Levy-Hara G, Littman J, Malhotra-Kumar S, Manchanda V, Moja L, Ndoye B, Pan A, Paterson DL, Paul M, Qiu H, Ramon-Pardo P, Rodríguez-Baño J, Sanguinetti M, Sengupta S, Sharland M, Si-Mehand M, Silver LL, Song W, Steinbakk M, Thomsen J, Thwaites GE, van der Meer JW, Van Kinh N, Vega S, Villegas MV, Wechsler-Fördös A, Wertheim HFL, Wesangula E, Woodford N, Yilmaz FO, Zorzet A. 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-

450 resistant bacteria and tuberculosis. *The Lancet Infectious Diseases* 18:318–327. DOI:
451 10.1016/S1473-3099(17)30753-3.
452 Tada T, Miyoshi-Akiyama T, Shimada K, Nga TTT, Thu LTA, Son NT, Ohmagari N, Kirikae T. 2015.
453 Dissemination of clonal complex 2 Acinetobacter baumannii strains co-producing
454 carbapenemases and 16S rRNA methylase ArmA in Vietnam. *BMC Infectious Diseases* 15:433.
455 DOI: 10.1186/s12879-015-1171-x.
456 Tsai Y-C, Tang C-C, Wu H-H, Wang Y-S, Chen Y-F. 2020. Antibacterial Activity of Cysteine-Derived
457 Cationic Dipeptides. *International Journal of Peptide Research and Therapeutics* 26:1107–1114.
458 DOI: 10.1007/s10989-019-09913-4.
459 Uchida H, Tada T, Sugahara Y, Kato A, Miyairi I, Kirikae T. 2018. A clinical isolate of Escherichia coli
460 co-harbouring mcr-1 and bla NDM-5 in Japan. *Journal of Medical Microbiology* 67:1047–1049.
461 DOI: 10.1099/jmm.0.000793.
462 Wang H, Qian J, Gu J, Yan W, Zhang J. 2019. Steric configuration-enabled selective antimicrobial
463 activity of chiral cysteine. *Biochemical and Biophysical Research Communications* 512:505–510.
464 DOI: 10.1016/j.bbrc.2019.03.080.
465 Weinstein MP. 2018. *M100-performance standards for antimicrobial susceptibility testing, 28th edition.*
466 Place of publication not identified: Clinical And Laboratory.
467 Xu Q, Gu J, Zhao Y, Ke X, Liu X. 2017. Antibacterial cotton fabric with enhanced durability prepared
468 using L-cysteine and silver nanoparticles. *Fibers and Polymers* 18:2204–2211. DOI:
469 10.1007/s12221-017-7567-z.
470 Yoneyama H, Ocaktan A, Tsuda M, Nakae T. 1997. The Role of mex-Gene Products in Antibiotic
471 Extrusion in Pseudomonas aeruginosa. *Biochemical and Biophysical Research Communications*
472 233:8. DOI: 10.1006/bbrc.1997.6506.

473
474
475
476

477
478
479

Table 1(on next page)

MICs of antibiotics and antimicrobial peptides against *Pseudomonas aeruginosa* strains.

The Mann-Whitney U test was used to compare the MIC values of C-DP, DP-C, and DP-C dimers with DP in *P. aeruginosa*. P-values less than 0.05 were considered statistically significant (*$p < 0.05$).

1

Strains of *P. aeruginosa*[a]	MIC (μg/mL)							
	Antibiotics				Antimicrobial peptides			
	Amikacin	Colistin	Meropenem	Ofloxacin	DP	C-DP*	DP-C*	DP-C Dimer*[b]
PAO-1	4	1	2	0.5	300	64	16	16
NCGM2.S1	128	1	>512	64	300	128	32	32
OCR1	8	1	8	2	>300	128	16	16
PAO4290	4	1	1	1	300	128	16	16

2

3 [a] *P. aeruginosa* strains used in this study were wild type PAO-1 (Weinstein, 2018), the MDR

4 clinical strain NCGM2.S1 (Miyoshi-Akiyama *et al*., 2011), the OprM overexpressing mutant

5 OCR1 (Poole *et al*., 1996) and PAO4290 (Yoneyama *et al*., 1997) which expressed a wild-type

6 level of MexAB-OprM.

7 [b] Generated by heating DP-C at 60 °C for 30 minutes to convert cysteine to cystine.

8

Table 2(on next page)

MICs of antimicrobial peptides against strains of bacteria.

The Mann-Whitney U test was used to compare the MIC values of C-DP and DP-C with DP in species. P-values less than 0.05 were considered statistically significant (*$p < 0.05$).

1

Strains[a]	MIC (μg/ml)			Genes or mutations associated with drug resistance		
	DP	C-DP	DP-C	β-lactamase(s)	16S rRNA methylase	colistin-resistance gene
Acinetobacter baumannii*						
ATCC15308	300	8	8			
IOMTU433	64	4	8	$bla_{\text{NDM -1}}$, $bla_{\text{OXA -23}}$, $bla_{\text{PER -7}}$		
NCGM237	128	4	8	$bla_{\text{OXA-23}}$	*armA*	
NCGM253	128	4	8	$bla_{\text{OXA-72}}$		
Escherichia coli						
ATCC 25922	>300	16	8			
NCCHD1261-5	64	4	8	$bla_{\text{NDM-5}}$		*mcr-1*
Klebsiella pneumoniae						
ATCC 15380	>128	>128	128			
ATCC BAA-2146	>128	16	16	$bla_{\text{NDM-1}}$		
Serratia marcescens						
NBRC102204	>256	>256	>256			
Staphylococcus aureus						
ATCC 25923	>128	>128	>128			

2 [a] *A. baumannii* strains were wild-type strain ATCC 15308 and multi-drug resistant strains
3 IOMTU433 (Tada *et al*., 2015) (GenBank accession no. AP014649), NCGM237 (Tada *et al*.,
4 2015) (GenBank accession no. AP013357) and NCGM253 (Tada *et al*., 2015) (GenBank accession
5 no. AB823544). *E. coli* strains were wild-type strain ATCC25922 and multi-drug resistant strain
6 NCCHD1261-5 (Uchida *et al*., 2018). *K. pneumonia*e strains were multidrug-resistant strain
7 ATCC15380 (Reading & Cole, 1977) and the penicillin resistant strain ATCC-BAA-2146, a
8 resistance caused by the production of β-lactamase. The *S. marcescens* strain NBRC102204 and
9 the *S. aureus* strain ATCC 25923 were wild-type strain.

Table 3(on next page)

FIC index of combinations of antibiotics and antimicrobial peptide against *P. aeruginosa*.

The synergistic effects of DP, C-DP or DP-C and antibiotics against *P. aeruginosa* NCGM2.S1 were analyzed by the checkerboard dilution method and the FIC index for each combination was calculated.

1

Combination	FIC index[a]	Interpretation
DP-C + Amikacin	1.5	Additive/ Indifference
DP + Colistin	**0.31**	**Synergy**
C-DP + Colistin	**0.38**	**Synergy**
DP-C + Colistin	**0.38**	**Synergy**
DP-C + Meropenem	0.67	Additive/ Indifference
DP-C + Ofloxacin	0.75	Additive/ Indifference
DP-C + Rifampicin	0.56	Additive/ Indifference

2

3 [a] FIC ≤0.5, synergistic; 0.5< FIC ≤4.0, additive or unrelated; FIC >4.0: antagonistic.

4

5

Figure 1

Cytotoxicity of colistin and antimicrobial peptides against HepG2 cells. HepG2 cells.

HepG2 cells were seeded at 3,000 cells/well in 96-well microtiter plates. After incubation for 48 h, DP-C and colistin were added and incubated for an additional 48 h. Cell viability was determined using a Cell Counting Kit-8, with colorimetric changes assessed at OD_{450} nm with a microplate reader.