A new class of antimicrobial therapeutics targeting the envelope stress response of Gram-negative bacteria: abridged secondary publication

SW Tang, SH Kwok, X Li, KH Tang, JA Kubi, AS Brah, K Yeung, M Dong, YW Lam *

KEY MESSAGES

1. BING is a novel amphipathic peptide with broad-spectrum antibacterial activity. Genome-wide transcriptomic analysis suggests that BING represents a new class of antibiotics.
2. BING suppresses the expression of genes involved in flagellar biosynthesis and chemotaxis, thereby inhibiting motility, in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium.
3. BING has synergistic effects with ampicillin, amoxicillin, and novobiocin in *E. coli* and *Pseudomonas aeruginosa*; it can suppress ampicillin resistance in *P. aeruginosa*.
4. Amidation and D-amino acid substitution can stabilise BING against serum degradation and heat inactivation.
5. BING and its derivatives are non-haemolytic and exhibit low in vivo toxicity.

Hong Kong Med J 2023;29(Suppl 4):S39-44
HMRF project number: 18170572

[1] SW Tang, [1] SH Kwok, [1] X Li, [1] KH Tang, [2] JA Kubi, [2] AS Brah, [2] K Yeung, [1] M Dong, [3] YW Lam

[1] *Department of Chemistry, City University of Hong Kong, Hong Kong SAR, China*
[2] *Department of Orthopaedics and Traumatology, The University of Hong Kong, Hong Kong SAR, China*
[3] *School of Applied Science, University of Huddersfield, United Kingdom*

* Principal applicant and corresponding author: y.lam@hud.ac.uk

Introduction

BING is a novel antimicrobial peptide (AMP) discovered by proteomic characterisation of plasma peptides in medaka fish.[1] This 13-mer amphipathic peptide displays broad-spectrum toxicity against pathogenic bacteria—including drug-resistant strains—at concentrations that are relatively non-toxic to mammalian cell lines. BING suppresses the expression of *cpxR*,[1] an upstream regulator of envelope stress responses that plays important roles in antimicrobial resistance (eg, by regulating drug efflux genes). In this study, we investigated the effects of BING-mediated *cpxR* downregulation by performing genome-wide transcriptomic analysis of BING-treated bacteria. We tested the combined toxicities of BING and other classes of antibiotics, mixed at various ratios, against pathogenic bacteria; we also investigated the effects of sublethal doses of BING on the generation of antibiotic resistance in serial passage experiments. Furthermore, we performed lead optimisation by introducing C-terminal amidation and D-amino acid substitution, then analysing how these modifications influenced antibacterial activity and stability at high temperature and in animal serum. Finally, to facilitate future clinical applications of BING, we conducted haemolytic assays and in vivo toxicity tests.

Methods

Detailed procedures for bacterial culture, determination of minimum inhibitory concentration (MIC), checkerboard experiments, and assessment of antibiotic resistance are described in our previous study.[1] RNA samples from BING-treated cultures and untreated control cultures (n=3 for each treatment) were isolated and reverse-transcribed as described. cDNA samples were analysed using an Illumina PE150 sequencer. To measure motility, we inoculated *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) on 0.3% soft swimming nutrient broth agar, then recorded colony diameters after incubation for 24 hours. Bacterial swimming behaviour was recorded at a rate of 10 frames per second. For assessments of haemolytic activity, human or mouse red blood cells were incubated with AMPs and antibiotics for 1 hour at 37°C, then centrifuged. The optical density of each supernatant was measured at 540 nm. For analyses of acute toxicity, a standard up-and-down procedure (Organisation for Economic Co-operation and Development test guideline 425) was performed on adult male C57BL/6 mice, using a starting dose of 62.5 mg CD-BING per 1 kg body weight.

Results

BING downregulates flagellar biosynthesis and affects bacterial motility

We conducted genome-wide RNA-Seq analysis of *E. coli* that had been treated with BING at the MIC for 60 minutes. We selected this treatment

time to observe initial responses to BING without interference from secondary effects (eg, cell stress or death). We found that BING induced differential expression (fold change >2, P<0.05) of 402 genes. Compared with the gene expression profiles of *E. coli* that had been separately treated with 37 antibiotics, representing the six classes of antibiotics currently in use,[2] transcriptomic changes induced by BING were highly unique (unpublished data). These findings suggest that the antibacterial mechanism of BING may be distinct from the mechanisms exhibited by other cationic AMPs. Consistent with this speculation, we did not detect any disruptive effects of BING on membrane integrity (Fig 1).

Additionally, BING caused widespread downregulation of flagellar and chemotactic genes. Reverse transcription polymerase chain reaction analysis confirmed that levels of *fliA* and *motA* were dramatically reduced in *E. coli* and *S.* Typhimurium after BING treatment; ampicillin treatment did not affect these genes (data not shown). We tested the effects of BING on motility in *E. coli* and *S.* Typhimurium by measuring surface colonisation rates and swimming trajectories; even at a sublethal dose (0.5× MIC), BING reduced bacterial motility to quasi-Brownian motion. Ampicillin did not significantly affect bacterial motility (unpublished data).

Structure of BING

Next, we investigated the structure of BING by circular dichroism spectroscopy (Fig 1). Because BING presumably exerts its effects through membrane interactions, we performed circular dichroism spectroscopy in hydrophobic environments. In 50% trifluoroethanol, BING displayed prominent changes in mean residue ellipticity at 191 and 215 nm. Deconvolution analysis revealed substantial β-sheet composition. Similar phenomena were observed in a membrane-mimetic condition containing sodium dodecyl sulphate. Structural modelling by PEP-FOLD42 indicated that a hydrophilic patch was formed by Arg-2, Arg-6, and Lys-12, whereas a hydrophobic patch was formed by Ile-3, Ala-9, Leu-10, and Ile-13 on the opposite surface; Glu-8 was located at the 'hinge' (Fig 1).

BING suppresses drug efflux expression and acts synergistically with antibiotics

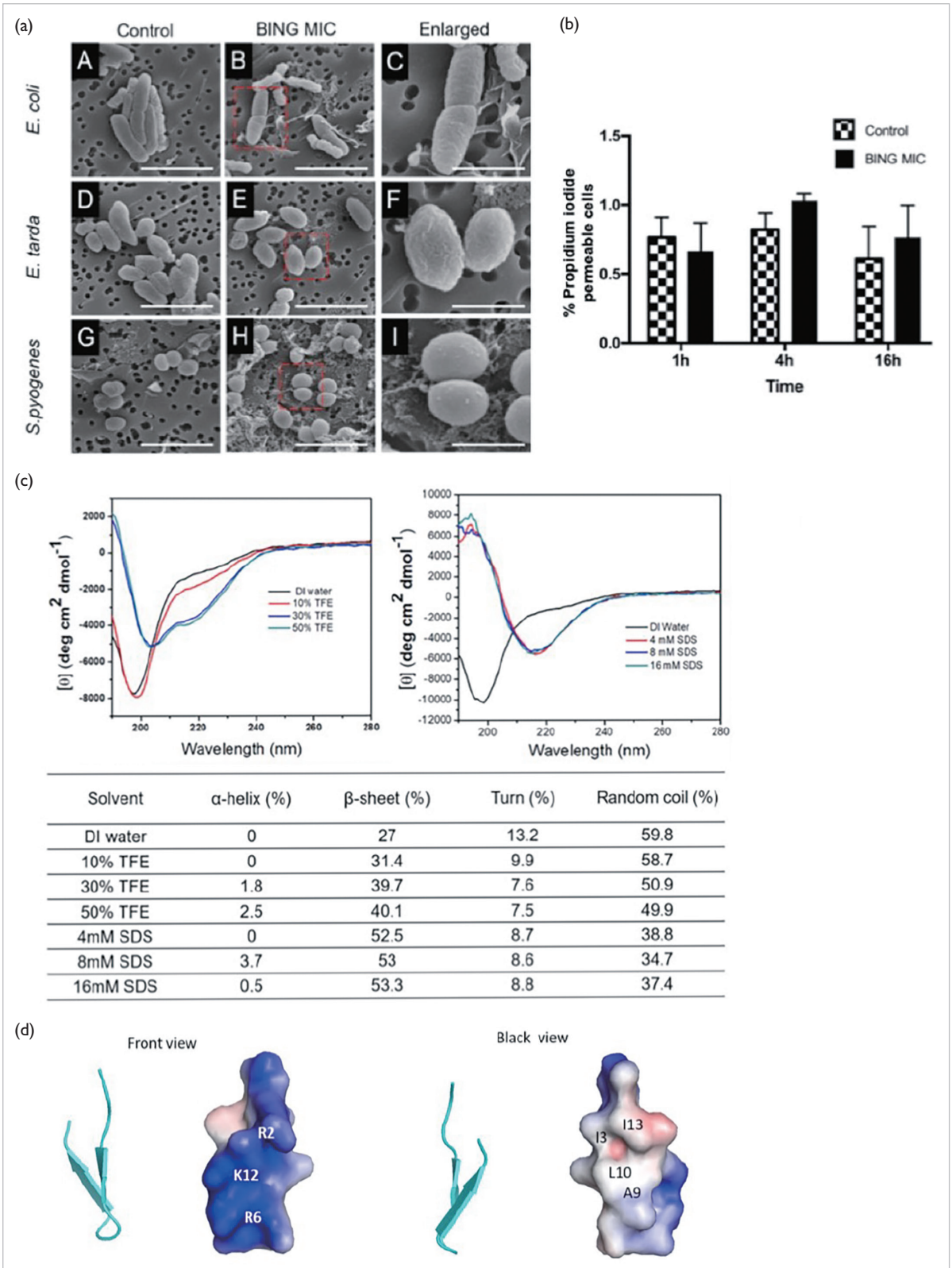
BING downregulates the RNA level of *cpxR*, a gene known to regulate bacterial drug efflux, in *P. aeruginosa* (Fig 2) and other Gram-negative bacteria (data not shown). We tested whether BING-induced downregulation of *cpxR* could alter the expression of efflux pump components. In *P. aeruginosa*, the expression levels of the transporter genes *mexB*, *mexY*, and *oprM* were significantly reduced after exposure to BING. In contrast, ampicillin, which upregulates *cpxR* (data not shown), enhanced the expression of these genes. Next, we investigated whether BING could sensitise bacteria to antibiotic toxicity, using two-dimensional checkerboard experiments to determine the combined effects of BING and antibiotics (Fig 2). The presence of BING significantly enhanced the effects of ampicillin (fractional inhibitory concentration index [FICI]=0.4), amoxicillin (FICI=0.39), and novobiocin (FICI=0.16). A synergistic effect of BING and ampicillin was also observed in *P. aeruginosa* that had previously been selected for ampicillin resistance (FICI=0.42), suggesting that BING can restore antibiotic sensitivity in drug-resistant bacteria.

Subsequently, we examined whether BING could attenuate the development of antimicrobial resistance. *E. coli* were passaged daily with increasing titres of kanamycin or ampicillin for 7 days, resulting in an increase in the kanamycin MIC by ≥8-fold and an increase in the ampicillin MIC by ≥4-fold (Fig 2). In the presence of a sublethal dose of BING (3.9 μg/mL) for 7 days, increases in the MICs of both antibiotics were limited, suggesting that exposure to BING can delay the development of antimicrobial resistance. The deletion of *cpxR* also led to a similar delayed onset of ampicillin resistance in *E. coli* even in the absence of BING, consistent with the notion that the effects of BING on antibiotic resistance are mediated by downregulation of *cpxR*. Similar results were observed in the development of resistance to ampicillin, meropenem, and imipenem in *P. aeruginosa*. Importantly, meropenem and imipenem belong to the carbapenem class of antibiotics; they are among the drugs of last resort.

In vivo toxicity of BING

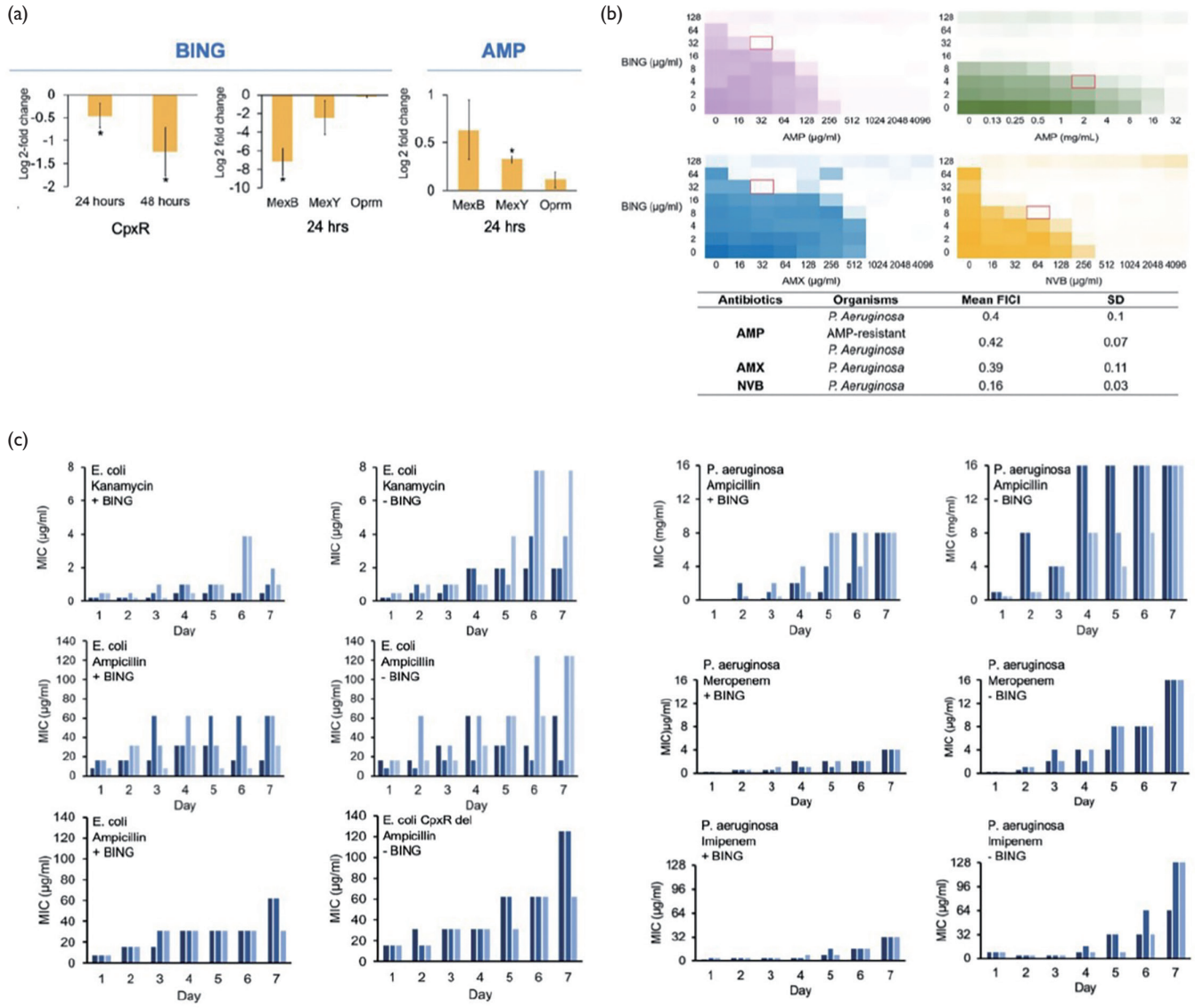
C-terminus amidation (C-BING), complete D-amino acid substitution (D-BING), and double modification (CD-BING) of BING increased its stability without compromising its antibacterial activity (Fig 3). We tested the potential haemolytic effects of BING and CD-BING. Neither peptide caused measurable haemolysis even at 200 mg/L, which is an order of magnitude greater than the MIC of each peptide for most pathogenic bacteria. Melittin, another AMP, induced significant haemolysis.

We then performed acute toxicity testing of CD-BING by intraperitoneal injection into adult mice. To reduce the use of experimental animals, we did not perform the test on BING; thus far, our data indicate that CD-BING is a more promising candidate for clinical translation. We used a standard up-and-down procedure (Organisation for Economic Co-operation and Development test guideline 425), which further minimised the number of animals required. At a dose of <200 mg/kg body weight, CD-BING did not cause any observable adverse effect for up to 5 days (data not shown). At 650 mg/kg



Solvent	α-helix (%)	β-sheet (%)	Turn (%)	Random coil (%)
DI water	0	27	13.2	59.8
10% TFE	0	31.4	9.9	58.7
30% TFE	1.8	39.7	7.6	50.9
50% TFE	2.5	40.1	7.5	49.9
4mM SDS	0	52.5	8.7	38.8
8mM SDS	3.7	53	8.6	34.7
16mM SDS	0.5	53.3	8.8	37.4

FIG 1. (a) Scanning electron micrographs of *Escherichia coli*, *Edwardsiella tarda*, and *Streptococcus pyogenes* incubated with 7.8, 10, and 50 μg/mL of BING or culture medium for 1 hour. (b) Percentages of unfixed bacterial cells labelled with propidium iodide after treatment with or without BING (at minimum inhibitory concentration of 4 ug/mL) for 1, 4, and 16 hours. (c) Secondary structure analysis of BING peptide by far-ultraviolet circular dichroism spectroscopy. The circular dichroism spectra of BING peptide were measured in aqueous solution with increasing concentrations of trifluoroethanol (TFE) and sodium dodecyl sulphate (SDS). The relative contents of secondary structure elements are presented as mean residue ellipticity (Θ). (d) Ribbon diagram of the BING peptide three-dimensional model and electrostatic potential mapped onto the accessible surface area of the model structure. Positive electrostatic surfaces are shown in blue and negative electrostatic surfaces are shown in red; all surfaces are drawn at ±3 kT/e.



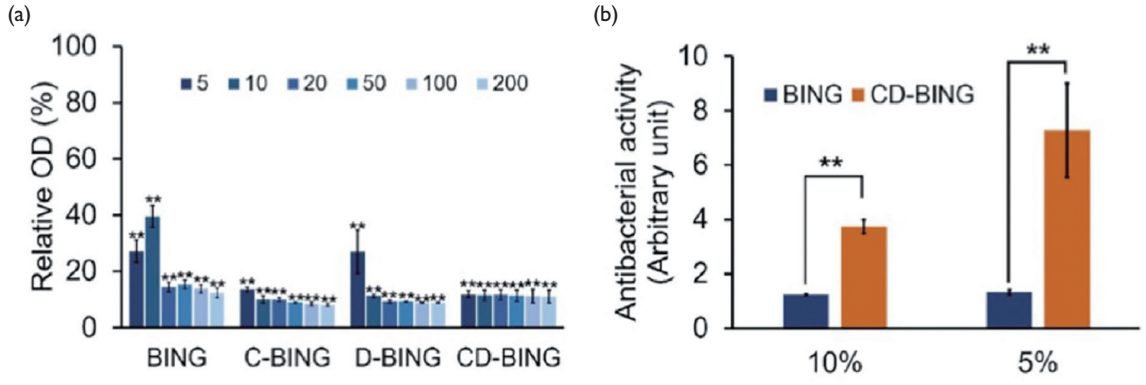
Antibiotics	Organisms	Mean FICI	SD
AMP	*P. Aeruginosa*	0.4	0.1
	AMP-resistant *P. Aeruginosa*	0.42	0.07
AMX	*P. Aeruginosa*	0.39	0.11
NVB	*P. Aeruginosa*	0.16	0.03

FIG 2. (a) *cpxR* gene expression in *Pseudomonas aeruginosa* treated with BING at 25 μg/mL for 24 hours and 48 hours, respectively. Expression levels of *mexB*, *mexY*, and *oprM* in *P. aeruginosa* after treatment with BING (25 μg/mL) and ampicillin (25 μg/mL) for 48 hours, respectively. (b) Combined effects of BING and antibiotics: checkerboard assays on the growth of *P. aeruginosa* or ampicillin-resistant *P. aeruginosa* treated with BING, ampicillin (AMP), amoxicillin (AMX), or novobiocin (NVB). Fractional inhibitory concentration indices (FICIs) of all checkerboards are shown. (c) Effect of BING on the development of antibiotic resistance in *Escherichia coli* and *P. aeruginosa*, which were cultured in increasing concentrations of antibiotics; cells that survived the highest antibiotic concentration were passaged daily for 7 days. Minimum inhibitory concentrations (MICs) of the selected cells on each day are shown.

body weight, CD-BING caused significant weight loss; lethality was observed at 2000 mg/kg body weight. Calculations using the Uniform Appraisal Dataset guideline indicated that the median lethal dose of CD-BING was 1200 mg/kg body weight (unpublished data).

Discussion

Antibiotic-resistant bacteria are generally sensitive to AMPs,[3] which makes AMPs promising candidates for combined antibiotic therapy. Similar to most known AMPs, BING is cationic with ~50% hydrophobic residues. This charge distribution presumably AMPs to disrupt bacterial membranes. However, our data suggest that BING inhibits bacterial growth through a different mechanism. First, BING did not cause detectable permeabilisation of bacterial membranes. Second, amino acid substitution experiments showed that the antibacterial activity of BING could be lost despite maintenance of its charge and hydrophobicity. The amide side chain at position



(c)

T(°C) \ t (hours)	Antimicrobial activity MIC (mg/L) BING 0.5	BING 3	BING 24	CD-BING 0.5	CD-BING 3	CD-BING 24
25	5	5	5	2.5	2.5	2.5
37	5	5	5	2.5	2.5	2.5
90	5	5	5	2.5	2.5	2.5

FIG 3. CD-BING is a stabilised form of BING with relatively low in vivo toxicity. (a) Relative viability (optical density [OD]) of *Escherichia coli* treated with BING, C-amidated BING (C-BING), D-isomer substituted BING (D-BING), and both C-amidated and D-isomer substituted BING (CD-BING) for 16 hours. (b) Minimum inhibitory concentrations (MICs) of BING and CD-BING against *E. coli* in the presence of 5% and 10% fetal bovine serum. (c) MICs of BING and CD-BING pre-incubated at different temperatures for 0.5, 3, and 24 hours (n=3).

8 is required for the antibacterial effect of BING. This degree of sequence dependence suggests that BING exerts its antibacterial effect through interactions with intracellular molecules in a specific ligand-targeting manner.

Our RNA-Seq data indicated that BING causes significant suppression of flagellar and chemotactic gene expression, resulting in bacterial immobilisation. This is an interesting observation because motility and chemotaxis are associated with antibiotic evasion and pathogenicity.

We showed that C-terminus amidation and D-amino acid substitution, modifications known to increase peptide stability in vivo, could increase BING potency and its stability in animal serum. Importantly, BING and CD-BING were extremely thermostable, maintaining their MIC even after 24 hours at 90°C. The stability of BING may be related to its existence in fish blood where it is exposed to serum protease activities and changing environmental conditions. Our data from haemolysis assays and in vivo toxicity tests showed that CD-BING has relatively low toxicity, consistent with our cytotoxicity findings.[1] To our knowledge, CD-BING has much lower acute toxicity in mice, compared with any other AMP for which data are available. Overall, our findings indicate that CD-BING has potential for clinical translation. Future investigations will explore the use of CD-BING as a novel antibacterial agent in animal models.

Funding

This study was supported by the Health and Medical Research Fund, Health Bureau, Hong Kong SAR Government (#18170572). The full report is available from the Health and Medical Research Fund website (https://rfs2.healthbureau.gov.hk).

Disclosure

The results of this research have been previously published in:

1. Dong M, Kwok SH, Humble JL, et al. BING, a novel antimicrobial peptide isolated from Japanese medaka plasma, targets bacterial envelope stress response by suppressing cpxR expression. Sci Rep 2021;11:12219.

References

1. Dong M, Kwok SH, Humble JL, et al. BING, a novel antimicrobial peptide isolated from Japanese medaka plasma, targets bacterial envelope stress response by

suppressing cpxR expression. Sc Rep 2021;11:12219.
2. O'Rourke A, Beyhan S, Choi Y, et al. Mechanism-of-action classification of antibiotics by global transcriptome profiling. Antimicrob Agents Chemother 2020;64:e01207-19.
3. Lázár V, Martins A, Spohn R, et al. Antibiotic-resistant bacteria show widespread collateral sensitivity to antimicrobial peptides. Nat Microbiol 2018;3:718-31.