

CGD-1, a defensin-related peptide derived from marine Chinese medicine *Ostreae concha*, inhibits the Gram-negative bacteria by membrane attack

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Author contributions

Chun-Lei Li, and Xue-Kui Xia conceived and designed the experiments. Wei-Tao Wang, Qing-Zhou Meng and Long-Fen Li performed all of the experiments. Wei-Yi Meng, Xin Yin, Meng-Meng Wang, Jing-Li Ren and Pei-Pei Zhao analyzed the data. Chun-Lei Li, Xue-Kui Xia, and Wei-Tao Wang wrote the manuscript. Chun-Lei Li revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

LB, Luria-Bertani; PI, Propidium iodide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; ONPG, o-nitrophenyl-β-D-galactopyranoside; NPN, N-phenyl-1-naphthylamine; MIC, minimum inhibitory concentration; SEM, scanning electron microscope; CD, circular dichroism; TFE, Trifluoroethanol.

Citation

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Abstract

There is an increasing interest in discovering new antibacterial agents derived from nature to enhance the treatment of various bacterial infections. Defensins and their derived peptide fragments exhibit significant antibacterial activity without any cytotoxic effects, making them attractive features for potential novel antibacterial therapeutics. *Crassostrea gigas*, a traditional seafood that has been used worldwide for centuries, has its shells applied in Chinese medicine as *Ostreae concha*. In this study, bioinformatics analysis was used to obtain a novel antibacterial peptide, CGD-1, derived from marine Chinese medicine *Ostreae concha*. The physicochemical characterization and circular dichroism analysis results demonstrated that CGD-1 assembled into an α-helical structure in a simulated membrane environment, and it displayed antibacterial action against Gram-negative bacteria. The minimal inhibitory concentrations against both *Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922 were 25 μM. CGD-1 was able to efficiently permeate the cell membrane. Changes in bacterial cell morphology were evaluated using a field emission scanning electron microscope. The results suggested that CGD-1 exerted its antibacterial activity through permeabilizing and disrupting the bacterial cell membrane. Therefore, CGD-1 may have potential applications in fighting against pathogenic bacteria such as *P. aeruginosa* and *E. coli*.

Keywords: defensin; antibacterial activity; hemolymph; *Crassostrea gigas*; *Ostreae concha*

Introduction

The use of antibiotics is leading to an increase in drug-resistant bacteria worldwide, which is compromising the effectiveness and availability of antibiotics. The efficacy of classical antibiotics is currently under severe pressure from antibacterial drug resistance [1]. After several decades of successful antibiotic treatment, bacterial infections have once again become a threat [2]. In recent years, Gram-negative bacterial infections have received more attention due to the unpredictable and escalating development of bacterial resistance to these infections, with new resistance mechanisms emerging and spreading globally [3]. These organisms employ multiple mechanisms against antibiotics, such as loss of porins and overexpression of transmembrane efflux pumps, by mutating or up-regulating genes that code for enabling antibiotic resistance [4]. The World Health Organization has published a list of the most hazardous superbugs with the highest priority needs for new antibiotics, and *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*, such as *Escherichia coli* and *Salmonella*, which are three species of Gram-negative pathogens that can cause severe infections and high mortality, were the only bacteria with a critical priority [5]. The pharmaceutical industry has previously focused more on developing antibiotic agents against Gram-positive bacteria, perhaps because anti-Gram-positive agents are less costly to develop. Therefore, there is a lack of novel antibiotics specific to Gram-negative pathogens. Compared with Gram-positive pathogens that mostly exist naturally outside the human body, Gram-negative bacteria typically live in the gastrointestinal tract, where pathogens may cause serious bloodstream infections or urinary tract infections. Data from the U.S. National Healthcare Safety Network showed that Gram-negative bacteria were responsible for more than 30% of hospital-acquired infections, as well as 47% of ventilator-associated pneumonia and 45% of urinary tract infections [6]. Hence, there is an urgent need for new antibiotics, particularly those for treating Gram-negative infections.

Antibacterial peptides, also known as host defense peptides, are produced by the host organism as part of its innate immunological defense, which is present in all forms of life. An abundance of antibacterial peptides, which are at the forefront of the host's defense against pathogen infections, have been discovered in the hemolymph from marine invertebrates. Studies suggest that the hemolymph of various species of healthy invertebrates is unsterile [7]. Investigations into hemolymph microbiota homeostasis indicate that the host suppresses hemolymph microbiota proliferation by producing critical antibacterial peptides and proteins [8]. Hence, the hemolymph of marine invertebrates usually exhibits antibacterial activity. Several different antibacterial peptide/protein groups have been found in marine invertebrates, including penaeidins, crustins, lectins, anti-lipopolysaccharide factors, defensins, and lysozymes [9–19]. Defensins are one of the most well-studied classes of AMPs found in animals, plants, and fungi. Most defensins are short (18–45 amino acid) peptides possessing an amphipathic structure. Defensins have a -hairpin motif as their primary secondary structure, but they can also have a -helix. Defensins exhibit antibacterial action against a wide range of pathogens, including enveloped viruses, protozoa, fungi, and bacteria. Defensins have been discovered in the hemolymph of marine invertebrates, including oysters, disk abalones, and mussels. To date, more than 50 defensin homologs exhibiting antibacterial activity have been identified. In addition to these typical antibacterial peptides/proteins, the degradation fragments of some large proteins in hemolymph, such as hemoglobin and defensin, also exhibit an appreciable antibacterial function and play a role in inhibiting or killing pathogenic bacteria [7]. Previously, we isolated and identified big defensin 1 from marine Chinese medicine *Ostrea concha*, but the potential peptide fragments possessing activity in big defensin 1 were not fully revealed. Therefore, we further identified an AMP fragment from *Ostrea gigas* big defensin 1, which was the first helical motif of big defensin 1 with fair positive net charges and amphipathicity,

named CGD-1.

Ostrea concha, a marine Chinese medicine, was first mentioned in “Shen Nong’s Herbal Classic” during the Han Dynasty and has been recorded in classic Chinese medicine texts of all dynasties. The Chinese Pharmacopoeia lists three sources of *Ostrea concha*: the shells of *Ostrea talienwhanensis* Crosse, *Ostrea gigas* Thunberg, or *Ostrea rivularis* Gould. It is one of the most economically important bivalves and is widespread in the western Pacific Ocean. In China, *Ostrea gigas* is regarded as a salutary food material and a traditional remedy for alleviating stomach pain, indigestion, and anemia. Several defensins that exert innate immunity effects from *Ostrea gigas* have been reported [20]. However, to our best knowledge, there is little information available concerning the antibacterial peptides and the mechanism of antibacterial action of Chinese materia medica *Ostrea concha*.

This study aimed to evaluate the antibacterial activity, physicochemical, and structural properties of the antibacterial peptide, CGD-1. Furthermore, the preliminary mechanism of CGD-1’s antibacterial action is revealed.

Materials and methods

Materials

Fetal bovine serum, Luria-Bertani (LB) broth, and RPMI-1640 were procured from GIBCO Invitrogen Corporation (USA). Propidium iodide (PI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide, streptomycin, penicillin, polymyxin B, o-nitrophenyl-β-D-galactopyranoside (ONPG), N-phenyl-1-naphthylamine (NPN), and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chemical Co. (USA). Ciprofloxacin was obtained from Guangzhou Baiyunshan Pharmaceutical Co., Ltd. (China). Other commercially available chemicals and reagents were of analytical grade. Unless otherwise specified, bacteria were cultured in LB broth at 37 °C. All bacteria were preserved in suitable culture media with 30% (v/v) glycerol at –80 °C.

Peptide synthesis

CGD-1 was synthesized by Shanghai GL Biochem Ltd. (China), and the peptides’ purity was determined using reversed-phase high-performance liquid chromatography and mass spectrometry. The purity of CGD-1 was found to be greater than 95% (Figure 1).

Antibacterial spectrum of CGD-1

The antibacterial activity of CGD-1 was assessed against several bacterial strains, including *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *P. aeruginosa* clinical isolate, *E. faecalis* ATCC 29212, *S. epidermidis* ATCC12228, *S. aureus* ATCC 25923, and *B. subtilis* ATCC 6633. The minimum inhibitory concentration (MIC) values of CGD-1 against pathogenic strains were determined using a modified NCCLS broth microdilution method [21]. The bacterial pellets were incubated to mid-logarithmic phase in LB broth at 37 °C. Serial concentrations of CGD-1 dissolved in sterile normal saline were added to the suspended bacteria solution in sterile 96-well plates and further incubated at 37 °C for 18 h. Ciprofloxacin was used as a control. The OD600 was measured using a microplate reader. The concentration at which the OD did not increase from the start of the assay was considered as the MIC.

Hemolytic and cell viability assays

Following the previously described method, erythrocytes from murine blood were washed thrice and added to serial dilutions of CGD-1 placed in 96-well plates and incubated at 37 °C for an hour [21]. A 1% Triton X-100 solution and saline solution were used as control samples for total and no hemolysis, respectively. Hemolysis was evaluated by determining OD570 using a microplate reader [21, 22]. To evaluate the cell viability of CGD-1, human embryonic kidney cells (HEK293 cells) at a concentration of 1×10^5 cells/mL were used for MTT reduction assays [21]. All experiments were performed in triplicate.

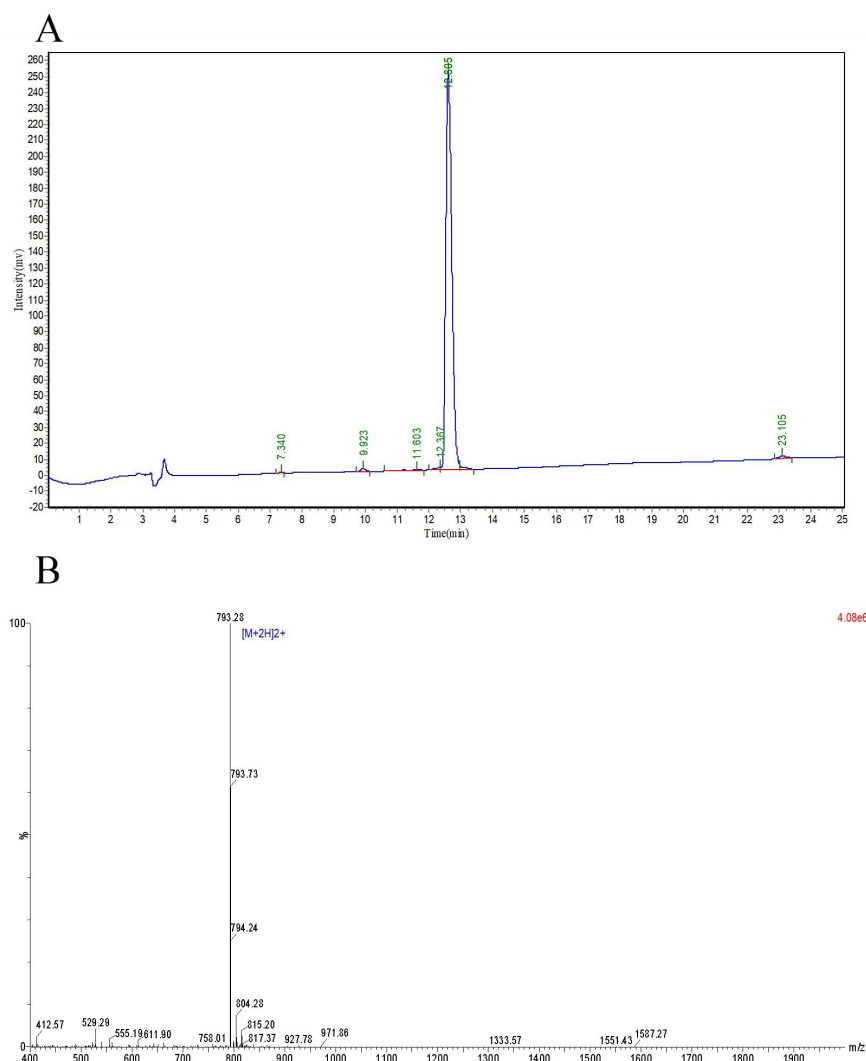


Figure 1 The purity confirmation of AI-hemocidin 1. (A) The result of RP-HPLC; (B) The result of Mass spectrometry.

Membrane permeability assay

P. aeruginosa and *E. coli* bacteria were utilized to assess the cell membrane permeability induced by CGD-1. The outer membrane permeability assay was conducted by measuring the NPN uptake, as previously described [23]. In brief, the bacterial cells were suspended in 5 mM HEPES buffer, and 1 mL of the bacterial suspension was mixed with 10 μ M NPN buffer in a quartz cuvette. The fluorescence increase caused by the introduction of NPN into the outer membrane was measured using a fluorescence spectrometer (HITACHI F-4500, Japan) until there was no further rise in fluorescence. The measurements were taken 360 seconds after CGD-1 treatment. The percentage of NPN uptake was used to evaluate the outer membrane permeability.

$$\% \text{ NPN uptake} = (A_{\text{obs}} - A_0) / (A_{100} - A_0) \times 100$$

In this equation, A_{obs} represents the fluorescence observed after CGD-1 treatment, A_0 represents the original fluorescence of NPN with only bacteria, and A_{100} , considered as 100% outer membrane permeability, represents the fluorescence observed with the positive control, 10 μ g/mL polymyxin B, which is known for its exceptional outer membrane permeabilizing ability.

To determine the permeability of the *E. coli* inner cell membrane, the transformation from ONPG to o-nitrophenol was assessed, as reported previously [24]. The bacterial cells were treated with a solution of ONPG with a final concentration of 1.5 mM and CGD-1 at 1 or 3 MIC. The conversion of ONPG to o-nitrophenol was measured every 30 minutes at a wavelength of 405 nm using a microplate

reader. A suspension of bacterial cells without peptide was used as a negative control.

Effects on the bacterial membrane integrity

To examine the bacterial membrane integrity, a PI uptake assay was conducted using flow cytometry [25]. The bacterial density of *E. coli* and *P. aeruginosa* was adjusted to 1×10^6 CFU/mL and mixed with CGD-1 at 1 and 3 $\times$ MIC for an hour of incubation. After centrifugation, the bacterial pellets were cultured with 100 μ M PI for 10 minutes on ice. Flow cytometry was used to collect 40,000 events per sample, with excitation and emission occurring at 488 and 525 nm, respectively.

Bacterial membrane damage detection by scanning electron microscope (SEM)

The bacterial cells were suspended and their density was adjusted to an OD₆₀₀ of 0.2. The cells were then incubated with CGD-1 at 1 $\times$ MIC for an hour and washed three times. Next, the cells were fixed with 2.5% (v/v) glutaraldehyde at 4 $^{\circ}$ C for 12 hours and dehydrated in a series of ethanol concentrations (50, 60, 70, 80, 90, and 100%) for 10 minutes each. The dehydrated cell pellets were added to a mixture of isoamyl acetate and ethanol (1:1, v/v) for 20 minutes. The samples were dried using a critical point dryer, coated with gold, and examined using a field emission SEM (Carl Zeiss Ultra 55, Germany) [25].

Physicochemical characterization and secondary structure analysis of CGD-1

The physicochemical parameters of CGD-1 were analyzed using the ExPASy Bioinformatics Resource Portal (<http://www.expasy.org/tools/>). The secondary structure of CGD-1 was detected using a circular dichroism (CD) spectrometer (Chirascan, UK) under aqueous and hydrophobic conditions represented by 10 mM PBS and 50% trifluoroethanol (TFE), respectively [26]. The scanning range was recorded from 195 to 250 nm. The secondary structure of CGD-1 in three dimensions was established using the online server I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>), and the helical structure was evaluated using HeliQuest (<http://heliquest.ipmc.cnrs.fr/>).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software, CA, USA). The experimental data are presented as the mean $\pm$ standard deviation, unless otherwise indicated. A P-value of less than 0.05 was considered statistically significant.

Results and discussion

Bioactivity evaluation of CGD-1

The antibacterial activity was investigated, and the MIC of CGD-1 was found to be 25 μ M against three pathogenic bacteria, namely *E. coli* ATCC25922, *P. aeruginosa* clinical isolate, and ATCC 27853 strain (Table 1). However, the MIC values of CGD-1 were more than 200 μ M against several Gram-positive bacterial strains, including *Staphylococcus aureus* ATCC 25923, *B. subtilis* ATCC 6633, *S. epidermidis* ATCC12228, and *E. faecalis* ATCC 29212. When compared with the MIC values of the Gram-positive drug ciprofloxacin, which were 3.125 μ M and 6.25 μ M against *E. coli* and *P. aeruginosa*, respectively, CGD-1 showed considerable inhibition against these Gram-negative bacterial strains, but no inhibition against Gram-positive strains (up to 100 μ M). Additionally, CGD-1 exhibited antibacterial activity against a *P. aeruginosa* clinical isolate. It is worth noting that several antibiotics have been discovered to inhibit Gram-positive pathogenic bacteria, but there is a dearth of antibiotics against Gram-negative bacteria. CGD-1 was discovered from *Crassostrea gigas*, which thrives in a bacteria-enriched environment. Studies have shown that macromolecules, such as hemoglobin in the hemolymph, might be digested by endogenous enzymes and produce molecules with antibacterial activity to combat bacterial invasion [7].

Table 2 shows that CGD-1 had minimal effect on the lysis of murine red blood cells. The maximum hemolysis observed was $7.54 \pm 0.32\%$ with a concentration of CGD-1 of 250 μ M. Cell viability was assessed in HEK293 cells, and the results indicated that $88.67 \pm 4.02\%$ of HEK293 cells survived after treatment with CGD-1 at 125 μ M (Table 2). Furthermore, the median inhibitory concentration for cell viability of CGD-1 was greater than 500 μ M. The concentrations of CGD-1 that resulted in cytotoxicity were much higher than the MIC values, indicating that CGD-1 exhibits good bacterial selectivity (Table 2). Some antibacterial peptides possess potent antibacterial effects, but also exhibit high cytotoxicity and hemolytic activity. For instance, melittin, a peptide purified from honey bee venom, exhibits both potent antibacterial activity and vigorous lytic activity against eukaryotic cells, including red blood cells [27]. The antibacterial action of melittin is achieved by disrupting the cell membranes. Similarly, the antibacterial peptide piscidin 1, derived from hybrid striped bass, exerts strong inhibitory effects on bacteria, as well as on normal human cell lines [28]. However, the use of melittin and piscidin 1 is limited due to their cytotoxic effects. In contrast, defensin-derived peptides exhibit antibacterial activity without obvious cytotoxicity. CGD-1 shows promise as a biocide candidate because it possesses antibacterial activity coupled with low cell toxicity and low hemolytic activity.

Physicochemical properties and secondary structure

characterization of CGD-1

Table 3 presents the physicochemical properties of CGD-1. CGD-1 possesses a net positive charge of +3, which classifies it as a cationic peptide. Additionally, CGD-1 has a moderate hydrophobicity of 0.797. The putative helical structure of CGD-1 is illustrated in Figure 2A, which demonstrates a stable amphipathic structure consisting of a positively charged surface and a hydrophobic lateral area. Generally, antibacterial peptides share several common features that are associated with their antibacterial activity [29, 30]. One of the most important features is their positive charge, which facilitates electrostatic interactions with negatively charged bacterial membranes [31]. Another crucial feature is having more than 50% hydrophobic residues, which are conducive to disrupting the bacterial membrane. Therefore, the presence of an ordered amphipathic structure is critical for peptides to exert antibacterial effects [32]. CGD-1 may possess antibacterial activity due to its ordered amphipathic structure, which consists of positively charged and hydrophobic side surfaces, in addition to proline residues.

Table 1 Antibacterial activity of AI-hemocidin-1

Bacteria	MIC Value (μ g/mL)	
	CGD-1	ciprofloxacin
<i>E. coli</i> ATCC25922	25	3.125
<i>P. aeruginosa</i> ATCC 27853	25	6.25
<i>P. aeruginosa</i> Clinical isolate	25	6.25
<i>S. aureus</i> ATCC 25923	> 100	3.125
<i>B. subtilis</i> ATCC 6633	> 100	3.125
<i>S. epidermidis</i> ATCC12228	> 100	3.125
<i>E. faecalis</i> ATCC 29212	> 100	6.25

MIC, minimum inhibitory concentration.

Table 2 Cytotoxicity and hemolytic activity of CGD-1

Sample	AI-hemocidin-1
MIC ^a (μ M)	25
HC ₁₀ ^b (μ M)	> 250
Hmax ^c	7.54 ± 0.32
% Cell viability at 125 μ M	88.67 ± 4.02
IC ₅₀ ^d (μ M)	> 500
SI value ^e	20

^a, MIC against *P. aeruginosa* and *E. coli*; ^b, HC₁₀ is the CGD-1 concentration induced 10% human erythrocytes lysis; ^c, Hmax is the percentage of hemolysis treated with 250 μ M CGD-1; ^d, IC₅₀ is the concentration causing half maximal inhibitory effect of HEK293 cell growth; ^e, Selectivity index (in vitro) is the ratio of IC₅₀ to MIC; IC₅₀ taken at the maximum of 500 μ M. MIC, minimum inhibitory concentration.

Table 3 Physicochemical properties of CGD-1

Peptide	Sequence	Length	pI	Mw	NC	H	PR/n%
AI-1	RKSFLSA IFIVLLVK	15	11.0	1734.20	+3	0.797	5/33.33%

pI, represents the isoelectric point; NC, represents net charge; H, represents hydrophobicity; PR, represents polar residues.

The secondary structure of CGD-1 was determined using CD spectroscopy in different environments, including a 50% TFE solution which mimics a hydrophobic environment, and 10 mM phosphate buffer saline (PBS) [25]. As shown in Figure 2B, CGD-1 adopted a random coil conformation in an aqueous environment, but displayed an α -helix conformation in a membrane environment mimicked by the 50% TFE solution. The CD spectrum of the α -helix formation was consistent with the results demonstrated by the helical wheel diagram (Figure 2A). The 3D structure of CGD-1 (Figure 2C) was predicted using I-TASSER. A structure with a high degree of α -helix formation enables antibacterial peptides to insert into bacterial membranes and alter the permeability of the bacterial membrane, eventually leading to bacterial cell lysis [33]. Therefore, CGD-1 exhibited sensitive antibacterial action through its α -helix structure.

Effects of CGD-1 on bacterial membrane permeability

A common mechanism by which antibacterial peptides inhibit bacteria is by increasing bacterial membrane permeability. The outer membrane permeability of CGD-1 was measured using a NPN uptake assay. After destabilization of the bacterial cell membrane induced by antibacterial peptides, NPN can insert into the injured membrane and emit an intense fluorescence signal in the hydrophobic interior of bacterial membranes [34]. As shown in Figure 3A, 25 μ M ($1 \times$ MIC) of CGD-1 caused $61.07 \pm 6.21\%$ and $65.283 \pm 3.01\%$ NPN uptake, at 360 s after peptide treatment, in *P. aeruginosa* and *E. coli*, respectively. Furthermore, 75 μ M ($3 \times$ MIC) of CGD-1 induced $71.84 \pm 5.07\%$ and $78.22 \pm 3.65\%$ NPN uptake in *P. aeruginosa* and *E. coli*, respectively. These results indicated that CGD-1 was able to permeabilize the outer membrane in a dose-dependent manner. The inner cytoplasmic

membrane permeability of CGD-1 was assessed using a β -galactosidase assay. β -Galactosidase leakage after treatment with CGD-1 was examined in *E. coli* (Figure 3B). No absorbance change was detected in the control cell medium over time. Despite the existence of multiple mechanisms for antibacterial action, most peptides with an α -helical conformation have been reported to exert antibacterial action via interaction with bacterial membranes, which contain specific membrane components [36, 37]. According to the self-promoted uptake theory, antibacterial peptides possessing an α -helix structure interact with the outer acidic surfaces of bacteria by displacing cations, such as Ca^{2+} and Mg^{2+} , thereby generating an electrostatic association with the cytoplasmic membrane [29]. Subsequently, the antibacterial peptides cause peptide-membrane lipid aggregates and membrane pores, ultimately resulting in bacterial cell death [38, 39]. Similar to other hemoglobin-related peptides, such as batenecin from bovine sources and SH β AP from skipjack tuna, CGD-1, possessing a positive net charge and an α -helix structure, displayed antibacterial activity by permeabilizing bacterial membranes in a rapid and reproducible manner [28, 35].

PI uptake assay

PI, a fluorescent intercalating agent used for staining DNA, is commonly used to determine cell death and cell membrane integrity by flow cytometry [40]. The results of the PI uptake assay are presented in Figure 4. In the absence of CGD-1, a low level of PI uptake in *P. aeruginosa* and *E. coli* cells was observed, suggesting that the bacterial cells were surviving with intact cell membranes. However, the amount of PI uptake in *P. aeruginosa* and *E. coli* cells was significantly increased after treatment with $1 \times$ and $3 \times$ the MIC of

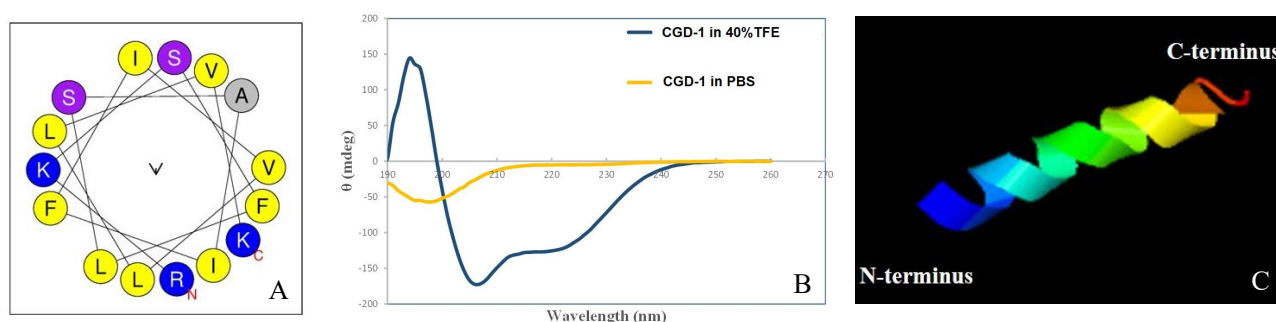


Figure 2 Secondary structure characterization of CGD-1. (A) Putative Schiffer & Edmundson helical wheel for CGD-1. The neutral residues are colored gray, polar residues are colored purple, hydrophobic residues are colored yellow, negatively charged hydrophilic residues are colored red, positively charged hydrophilic residues are colored blue, proline residues are colored green, and glutamine and asparagine are colored pink; (B) CD spectrum of CGD-1; (C) three-dimensional structure prediction of CGD-1. The colors are meaningless.

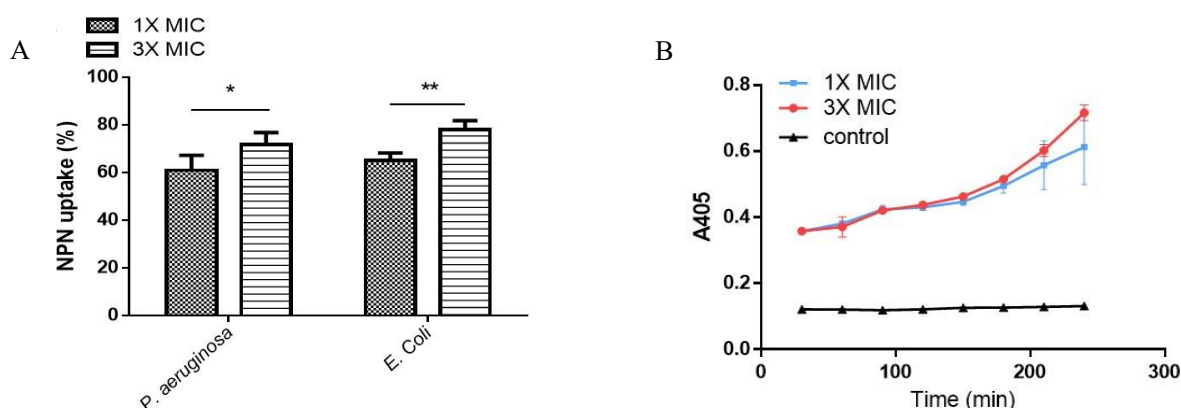


Figure 3 Bacterial membrane permeability effect of CGD-1. (A) *P. aeruginosa* and *E. coli* outer membrane permeabilization induced by CGD-1, NPN uptake values are presented as a percentage compared with polymyxin B (positive control), *, $P < 0.05$, **, $P < 0.01$; (B) *E. coli* cytoplasmic membrane permeabilization induced by CGD-1.

CGD-1. A dose-dependent increase in PI fluorescence demonstrated that the *P. aeruginosa* and *E. coli* cells were damaged by CGD-1. Taken together with the results of the membrane permeability assay, CGD-1 was shown to have the ability to permeabilize and disrupt bacterial cell membranes, resulting in cell integrity damage and cell death. Most α -helical antibacterial peptides exhibit their antibacterial function through bacterial membrane attack. One mechanism for the membrane attack of α -helical antibacterial peptides is the traditional toroidal model. In this model, it is hypothesized that the lytic pores in damaged bacterial membranes are attached to the hydrophilic side chains of peptides, and these are concurrently interacted with the lipid groups of antibacterial peptides [41, 42]. Recently, an alternative mechanism for membrane attack induced by α -helical peptides has been proposed. Based on the results of molecular modelling and dynamic simulations, it was hypothesized that antibacterial peptides with an α -helix structure may insert a partial sequence into the cytoplasmic membrane, ultimately contributing to an arrangement of tiny and dynamic toroidal pore aggregates, compared with the putative larger lytic pores in the traditional toroidal model [38, 42]. Both of these hypotheses are based on interactions between antibacterial peptides and the bacterial membrane, ultimately resulting in membrane damage and bacterial cell death. The results of the PI uptake and bacterial membrane permeability assays indicated that CGD-1 can increase cell membrane permeability, resulting in damage to cell integrity.

Examination of bacterial morphologic changes by SEM

Cellular damage and morphological changes in *E. coli* and *P. aeruginosa* were assessed using SEM following exposure to CGD-1. Treatment with CGD-1 resulted in altered bacterial cell morphology. Specifically, the *E. coli* cell membrane was partially impaired after treatment with CGD-1 at $1 \times \text{MIC}$ for 1 hour, as shown in Figure 5B, in contrast to the control (Figure 5A). However, after 3 hours of co-incubation, ruptured membranes and leakage of cellular components were observed, as depicted in Figure 5C.

The antibacterial effects of CGD-1 against *P. aeruginosa* were observed and documented in Figure 5D–F. After only 1 hour of treatment with CGD-1, roughness and wrinkling of the bacterial membrane surface were immediately apparent, as shown in Figure 5E,

in contrast to the smooth and intact appearance of control bacterial cells (Figure 5D). Furthermore, severe membrane corrugation was observed after 3 hours of peptide treatment (Figure 5F). Interestingly, CGD-1 was found to deform not only the cytoplasmic membrane, but also the outer membranes of *P. aeruginosa* cells. As depicted in Figure 5D–F, the smooth outer membranes of *P. aeruginosa* cells became furrowed after CGD-1 treatment. These results suggest that CGD-1 is capable of damaging the cellular integrity of bacteria and promoting significant distortions in cellular morphology. These morphological changes in the bacterial cell surface caused by CGD-1 are consistent with the findings of previous studies [43, 44]. The negatively charged components on the outer acidic surfaces of the bacterial membrane, such as peptidoglycans and lipopolysaccharides, may bind to the peptides, causing membrane instability [45]. The results of this study demonstrate that CGD-1 may exert its antibacterial effect by permeabilizing and disrupting the bacterial cell membrane integrity.

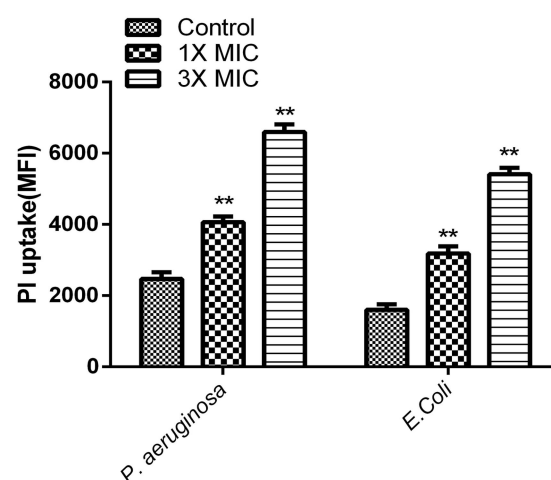


Figure 4 CGD-1 affected the membrane integrity of *P. aeruginosa* and *E. coli*. PI uptake is shown with mean fluorescence intensity (MFI) values. **, $P < 0.0001$ versus control. PI, Propidium iodide.

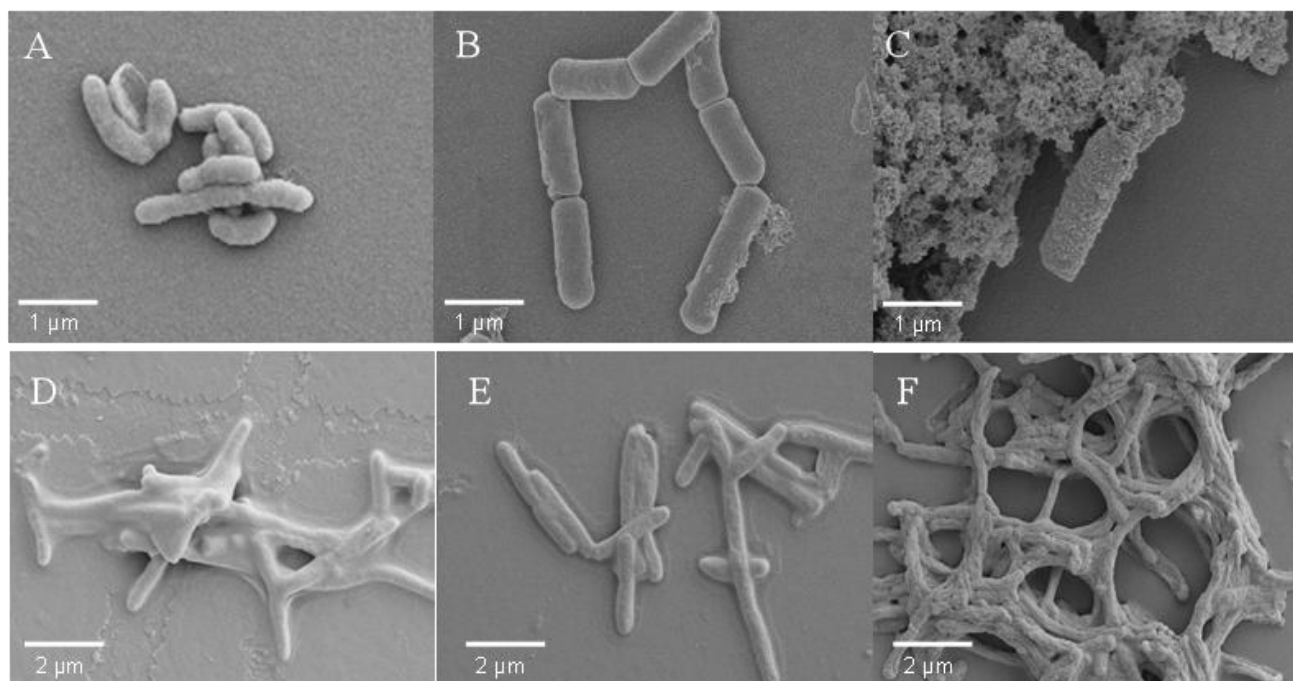


Figure 5 Cell morphology changes of *E. coli* and *P. aeruginosa* with CGD-1 treatment. (A) control, *E. coli* cells only; (B) *E. coli* treated with CGD-1 at $1 \times \text{MIC}$ for 1 h; (C) *E. coli* treated with CGD-1 at $1 \times \text{MIC}$ for 3 h; (D) control, *P. aeruginosa* cells only; (E) *P. aeruginosa* treated with CGD-1 at $1 \times \text{MIC}$ for 1 h; (F) *P. aeruginosa* treated with CGD-1 at $1 \times \text{MIC}$ for 3 h. MIC, minimum inhibitory concentration.

Conclusions

CGD-1 is a defensin-related peptide derived from the marine Chinese medicine *Ostrea concha*, which has been found to exhibit antibacterial activity against the Gram-negative bacteria *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853, with low hydrolysis activity and cytotoxicity. In a simulated membrane environment, CGD-1 formed a representative α -helical structure. The results of the membrane permeability determination assay and SEM examination suggested that the potential mechanism for the antibacterial action of CGD-1 might be the induction of bacterial cell death by permeabilizing and disrupting the bacterial membrane. These findings suggest that defensin-related peptides may serve as a source of nature-derived biocide agents against pathogenic bacteria.

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