Identification and functional characterization of an uncharacterized

antimicrobial peptide from a ciliate *Paramecium caudatum*

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Abstract The global ever-growing concerns about multi-drug resistant (MDR) microbes leads to urgent demands for exploration of new antibiotics including antimicrobial peptides (AMPs). Here we demonstrated that a cDNA from Ciliata *Paramecium caudatum*, designated *Pcamp1*, coded for a protein with features characteristic of AMPs, which is not homologous to any AMPs currently known. Both the C-terminal 91 amino acid residues of PcAMP1, cPcAMP1, expressed in *E. coli* and the C-terminal 26 amino acid residues (predicted mature AMP), cPcAMP1/26, synthesized, underwent a coil-to-helix transition in the presence of TFE, SDS or DPC. Functional assays revealed that cPcAMP1 and cPcAMP1/26 were both able to kill *A. hydrophila* and *S. aureus*. ELISA showed that cPcAMP1 and cPcAMP1/26 were able to bind to microbe-associated molecular pattern molecules LPS and LTA, which was further corroborated by the observations that cPcAMP1 could deposit onto the bacterial membranes. Importantly, both cPcAMP1 and cPcAMP1/26 were able to induce bacterial membrane permeabilization and depolarization, and to increase intracellular ROS levels. Additionally, cPcAMP1 and cPcAMP1/26 were not cytotoxic to mammalian cells. Taken together, our results show that PcAMP1 is a potential AMP with a membrane selectivity towards bacterial cells, which renders it a promising template for the design of novel peptide antibiotics against MDR microbes. It also shows that use of signal conserved sequence of AMPs can be an effective tool to identify potential AMPs across different animal classes.

Keywords: Multi-drug resistant microbes; Antibiotics; Antimicrobial peptides; Ciliate; *Paramecium*

1. Introduction

Antibiotics have been successfully used for the treatment of bacterial infections in humans, preventing bacterial epidemics for nearly 70 years. However, the emergence of multi-drug resistant (MDR) microbes caused by overuse of antibiotics has resulted in the less efficacy of major antimicrobial drugs used in clinical settings (Huh and Kwon, 2011). With the global ever-growing concerns about multi- or pan-resistant pathogens, there occur urgent demands for exploration of new antibiotics, such as phytochemicals, synthetic antibiotics, antimicrobial peptides (AMPs), and inhibitors for drug-efflux pumps (Davies et al., 2010; Dossey, 2010; Mangoni, 2006; Nguyen et al., 2011; Theopold et al., 2004; Torres-Larios et al., 2000; Wright, 2005). Among them, AMPs are endogenous components of innate immunity that act as a first line of defense of hosts against pathogen infection, and thus a promising class of compounds with a great potential pharmaceutical application. AMPs are usually cationic and amphipathic molecules which interact with microbial membranes, and kill microbes by direct disruption of cellular components, including the microbial membrane and DNA (Epand and Vogel, 1999; Wang et al., 2015), and thus the acquisition of resistance against AMPs is very rare, in sharp contrast to conventional antibiotics (Hancock and Sahl, 2006). Accordingly, AMPs have attracted great attention for overcoming MDR microbes.

According to the online updated Antimicrobial Peptide Database (APD) (Wang et al., 2009; Wang and Wang, 2004), more than 2,600 AMPs have been isolated and characterized currently. AMPs are generally short, gene-coded polypeptides that can

be constitutively expressed or induced to attack invading microbes. They are usually present in natural sources, such as the skin mucosa of aquatic animals, only in small quantities, thus their isolation and characterization are often rather laborious and time-consuming (Pukala et al., 2006). In addition, they are often produced or induced in exotic animals or their tissues as inactive precursors, complicating sample collection and assay-based identification methods. An alternative approach is to identify the genes encoding AMPs, either by directly isolating genomic DNA from small tissue samples, or by mining the vast amount of sequence information already deposited in genomic or expressed sequence tag (EST) databases (Tessera et al., 2012).

Identification of novel AMPs in databases is basically dependent on the presence of a sufficient sequence similarity, substantial number of EST data and a query sequence of a known AMP (Fedders and Leippe, 2008). However, the sequences between orthologous AMPs are considerably divergent because they are at the interface between the host and a complex and ever changing microbial biota, and are thus under strong positive selection for variation in many animal taxa (Tennessen, 2008), leading to extremely lower similarity between orthologous AMPs of even closely related species. Fortunately, the sequences of signal peptides and proregions that AMPs have in most cases tend to be significantly more conserved than mature AMPs or full-length AMPs themselves. Tessera et al. (2012) and Juretić et al. (2011) have taken this advantage successfully to search for and identify novel AMPs from databases within the same lineages of fish and amphibians, respectively. Whether

such an approach can apply to animals from different lineages of more divergent species or not remains to be tested. Therefore, this study was performed to examine the possibility via using the signal peptide of the pore-forming peptide ameobapore A, a known AMP from *Entamoeba histolytica*, a species of Amoebozoa (Leippe et al., 2005; Andrä et al., 2003; Zhai et al., 2000), as a query to search for AMP candidates from *Paramecium* databases. Here we report the identification of an uncharacterized AMP from a species of Ciliata *Paramecium caudatum*, named as PcAMP1 hereafter, its bactericidal activity and modes of action, proving that conserved sequences of signal peptides of AMPs can be used to identify potential AMPs across different animal classes.

2. Materials and methods

2.1. Database searching

Use of conserved signal sequences as queries has been shown to be effective in identifying putative AMPs in both teleost fish and anurans from the EST database (Tessera et al., 2012; Juretić et al., 2011). BLASTP 2.2.31+ was used to search the non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF for protein sequences using the sequence MKAIVFVLIFAVAFVTATHQ as probe, with search set, organism *Paramecium* (taxid:5884) and algorithm parameters word size: 3, matrix: BLOSUM62, gap costs: existence 11, extension 1 (Tessera et al., 2012; Sun et al., 2007). This query is the signal sequence of pore-forming peptide ameobapore A precursor (GenBank XP_653265.1), an AMP from a parasite *E. histolytica*. Sequences in the output list were inspected via examining the conservation of signal peptides,

and resulting 2 hits were used as queries to further search protein databases of *Paramecium tetraurelia*, which resulted in identification of two hypothetical proteins (XP_001431782.1 and XP_001452803.1) that were predicted to contain 202 and 220 amino acid residues, respectively, and showed the characteristics of putative AMPs.

2.2. Cloning of *Pcamp1* cDNA

Based on analyses above, we tried to clone the genes encoding the homologues of XP_001431782.1 and XP_001452803.1 from *P. caudatum*, which was cultured in yeast medium as described by Yuan (2010). Total RNAs were extracted from *P. caudatum* with Trizol (Invitrogen) according to the manufacturer's instructions. After digestion with recombinant RNase-free DNase (TaKaRa) to eliminate the genomic contamination, the first-strand cDNA was synthesized with reverse transcription system (Promega) using oligo d(T) primer, and used as PCR template. The fragment of XP_001431782.1 homologue, named as *Pcamp1* hereafter, was amplified by PCR with the primer pairs P1-S and P1-AS (Table 1) that were designed using Primer Premier 5.0 program on the basis of relative sequence identified in *P. tetraurelia*. After determination of the partial cDNA sequence, rapid amplification of cDNA ends (RACE) was employed to obtain the full-length cDNA. The gene-specific primer pairs P2 and P3 (Table 1) were used in RACE reactions for the cloning of 3'-end cDNAs. The 3'-RACE-Ready cDNAs were synthesized from the total RNAs using the 3' Full RACE Core Set (TaKaRa, Dalian, China) according to the manufacturer's instructions. The products of 3'-RACE were gel-purified, sub-cloned and sequenced, and *Pcamp1* cDNA was obtained by assembling the overlapping sequences.

Unfortunately, we have failed to clone XP_001452803.1 homologue although we have tried three different pairs of primers.

2.3. Sequence analyses

Pcamp1 cDNA obtained was analyzed for coding probability with the EditSeq in DNASTAR software package (DNASTAR Inc., USA), and sequence comparison was performed using the MegAlign program by CLUSTAL W method (Chenna et al., 2003). The SMART program (<http://smart.embl-heidelberg.de/>) was used to predict the functional sites and domains, and the SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>) used to predict the signal peptide. The molecular mass (MW) and isoelectric point (pI) of the predicted mature peptide were calculated by ProtParam (<http://www.expasy.ch/tools/protparam.html>), and the cut site and the position of the mature peptide predicted by PeptideCutter (http://web.expasy.org/peptide_cutter/) and CAMP (<http://www.camp.bicnirrh.res.in/predict/>). The Jpred program (<http://www.compbio.dundee.ac.uk/www-jpred/index.html>) was used to predict the secondary structure, and the QUARK ONLINE (<http://zhanglab.ccmb.med.umich.edu/QUARK/>) to generate three-dimensional (3D) structure model. Antimicrobial Peptide Calculator and Predictor at APD (<http://aps.unmc.edu/AP/main.php>) were used to calculate the total hydrophobic ratio and net charge.

Helical wheel of the predicted mature AMP was created by the internet site “helical wheel projection” (<http://rzlab.ucr.edu/scripts/wheel/wheel.cgi>) developed by

Armstrong and Zidovetzki (Appadu, 2012).

2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to assay the expression of *Pcamp1* in response to challenge with the microbial signature molecule LPS, as described by Wang et al. (2009). *P. caudatum* was cultured in sterilized distilled water with 10 µg/ml LPS (Sigma, USA), and sampled at 0, 2, 8, 16, 32 and 48 h. Total RNAs were extracted from *P. caudatum* sampled with Trizol (Invitrogen). After digestion with RNase-free DNase (Promega) to eliminate the genomic contamination, cDNAs were synthesized and used as template for reverse transcription system using oligo d(T) primer. The PCR primers P4-S and P4-AS specific of *Pcamp1* and the primers P5-S and P5-AS specific of beta-actin gene (Table 3) were designed using the primer premier 5.0 program. The reaction mixture (final volume 20 µl) consisted of 10 µl of SYBR Premix ExTaq™ (Tli RNaseH Plus), 0.4 µl of ROX Reference Dye II, 0.5 µl of template and 200 nM of each sense and antisense primers. The beta-actin gene was chosen as the reference for internal standardization. qRT-PCR experiments were conducted in triplicate. The amplification was performed on ABI 7500 real-time PCR system (Applied Biosystems) at 95 °C for 15 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s, and 72 °C for 30 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression levels of *Pcamp1* relative to that of beta-actin gene were calculated by the comparative CT method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001).

2.5. Expression and purification of recombinant cPcAMP1

Huntington and Stein has reported that ovalbumin has an internal signal sequence between residues 21 and 47 (Huntington and Stein, 2001). Interestingly, the deduced protein encoded by *Pcamp1* cDNA, consisting of 201 amino acid residues, also has a putative internal signal sequence of 18 amino acid residues positioned between residues 93 and 110 (See below). As the N-terminal 92-residue fragment of PcAMP1 showed no characteristics of AMPs (See below), while its C-terminal 91-residue fragment after the predicted signal sequence did, therefore, the cDNA encoding the C-terminal 91 residues, named as *cPcamp1* hereafter, was amplified by PCR using the sense primer P6 (*EcoR I* site is underlined) and the antisense primer P7 (*Xho I* site is underlined) (Table 1). The reaction was carried out under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 35 s, and an additional extension at 72°C for 7 min. The PCR products were digested with *EcoR I* and *Xho I* and sub-cloned into the pET32a expression vector (Novagen, Darmstadt, Germany) digested previously with the same restriction enzymes. The inserts were verified by DNA sequencing, and the expression construct was designated *pET32a/cPcamp1*.

The cells of *Escherichia coli* BL21 (DE3) were transformed with the plasmid *pET32a/cPcamp1* and then cultured in LB broth containing Ampicillin Na (100 µg/ml) overnight. The cultures were diluted 1:100 with LB broth and subjected to further incubation until OD₆₀₀ reached about 1.0 at 37°C. The expression of *pET32a/cPcamp1* was induced by adding isopropyl β-D-thiogalactoside (IPTG) to the

cultures at a final concentration of 0.5 mM. After further incubation at 28°C for about 12 h, the bacterial cells were harvested by centrifugation at 5000 g at 4°C for 20 min, re-suspended in ice-cold 50 mM Tris-HCl buffer containing 100 mM NaCl (pH8.2) and sonicated on ice. The homogenates were centrifuged at 12000 g at 4°C for 30 min and the supernatants collected. The recombinant cPcAMP1 was purified by Ni-nitrilotriacetic acid resin column (GE Healthcare), and the purity of cPcAMP1 purified was analyzed by SDS-PAGE on a 12% gel, and stained with Coomassie Brilliant Blue R-250. In parallel, the purified cPcAMP1 was analyzed by Western blotting using mouse anti His-tag monoclonal antibody (CWBIO). Protein concentration was determined by the method of BCA using bovine serum albumin (BSA) as a standard (CWBIO, China).

2.6. Peptide synthesis

As analyses by PeptideCutter and CAMP predicted the C-terminal 26-residue fragment (PPHKKKLAVYPVFLFYLFSLWFSIV) being a potential mature AMP, named as cPcAMP1/26, it was thus synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, using standard solid-phase Fmoc method, and used for functional characterization. Because C-terminal amidation is common in AMPs (Lee et al., 1997), the C-terminus of the synthetic cPcAMP1/26 was thus amidated (PPHKKKLAVYPVFLFYLFSLWFSIV-NH₂). The peptide synthesized was purified to >95% by high-performance liquid chromatography (HPLC) and verified by mass spectrometer (lcms-2010a, Shimadzu, Japan) as well as by MS/MS to further confirm the sequence. The peptide was dissolved in 1% (v/v)

2,2,2-trifluoroethanol water solution (2 mg/ml) and stored at -80°C till used.

2.7. Circular dichroism spectroscopy

The propensities of cPcAMP1 and cPcAMP1/26 to assume a helical conformation were both probed by CD spectroscopy Jasco J-810 spectropolarimeter (Tokyo, Japan) in a 1-mm path-length quartz cells and with a peptide concentration of 100 µg/ml (Tessera et al., 2012; Lee et al., 2015). Spectra were measured in the presence of 50 mM Tris-HCl containing 100 mM NaCl (pH8.2), phosphate-buffered saline (PBS), 50% 2,2,2-trifluoroethanol (TFE), 50 mM SDS micelles, and 50 mM dodecylphosphocholine (DPC) micelles at room temperature. The spectra were averaged over three scans.

2.8. Antimicrobial activity assay

A colony formation assay was used to test the antimicrobial activity of cPcAMP1 and cPcAMP1/26 against *Aeromonas hydrophila* and *Staphylococcus aureus* as described by Shi et al. (2006) with slight modification. In brief, the strains of *A. hydrophila* and *S. aureus* were cultured in Tryptic Soy Broth (TSB) and Luria-Bertani (LB) media, respectively. They were cultured to mid-logarithmic phase, and then collected by centrifugation at 6000 g at room temperature for 10 min. The bacterial pellets were washed in 50 mM Tris-HCl buffer containing 100 mM NaCl or PBS three times, re-suspended in Tris-HCl buffer or PBS and adjusted to a density of 3×10^4 cells/ml. Totally, 50 µl of *A. hydrophila* and *S. aureus* suspensions was each pre-incubated at 25°C for 1 h with 50 µl of recombinant cPcAMP1 (12.5, 25 and 50 µg/ml) or cPcAMP1/26 (12.5, 25 and 50 µg/ml) solutions, yielding final

concentrations of 6.25, 12.5 and 25 µg/ml, respectively, and then each of these bacterial suspensions was plated onto 3 agar plates (30 µl/plate). After incubation at 37°C for 12 h, the resulting bacterial colonies in each plate were counted. The percent of bactericidal activity was calculated as follows: [number of colonies (control-test)/number of colonies (control)] ×100 (n=3). The control was processed similarly except that Tris-HCl buffer or PBS was used instead of the peptide solutions.

2.9. Transmission electron microscopy (TEM)

TEM was performed to test the effects of cPcAMP1 and cPcAMP1/26 on bacterial cell walls. Briefly, aliquots of 500 µl of *A. hydrophila* or *S. aureus* suspensions containing 5×10^7 cells/ml were mixed with 500 µl of 50 µg/ml recombinant cPcAMP1 or cPcAMP1/26. In parallel, aliquots of 500 µl of the bacterial suspensions were mixed with 500 µl of 50 mM Tris-HCl containing 100 mM NaCl or PBS as control. The mixtures were incubated at 25°C for 1 h, fixed in 2.5% glutaraldehyde in 100 mM PBS, and then dropped onto 400-mesh carbon-coated grids and allowed to stand at room temperature for 3 min for negative staining. Excess fluid was removed by touching the edge of filter paper. The grids were then put into 2% phosphotungstic acid for 3 min and dried by filter paper. Observation was performed under a JEOL JSM-840 transmission electron microscope.

2.10. Assay for binding of cPcAMP1 and cPcAMP1/26 to LPS and LTA

An ELISA was performed to test if cPcAMP1 and cPcAMP1/26 could bind to lipopolysaccharide (LPS) and lipoteichoic acid (LTA). Both LPS and LTA were labeled with biotin hydrazide (Sigma-Aldrich) as described by Wang et al. (2011).

Aliquots of 50 μ l of 100 μ g/ml recombinant cPcAMP1, cPcAMP1/26 and BSA (control) were applied to each well of a 96-well microplate and air-dried at 25°C overnight. The plate was incubated at 60°C for 30 min to fix the peptide, and then each well was blocked with 200 μ l of 40 mg/ml BSA in PBS (pH7.4) at 37 °C for 2 h. After washing five times with 200 μ l of PBS (pH7.4) containing 0.5% Tween-20 (PBST), a total of 50 μ l PBS containing 1 mg/ml BSA plus different concentrations of biotin-labeled LPS (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, and 5 μ g/ml) or LTA (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, and 5 μ g/ml) was added into each well and incubated at 25°C for 3 h. The wells were each washed five times with 200 μ l of PBST, added with 100 μ l of streptavidin-HRP (CWBIO) diluted to 1: 2000 with 1 mg/ml BSA and incubated at 25°C for 1 h. Subsequently, the wells were each washed five times with 200 μ l of PBST, added with 100 μ l of 0.4 mg/ml ophenylenediamine (Amresco) in the buffer consisting of 51.4 mM Na₂HPO₄, 24.3 mM citric acid, and 0.045% H₂O₂ (pH5.0), and reacted at 37°C for 20 min. Finally, 50 μ l of 2 M H₂SO₄ was added into each well to terminate the reaction, and the absorbance at 492 nm was monitored by a microplate reader (Multiskan GO; Thermo Scientific).

2.11. Assay for interaction of cPcAMP1 with bacterial membranes

The membrane proteins were extracted with Membrane and Cytosol Protein Extraction Kit (Beyotime, China) according to the manufacturer's instructions. The 32a-His-tag was expressed and purified as described by Wang et al. (2011). Totally, 50 μ g of purified recombinant cPcAMP1 or 32a-His-tag (negative control) was added into 1 ml of the bacterial suspension (1×10^8 cells/ml) of *A. hydrophila* or *S. aureus*,

and incubated at 25°C for 1 h, and centrifuged at 6000 g at 4°C for 10 min. The bacterial pellets were washed three times in 50 mM Tris-HCl containing 100 mM NaCl, re-suspended in ice-cold Protein Extraction Reagent A containing 2 µl PMSF and sonicated on ice. The homogenates were centrifuged at 700 g at 4°C for 10 min to remove the cell wall components and unbroken cells, and the supernatants were collected. After centrifugation at 14000 g at 4°C for 30 min, the pellets were pooled, re-suspended in 100 µl of Protein Extraction Reagent B, and loaded onto 12% SDS-PAGE gel. After electrophoresis, the proteins on the gel were electroblotted onto PVDF membrane (Amersham) by a semi-dry technique. The membrane was then blocked with 4% bovine serum albumin (BSA) in PBS at room temperature for 2 h, followed by incubation with mouse anti His-tag monoclonal antibody (CWBIO) diluted 1: 3000 with PBS at 4°C overnight. After thorough washing in PBS containing 0.5% Tween-20, the membrane was incubated in horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (CWBIO, China) diluted 1:8000 at room temperature for 2 h. The bands were visualized using DAB (CWBIO, China) according to the manufacturer's instruction.

2.12. Membrane depolarization assay

The assay for membrane depolarization activity of cPcAMP1 and cPcAMP1/26 was performed with the membrane potential-sensitive dye DiSC₃-5 (DiSC₃-5; Sigma-Aldrich) and the Gram-negative bacterium *A. hydrophila* and the Gram-positive bacterium *S. aureus* (Lee et al., 2015). The bacterial cells in the mid-logarithmic phase were harvested by centrifugation at 6000 g for 10 min, washed

in 5 mM HEPES buffer (pH7.3) containing 20 mM glucose, and re-suspended in 5 mM HEPES buffer containing 20 mM glucose and 100 mM KCl to an OD₆₀₀ of 0.05. Aliquots of 100 µl of the bacterial suspensions were applied to each well of a 96-well flat bottom white microplate. A stock solution of diSC₃-5 was added to the bacterial suspensions, yielding a final concentration of 0.5 µM, and incubated at room temperature for 30 min to get a steady baseline of fluorescence intensity. The bacterial suspensions were then mixed with 100 µl of different concentrations of recombinant cPcAMP1 (50 and 100 µg/ml) or cPcAMP1/26 (50 and 100 µg/ml), giving the desired concentrations of 25 µg/ml and 50 µg/ml, respectively. HEPES buffer containing 20 mM glucose was used as control. Changes in fluorescence intensity were continuously recorded for 30 min with a TECAN-GENios plus spectrofluorimeter at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

2.13. Assay for permeability of microbial cells

The assay for permeabilization activity of cPcAMP1 and cPcAMP1/26 was carried out using *A. hydrophila* and *S. aureus* cells by flow cytometry as described by Hu et al. (2013). The bacteria *A. hydrophila* and *S. aureus* were cultured in TSB medium and LB medium, respectively, to mid-logarithmic phase, and then collected by centrifugation at 6000 g at room temperature for 10 min. The bacterial pellets were washed in 50 mM Tris-HCl containing 100 mM NaCl (pH8.2) or PBS three times, re-suspended in Tris-HCl buffer or PBS and adjusted to a density of 1×10^8 cells/ml. Aliquots of 300 µl of the bacterial suspensions were mixed with 300 µl of different concentrations of recombinant cPcAMP1 (25 and 50 µg/ml) or cPcAMP1/26 (25 and

50 µg/ml), yielding final concentrations of 12.5 µg/ml and 25 µg/ml, respectively. For control, the bacteria were mixed with 50 mM Tris-HCl containing 100 mM NaCl or PBS alone. The mixtures were incubated at 37°C for 1 h, and fixed with 20 µM of propidium iodide (PI; a DNA-intercalating agent and a fluorescent molecule) under dark at 4°C for 15 min. The bacterial cells staining by PI was examined using a FC500 MPL flow cytometer (Beckman). Data was analyzed using WinMDI v.2.9 software (Scripps Research Institute, San Diego, CA).

2.14. Reactive oxygen species assay

The levels of reactive oxygen species (ROS) were measured based on the intracellular peroxide-dependent oxidation of 2',7'-dichlorofluorescein-diacetate (DCFH2-DA), which is a non-fluorescent lipophilic probe that can cross the cell membrane. Inside the cell, DCFH2-DA deacetylates to form DCFH2, which is also a non-fluorescent probe but cannot diffuse freely across the cell membrane. DCFH2 reacts with intracellular ROS to yield the highly fluorescent compound 2',7'-dichlorofluorescein (DCF), which can reflect the levels of ROS in the cells. *A. hydrophila* and *S. aureus* were cultured to mid-logarithmic phase, and re-suspended in corresponding culture medium containing 10 µM DCFH2-DA, yielding a density of 10^7 cells/ml. After incubation at 37°C for 30 min, the bacteria were collected by centrifugation at 6000 g at room temperature for 10 min. The bacterial cells were washed three times in 50 mM Tris-HCl containing 100 mM NaCl or PBS, re-suspended in 1 ml of 25 µg/ml recombinant cPcAMP1 or cPcAMP1/26. For positive control, the bacterial cells were re-suspended in 1 ml of 50 mM Tris-HCl

containing 100 mM NaCl and 50 µg/ml Rosup (A compound mixture which is able to significantly increase ROS levels in cells within 30 min) or PBS containing 50 µg/ml Rosup (Beyotime, China). For blank control, the cells were re-suspended in 1 ml of 50 mM Tris-HCl containing 100 mM NaCl or PBS alone. The bacterial suspensions were incubated at 25°C for 1 h and the fluorescence intensities were recorded immediately with a TECAN-GENios plus spectrofluorimeter at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.15. Hemolytic activity assay

Human red blood cells (RBCs) were used to test the hemolytic activity of recombinant cPcAMP1 and cPcAMP1/26 as described by Hu et al. (2013). Healthy human blood was obtained into a EDTA anticoagulant tube, and RBCs were collected by centrifugation at room temperature at 1000 g for 10 min. After washing three times with 50 mM Tris-HCl buffer, RBCs were suspended in 50 mM Tris-HCl containing 100 mM NaCl or PBS to give a concentration of 4% (v/v). Aliquots of 200 µl RBCs suspension were mixed with 200 µl of different concentrations of cPcAMP1 (25, 50, 100 and 200 µg/ml) or cPcAMP1/26 (25, 50, 100 and 200 µg/ml), giving final concentrations of 12.5, 25, 50 and 100 µg/ml. After incubation at 37°C for 1 h, the mixtures were centrifuged at room temperature at 1000 g for 10 min. The supernatants were collected and added into a 96-well plate. The absorbance was measured at 540 nm under a microplate reader (Multiskan GO; Thermo Scientific). RBCs incubated with Tris-HCl buffer, PBS, BSA solution (100 µg/ml), and 0.1% Triton X-100 solution served as blank, negative and positive controls.

2.16. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To test if recombinant cPcAMP1 and cPcAMP1/26 are cytotoxic to murine RAW264.7 cells, MTT assay was performed as described by Hu et al. (2013). RAW264.7 cells were suspended in serum-free DMEM and aliquots of 180 μ l of the cell suspension (1×10^6 cells/ml) were sampled into a 96-well plate and cultured at 37°C with 5% CO₂ for 2 h. After removal of the medium, aliquots of 200 μ l serum-free DMEM with different concentrations of recombinant cPcAMP1 (12.5, 25, 50 and 100 μ g/ml) or cPcAMP1/26 (12.5, 25, 50 and 100 μ g/ml) were added to each well of a 96-well microplate, incubated for 4 h, and then 20 μ l of MTT solution (5 mg/ml) was added into each well. After incubation for another 4 h, the medium was removed and 150 μ l of dimethyl sulfoxide (DMSO) was added. The absorbance at 492 nm was measured under a microplate reader. The cells incubated in serum-free DMEM served as control. The percent viability against the control was calculated as follows: (OD of treated groups / OD of control groups) $\times$ 100% (n = 3).

2.17. Statistical analysis

All the experiments were performed in triplicate, and repeated three times except TEM experiment. Statistical analyses were performed using the GraphPad Prism 5. The significance of difference was determined by two-way ANOVA, and difference at $p < 0.05$ was considered significant. All the data were expressed as mean $\pm$ SEM.

3. Results

3.1. *Pcamp1* codes for a novel putative AMP

The application of a signal peptide from an Amoebozoa species (pore-forming

peptide amoebapore A from an microaerophilic parasitic protozoan) as a query in a BLASTP search of protein databases for *Paramecium* resulted in 2 hits. When these hits were used as queries in a BLASTP search of protein databases for *Paramecium tetraurelia*, it resulted in identification of two hypothetical proteins (XP_001431782.1 and XP_001452803.1) that were predicted to contain 202 and 220 amino acid residues, respectively, and showed characteristics of putative AMPs. One of the genes encoding the homologue of XP_001431782.1, *Pcamp1*, was then cloned from *P. caudatum*. The open reading frame (ORF) of *Pcamp1* was 606 bp long, and coded for a polypeptide of 201 amino acid residues (Supplementary data 1). Sequence alignment showed that PcAMP1 was 93% identical to the hypothetical protein sequence (XP_001431782.1) from *P. tetraurelia*, with 14 out of 201 residues being different (Supplementary data 2), suggesting that PcAMP1 was a putative AMP. Sequence analyses also showed that like ovalbumin which contains an internal signal sequence between residues 21 and 47, PcAMP1 also had a putative internal signal sequence of 18 amino acid residues positioned between residues 93 and 110. In addition, analysis by Antimicrobial Peptide Calculator and Predictor at APD revealed that the C-terminal 91 amino acid residues of PcAMP1, cPcAMP1, had a total hydrophobic ratio of 61% and a net charge of +4 (Fig. 1a), which are characteristic of AMPs, while the N-terminal 92 amino acid residues of PcAMP1 showed no such characteristics (Supplementary data 3). Interestingly, analysis by PeptideCutter and CAMP revealed the presence of a proteinase K cleavage site ¹⁷⁶PP¹⁷⁷ in the C-terminal region of cPcAMP1 (Fig. 1a), which implicates the release of a possible mature AMP consisting of the C-terminal

26 amino acid residues, cPcAMP1/26, with a total hydrophobic ratio of 57% and a net charge of +3 (Fig. 1a). The 3D modeling showed that cPcAMP1 and cPcAMP1/26 comprised 3 α -helices and 1 α -helix (Fig. 1b and c), respectively. Analysis by helical wheel projection indicated that both cPcAMP1 and cPcAMP1/26 had the properties to adopt an amphipathic α -helix structure (Fig. 1d). Collectively, these physicochemical and structural properties of cPcAMP1 and cPcAMP1/26 well fit into the characteristics of AMPs. Furthermore, comparison of cPcAMP1 and cPcAMP1/26 with all the sequences in the online updated Antimicrobial Peptide Database APD (<http://aps.unmc.edu/AP/main.php>) revealed that they showed no similarity at all to known AMPs, thus they were likely novel AMPs, and thus characterized functionally in the following experiments.

3.2. Induction of *Pcamp1* expression by LPS

The dissociation curve of amplification product showed a single peak, indicative of specific amplification (Data not shown). As shown in Fig. 2, challenge with LPS resulted in a significant up-regulation of *Pcamp1* expression at 32 to 48 h. This suggested that PcAMP1 was involved in the immune response elicited by LPS in *P. caudatum*, agreeing with our expectation that PcAMP1 was a potential AMP.

3.3. Structural characteristics of cPcAMP1 and cPcAMP1/26

Recombinant cPcAMP1 with the His tag expressed in *E. coli* BL21 was purified by chromatography on a Ni-NTA resin column, and analyzed by SDS-PAGE, which yielded a single band of approximately 28.2 kDa (Supplementary data 4), well matching the expected sizes (28.2 kDa calculated by Editseq). Western blotting

showed that recombinant cPcAMP1 reacted with mouse anti-His tag antibody, indicating that it was correctly expressed (Supplementary data 4). The sequence of cPcAMP1/26 synthesized using standard solid-phase Fmoc method was confirmed by MS/MS (Supplementary data 5).

To investigate alterations of the secondary structures of recombinant cPcAMP1 and synthesized cPcAMP1/26, CD spectroscopy was conducted in 50 mM Tris-HCl buffer with or without 2,2,2-trifluoroethanol (TFE), sodium dodecyl sulfate (SDS) micelles, and dodecylphosphocholine(DPC) micelles, or in PBS with or without TFE, SDS micelles, and DPC micelles. As shown in Fig. 3, both recombinant cPcAMP1 and synthesized cPcAMP1/26 showed α -helical properties in membrane-mimicking environments (50% TFE, 50 mM SDS or 50 mM DPC) but a random coil pattern in Tris-HCl buffer or PBS.

3.4. cPcAMP1 and cPcAMP1/26 are bactericidal agents

We then tested the antimicrobial activity of recombinant cPcAMP1 and synthesized cPcAMP1/26 against the Gram-negative bacterium *A. hydrophila* (ATCC 35654) and the Gram-positive bacterium *S. aureus* (ATCC 25923). It was found that recombinant cPcAMP1 was capable of significantly killing both *A. hydrophila* and *S. aureus* ($p<0.01$) at all the concentrations (6.25, 12.5 and 25 $\mu\text{g/ml}$) tested, with the bactericidal rates being 38.5%, 52.1% and 77.4% as well as 26.8%, 34.1% and 42.2%, respectively (Fig. 4a and b). It was clear that the bactericidal activity of recombinant cPcAMP1 was more active against *A. hydrophila* than *S. aureus*. Similarly, at concentrations of 6.25, 12.5 and 25 $\mu\text{g/ml}$, cPcAMP1/26 was capable of markedly

killing *A. hydrophila* and *S. aureus* ($p < 0.05$), with the bactericidal rates being 27.7%, 31.7% and 40.1% as well as 34.4%, 47.4% and 49.2%, respectively (Fig. 4c and d). These data together showed that recombinant cPcAMP1 and synthesized cPcAMP1/26 possessed bactericidal activity towards *A. hydrophila* and *S. aureus*.

Next, we determined the effects of recombinant cPcAMP1 and synthesized cPcAMP1/26 on fine structures of *A. hydrophila* or *S. aureus* cells by co-incubation with the peptides, followed by transmission electron microscopy (TEM) examination. It was found that recombinant cPcAMP1 and synthesized cPcAMP1/26 caused a direct damage to the cells of *A. hydrophila* and *S. aureus* (Fig. 4e-l), resulting in membrane disruption. Cytoplasmic leakage was also been observed in both bacteria treated with recombinant cPcAMP1 or cPcAMP1/26. These showed that recombinant cPcAMP1 and synthesized cPcAMP1/26 are bactericidal agents capable of directly killing *A. hydrophila* and *S. aureus*.

3.5. cPcAMP1 and cPcAMP1/26 bind to LPS and LTA

An enzyme linked immunosorbent assay (ELISA) was carried out to test the interaction of recombinant cPcAMP1 and synthesized cPcAMP1/26 with LPS and LTA. As shown in Fig. 5, recombinant cPcAMP1 and synthesized cPcAMP1/26 were both able to bind to both LPS and LTA, and the binding was dose-dependent and saturable, whereas bovine serum albumin (BSA) showed little affinity to LPS and LTA. These indicated that recombinant cPcAMP1 and synthesized cPcAMP1/26, in addition to being bactericidal agents, are also able to bind to microbe-associated molecular pattern molecules such as LPS and LTA.

3.6. cPcAMP1 interacts with bacterial membranes

To confirm the interaction of recombinant cPcAMP1 with the bacteria, we extracted the bacterial membrane proteins of *A. hydrophila* and *S. aureus* that had been treated with recombinant cPcAMP1 or 32a-His-tag, and subjected to Western blotting analysis. As shown in Fig. 5e, the extracted membrane proteins of *A. hydrophila* and *S. aureus* treated with cPcAMP1 were reactive with the anti-His tag mouse monoclonal antibody, while the membrane proteins of the same bacteria treated with 32a-His-tag was not. These suggested that recombinant cPcAMP1 could specifically interact with the membranes of *A. hydrophila* and *S. aureus*.

3.7. cPcAMP1 and cPcAMP1/26 causes membrane depolarization and permeabilization

The membrane depolarization activities of recombinant cPcAMP1 and synthesized cPcAMP1/26 were assayed using 3,3'-dipropylthiobarbituric acid iodide (DiSC₃₋₅, a potential-dependent distributional fluorescent dye). As shown in Fig. 6a-d, the fluorescence intensity of both *A. hydrophila* and *S. aureus* cells treated with recombinant cPcAMP1 or cPcAMP1/26 increased significantly, compared with control, indicating that recombinant cPcAMP1 and synthesized cPcAMP1/26 caused depolarization of the bacterial plasma membrane. Flow cytometry was then used to test if recombinant cPcAMP1 and synthesized cPcAMP1/26 can permeabilize the membranes of *A. hydrophila* and *S. aureus* cells. As shown in Fig. 7, few cells of *A. hydrophila* and *S. aureus* in control group showed propidium iodide (PI; a DNA-intercalating agent and a fluorescent molecule) fluorescent signal, suggesting

that they had intact and viable cell membranes. By contrast, a significant proportion of *A. hydrophila* and *S. aureus* cells treated with recombinant cPcAMP1 or cPcAMP1/26 displayed PI fluorescent signal, implicating that the membranes of *A. hydrophila* and *S. aureus* cells were permeabilized by recombinant cPcAMP1 or cPcAMP1/26. Collectively, these data showed that recombinant cPcAMP1 and synthesized cPcAMP1/26 disrupted the bacterial membranes by a membranolytic mechanism including a combined action of membrane depolarization and membrane permeabilization.

3.8. cPcAMP1 and cPcAMP1/26 increase intracellular ROS levels

High intracellular levels of ROS can cause apoptosis or necrosis. When *A. hydrophila* or *S. aureus* cells were treated with recombinant cPcAMP1 or cPcAMP1/26, their intracellular ROS levels were significantly increased (Fig. 8). These indicated that both recombinant cPcAMP1 and cPcAMP1/26 were possible to induce apoptosis/necrosis of the bacterial cells.

3.9. cPcAMP1 and cPcAMP1/26 are not toxic to mammalian cells

To test if recombinant cPcAMP1 and synthesized cPcAMP1/26 were cytotoxic, their hemolytic activities on human red blood cells (RBCs) were determined. As shown in Fig. 9, recombinant cPcAMP1 and synthesized cPcAMP1/26 showed little hemolytic activity towards human erythrocytes at all the concentrations tested. By contrast, RBCs incubated with 0.1% Triton X-100 (full lysis control) exhibited significant hemolysis. In addition, the cytotoxicity of recombinant cPcAMP1 and cPcAMP1/26 to murine RAW264.7 cells was also tested using MTT. As shown in

Table 2 and 3, recombinant cPcAMP1 and synthesized cPcAMP1/26 were not toxic to murine RAW264.7 cells at the concentrations tested. These data showed that recombinant cPcAMP1 and synthesized cPcAMP1/26 were non-cytotoxic to mammalian cells, suggesting that recombinant cPcAMP1 and synthesized cPcAMP1/26 have preference for bacterial membranes.

4. Discussion

Taking the advantage of signal sequence conservation of known AMPs to search for putative AMPs has been successfully applied in the same lineages of both fish and amphibians from the EST database (Tessera et al., 2012; Juretić et al., 2011). We show in this study that such an approach is applicable to the different lineages for we have identified a putative ciliate AMP (XP_001431782.1) from the database of *P. tetraurelia* using the signal sequence of pore-forming peptide ameobapore A precursor, an AMP from a Amoebozoa species *E. histolytica*, and accordingly cloned a gene encoding an AMP, PcAMP1, from a species of ciliate *P. caudatum*. The deduced protein PcAMP1 had a putative internal signal sequence of 18 amino acid residues positioned between residues 93 and 110, as reported in ovalbumin, and its C-terminal 91 amino acid residues cPcAMP1 had a total hydrophobic ratio of 61% and a net charge of +4. The predicted mature AMP, cPcAMP1/26, had a total hydrophobic ratio of 57% and a net charge of +3. Both cPcAMP1 and cPcAMP1/26 possessed the properties to adopt amphipathic α -helix structure, which is very common in AMPs available. As cPcAMP1 and cPcAMP1/26 showed no significant similarity to all known AMPs from related sources at amino acid level, thus they

likely represent an uncharacterized AMP.

CD spectroscopy revealed that both cPcAMP1 and cPcAMP1/26 displayed spectra consistent with random coil structure in Tris-HCl buffer and PBS, which underwent a coil-to-helix transition in the presence of TFE, SDS or DPC. This clearly confirms that our expectation that cPcAMP1 and cPcAMP1/26 have an α -helical properties, which often have critical roles for the binding to microorganisms, providing additional evidences that cPcAMP1 and cPcAMP1/26 are AMPs. Therefore, we have purified recombinant cPcAMP1 and synthesized cPcAMP1/26, and then subjected to antibacterial/bactericidal activity assay, which proved that they not only inhibited the growth of *A. hydrophila* and *S. aureus* but also caused direct damage to the bacteria. These data present functional evidences that show cPcAMP1 and cPcAMP1/26 are indeed AMPs with an antibacterial/bactrericidal activity.

AMPs kill microbes via several modes of actions, including binding to or inserting into microbial membranes, which had fatal depolarization of the normally polarized membrane, formation of physical pores, scrambling of the usual distribution of lipids between the leaflets of the bilayer, and damage to critical intracellular targets. We showed that cPcAMP1 and cPcAMP1/26 had strong affinity to LPS from Gram-negative bacteria and LTA from Gram-positive bacteria, suggesting they can bind to the microbial surfaces via interaction with LPS and LTA. This is apparently corroborated by the observations that cPcAMP1 was able to deposit onto the bacterial membranes of *A. hydrophila* and *S. aureus*. Using DiSC₃₋₅, an indicator of membrane potential, we demonstrated that the membranes of *A. hydrophila* and *S. aureus* cells

exposed to cPcAMP1 and cPcAMP1/26 were both depolarized. When *A. hydrophila* and *S. aureus* cells were incubated with PI, a fluorescent dye that intercalates with DNA but cannot cross intact cell membranes, an increase in fluorescence was observed in both cPcAMP1- and cPcAMP1/26-treated cells, indicating cell death via membrane permeabilization (Mortimer et al., 2000) and breach of the plasma membrane (Pilszczek et al., 2010). Moreover, treatment of *A. hydrophila* and *S. aureus* with cPcAMP1 or cPcAMP1/26 resulted in marked increase in their intracellular ROS levels, indicating induction of apoptosis/necrosis of *A. hydrophila* and *S. aureus* by cPcAMP1 and cPcAMP1/26. Taken together, our results suggest that cPcAMP1 and cPcAMP1/26 fulfill bactericidal functions at several levels: they bind to cytoplasmic membranes via interaction with LPS and LTA, cause membrane depolarization and membrane permeabilization, and induce apoptosis/necrosis, eventually leading to the bacterial death.

A crucial point regarding the development of antimicrobial therapeutics is that they must not destroy the membrane of mammalian cells. We showed here that compared with their antimicrobial activity, cPcAMP1 and cPcAMP1/26 have virtually no cytotoxic activity towards human RBCs and murine RAW264.7 cells. This implies that cPcAMP1 and cPcAMP1/26 have high membrane selectivity towards bacterial cells but not mammalian cells.

In summary, this study reports the identification of an uncharacterized AMP from *Paramecium* via *in silico* and functional analyses, presenting that the conserved sequences of signal peptides of AMPs can be used to identify potential AMPs across

different animal classes. This uncharacterized AMP as well as its predicted mature AMP can both kill the Gram-negative bacterium *A. hydrophila* and the Gram-positive bacterium *S. aureus* via several modes of actions including membrane active mechanism and increasing intracellular ROS levels, but they are non-cytotoxic to mammalian cells, rendering them promising templates for the design of novel peptide antibiotics against MDR microbes.

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Figure legends

Figure 1. Amino acid sequence, 3D molecular modeling and helical wheel projection.

(a) Amino acid composition distribution of cPcAMP1 and cPcAMP1/26. The putative cleavage site is indicated by (*). (b) 3D structure model of cPcAMP1. (c) 3D structure model of cPcAMP1/26. (d) The helical wheel projection of three α -helical fragments of cPcAMP1, demonstrating amphipathic potential of the peptide structure. Hydrophilic residues are presented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. Hydrophobicity is color coded: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic (uncharged) residue, and the amount of red decreasing proportionally to the hydrophilicity. The potentially charged residues are light blue.

Figure 2. LPS-induced expression of *Pcamp1*. *P. caudatum* were challenged with 10 μ g/ml LPS, and sampled at 0, 2, 8, 16, 32 and 48 h after the challenge. The expression of *Pcamp1* was determined by qRT-PCR. The expression level at 0 hour was set as 1. Values were shown as mean $\pm$ SEM (n = 3). The symbols (***) and (*) indicate $p < 0.001$ and $p < 0.05$, respectively, compared with the control group.

Figure 3. Circular dichroism spectra of cPcAMP1 and cPcAMP1/26 (100 μ g/ml) in the presence of 50% TFE, 50 mM SDS and 50 mM DPC. The peptides cPcAMP1 and cPcAMP1/26 had α -helical properties in the presence of 50% TFE, 50 mM SD and 50 mM DPC but a random coil pattern in Tris-HCl buffer or PBS.

Figure 4. Antibacterial activity of cPcAMP1 and cPcAMP1/26 and their effects on bacterial structures. (a) Antibacterial activity of cPcAMP1 against *A. hydrophila*. (b)

Antibacterial activity of cPcAMP1 against *S. aureus*. (c) Antibacterial activity of cPcAMP1/26 against *A. hydrophila*. (d) Antibacterial activity of cPcAMP1/26 against *S. aureus*. (e and f) *A. hydrophila* and *S. aureus* incubated with Tris-HCl buffer, without membrane disruption observed. (i and j) *A. hydrophila* and *S. aureus* incubated with cPcAMP1, with membrane disruption observed. (g and h) *A. hydrophila* and *S. aureus* incubated with PBS, without membrane disruption observed. (k and l) *A. hydrophila* and *S. aureus* incubated with cPcAMP1/26, with membrane disruption observed. The symbols (***), (**) and (*) indicate $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively, compared with the control group.

Figure 5. Binding of cPcAMP1 to LPS (a) and LTA (b). Binding of cPcAMP1/26 to LPS (c) and LTA (d). BSA was used as control. (e) Presence of cPcAMP1 in the extracted bacterial cell membrane proteins was revealed by Western blotting. (1) Recombinant cPcAMP1. (2) *S. aureus* treated with 32a-His-tag. (3) *S. aureus* treated with cPcAMP1. (4) *A. hydrophila* treated with 32a-His-tag. (5) *A. hydrophila* treated with cPcAMP1.

Figure 6. Bacterial membrane depolarization. Depolarization of *A. hydrophila* and *S. aureus* cell membranes were detected using DiSC₃₋₅ (excitation, 622 nm; emission, 670 nm).

Figure 7. Bacterial membrane permeabilization. Effects of cPcAMP1 and cPcAMP1/26 on the membrane integrity of *A. hydrophila* and *S. aureus* cells analyzed by flow cytometry. The proportion of integrated cells was shown near the R1.

Figure 8. Effects of cPcAMP1 and cPcAMP1/26 on intracellular ROS levels. (a) *A.*

hydrophila treated with cPcAMP1. (b) *S. aureus* treated with cPcAMP1. (c) *A. hydrophila* treated with cPcAMP1/26. (d) *S. aureus* treated with cPcAMP1/26. Rosup, a compound mixture, is able to significantly increase ROS levels in cells within 30 min.

Figure 9. Hemolytic activity of cPcAMP1 and cPcAMP1/26 to human blood cells (RBCs). Data were expressed as mean $\pm$ SEM (n = 3). The bars represent the standard error of the mean values. The symbol (***) indicates $p < 0.001$ compared with the Triton-X 100 treated group.

Tables

Table 1. Sequences of the primers used in this study.

Primers	Primer sequences (5'–3')
P1-S	TTCCCAAAGAACAAGAGG
P1-AS	ATGCCATAAAGCCAACAG
P2	TGGTCGGCATATTCATCT
P3	GCAGAATCAACTAATAGTACAAAAT
<u>P4-S</u>	<u>CTCTAAATGCTTAATTGCTAGGAGGG</u>
<u>P4-AS</u>	<u>AGCCAACAGATGAATATGCCGAC</u>
<u>P5-S</u>	<u>CACTTGCTCCATCATCCA</u>
<u>P5-AS</u>	<u>TTTCGTCGTATTCGCTTC</u> <u>CGGAATTCCAATTGCTAGGAGGGAA</u>
<u>P6</u>	AGTATCAT <u>CCGCTCGAGTACTATCAAAGAAAAC</u>
<u>P7</u>	CATGAC

Table 2. The percent viability of RAW264.7 cells in the presence of cPcAMP1.

Concentration (μ g/ml)	Percent viability (%)
0	100
12.5	106 $\pm$ 2

25	106 ± 1
50	105 ± 1
100	112 ± 4

Table 3. The percent viability of RAW264.7 cells in the presence of cPcAMP1/26.

Concentration (µg/ml)	Percent viability (%)
0	100
12.5	116 ± 7
25	112 ± 3
50	114 ± 8
100	110 ± 6

a cPcAMP1 QLLGGKVSFFQSVCLLGYCIPINIEAIVIAFFGSYLPFVVKLIPVFICFAWSAYSSVGFMASLVPPHKKKLAVYPVFLFYFLSWFSLIV-COOH
 1 66 91

cPcAMP1/26

PPHKKKLAVYPVFLFYFLSWFSLIV

b

c

d

N-terminal residues 1-35

N-terminal residues 36-65

N-terminal residues 66-91

Pcamp1 expression