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Research paper

Functional characterization of a synthetic hydrophilic antifungal peptide derived from the marine snail *Cenchritis muricatus*

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ABSTRACT

Antimicrobial peptides have been found in mollusks and other sea animals. In this report, a crude extract of the marine snail *Cenchritis muricatus* was evaluated against human pathogens responsible for multiple deleterious effects and diseases. A peptide of 1485.26 Da was purified by reversed-phase HPLC and functionally characterized. This trypsinized peptide was sequenced by MS/MS technology, and a sequence (SRSELIVHQR), named Cm-p1 was recovered, chemically synthesized and functionally characterized. This peptide demonstrated the capacity to prevent the development of yeasts and filamentous fungi. Otherwise, Cm-p1 displayed no toxic effects against mammalian cells. Molecular [illegible] probable [illegible] monstrate [illegible] could be useful in solving human health and agribusiness problems.

1. Introduction

In the last three decades, several agents of new infectious diseases have been identified, some of which are responsible for entirely novel and life-threatening disorders [1,2]. A lack of new antibiotics for treatment of illnesses associated with the appearance of multi-drug-resistant strains has demanded the urgent development of innovative strategies for the control of microorganisms [3]. Certain human fungal infections, such as those caused by *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Candida albicans*, are gaining importance due to the increasing number of immunocompromised patients [4]. Thus far, existing treatments for these infections are limited to only a small number of antifungal drugs, such as azoles, echinocandins, and polyenes [5]. Indeed, pathogenic fungi possess many complicated mechanisms for resisting these drugs, constituting a critical challenge for antifungal therapy. The development of novel therapeutic agents may overcome this problem [6]. Additionally, because the search for original and useful antifungals has been limited, especially for drug-resistant pathogenic fungi, screening for novel antifungal peptides in multiple biomes could help to reduce infections in plants and animals.

Antimicrobial peptides (AMPs) can exhibit a broad spectrum of activities against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria [7], yeasts [8], fungi [9], viruses [10], protozoa [11] and parasites, such as nematodes [12]. Several mechanisms of action have been proposed for these molecules [13], which indicate that many of them have more than one antimicrobial target at the cellular level [14]. Initially, peptides can interact with the cell membrane, causing an increase in permeability concomitant with a loss of membrane function. Moreover, peptides can affect intracellular targets, such as the nucleus and its DNA, leading to apoptosis [15].

There are multiple sources of antimicrobial peptides, including plants, mammals and invertebrates. Invertebrates base their

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humoral defense against infectious agents on a wide and effective repertoire of AMPs that may interact directly with microbes or with their toxic molecules [16]. Due to this fact, many researchers have used mollusks and other marine sea animals as sources for the development of novel antimicrobials [16]. Mollusks are an abundant and significant group in the trophic chain of the animal kingdom. Among the mollusks, gastropods, including snails and slugs, represent the most abundant class. Snails in particular are successful animals from an evolutionary point of view, due to their capacity to adapt to different environments and to reach dry land [17]. Although antimicrobial peptides have been purified from several marine invertebrates, only a few have been reported in mollusks [16]. Most research has focused on species such as *Mytilus edulis* Linnaeus, 1758 and *Mytilus galloprovincialis* Lamarck, 1819. These species are capable of synthesizing defensins, mytilins and the antifungal peptide mitomycin [16].

Here, we report the isolation and biochemical characterization of different forms of the antimicrobial peptide fragments from *C. muricatus*. To expand our functional analyses, a lower-molecular-weight peptide, Cm-p1, was synthesized and further evaluated regarding its antimicrobial actions toward multiple fungi and mammalian cells. An *in silico* evaluation was also conducted, which showed that the molecular surface and the presence of several residues could be essential for the peptide's antifungal activity.

2. Materials and methods

2.1. Extraction and isolation of proteinaceous compounds

C. muricatus Linnaeus, 1758 (Mollusca: Gastropoda) snails were hand-collected on the northern coast of Havana, Cuba. *C. muricatus* snails were homogenized in a solution containing 0.6 M NaCl and 0.1% HCl (2:1 [w/v]) in a blender. The homogenate was centrifuged at 10,000 × g for 30 min at 4 °C. Soluble proteins were precipitated with $(NH_4)_2SO_4$ at 100% saturation with constant stirring for 1 h at 4 °C. After centrifuging again at 10,000 × g for 30 min at 4 °C, the precipitate was resuspended in 10 mM Tris–HCl buffer, pH 8.0 and was further desalted using PD MidiTrap G-10 columns (GE Healthcare, USA). The resulting desalted, protein-rich fraction was separated into low- (<10 kDa) and high-molecular-weight fractions (>10 kDa) using Amicon Ultra-15 (10 K) centrifugal filter devices (Millipore, USA). The low-molecular-weight fraction was lyophilized, and 3.0 mg of the dry fraction was dissolved in 0.1% trifluoroacetic acid (TFA) and then applied onto a reversed-phase HPLC Vydac C18-TP analytical column (Hesperia, CA, USA) equilibrated with 0.1% TFA. Retained proteins were eluted with a linear methanol gradient (0–100%) at a flow rate of 1.0 ml min^{-1}. Protein detection was performed at 280 nm.

2.2. Protein identification by mass spectrometry

After elution, a peptide digestion was performed using sequencing grade modified trypsin (Promega, USA) according to [18] with minor modifications. Briefly, 600 ng of buffered trypsin was added to the peptide and incubated on ice for 30 min. Then 40 μL of 50 mM NH_4HCO_3 was added, and the solution was incubated at 37 °C for 22 h. The digestion supernatant was collected and stored at −20 °C. The peptides derived from the tryptic digestion were analyzed using an UltraFlex III MALDI-TOF/TOF (Bruker Daltonics) precisely calibrated with peptide calibration standard II (Bruker Daltonics). A 1 μL sample was mixed with 3 μL of matrix solution (1% [w/v] α-cyano-4-hydroxycinnamic acid, 3% [v/v] trifluoroacetic acid and 50% [v/v] acetonitrile) and applied onto a MALDI target plate (1 μL in duplicate). After crystallization at room temperature, samples were analyzed using a spectrometer operated in reflector mode for MS acquisitions and LIFT mode for tandem MS (MS/MS). Protein identification was achieved by peptide mass fingerprinting (PMF) and manual *de novo* sequencing. The sequenced peptide was directly aligned at the non-redundant protein database (NCBI) and APD data bank similarity searches with bioactive peptides.

2.3. Amino acid sequence analysis by automated Edman degradation

The amino acid sequences of the peptides were analyzed by automated Edman degradation. Microsequencing was performed using a Hewlett–Packard 1000A protein sequencer equipped with a HPLC system.

2.4. Peptide synthesis

Cm-p1 was synthesized by the solid-phase method using 9-fluorenyl-methoxycarbonyl chemistry [19], purified by reverse-phase high-performance liquid chromatography to >98% purity on an acetonitrile/H_2O-TFA gradient and confirmed by ion-spray mass spectrometry (Micromass, Manchester, United Kingdom).

2.5. Determination of protein concentration

Protein concentrations were estimated using Coomassie Blue staining [20]. Bovine serum albumin (BSA, 0.1 mg ml^{-1}) was used as the standard protein. All determinations were performed in triplicate.

2.6. Bioassays against bacteria

Pathogenic bacteria were cultured in 2.0 mL of LB (Luria-Bertani) broth (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract and 45 g L^{-1} bacto peptone) for 18–24 h at 37 °C. Protein-rich fractions, the purified peptides and Cm-p1 were resuspended in distilled water and filtered through 0.22-μm nylon membranes. Dilution series for all samples were prepared for an initial concentration of 200 μg ml^{-1}. Samples were incubated for 6 h at 37 °C with 5×10^6 CFU mL^{-1} of each bacterium tested. The assayed bacteria were *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* sp., and *Shigella* sp. These microorganisms were obtained from the microbiology collection of the Universidade Católica de Brasília. Sterile distilled water and chloramphenicol (40 μg mL^{-1}) were used as the negative and positive controls, respectively. Bacterial growth was determined spectrophotometrically at 595 nm every hour during the incubation period according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [21].

2.7. Bioassays against fungi

Bioassays against fungi were performed using the broth microdilution method [22]. *Fusarium oxysporum*, *Botrytis cinerea*, and *Aspergillus niger* were obtained from the Universidade Católica Collection and were grown on solid medium potato dextrose broth (Difco, USA). Liquid potato dextrose broth was used for the inhibition assays against these fungi. Amphotericin-B (30 μg ml^{-1}) was used as the positive control, and liquid potato dextrose broth was used as the negative control. The bioassays against human pathogenic yeast (*Candida parapsilosis* [ATCC 22019] and clinically isolated strains of *C. albicans* [01U, 38U], *C. neoformans* [L26, L30], *Trichophyton mentagrophytes* [28d, 28e] and *Trichophyton rubrum* [329]) were performed in RPMI-1640 medium (Gibco BRL, USA). Itraconazole and fluconazole (0.5–4 μg ml^{-1}) were used as the positive controls. Sterile distilled water was used as the negative

control. The minimum inhibitory concentrations (MICs) were obtained according to the CLSI guidelines [23]. All tests were conducted in triplicate.

2.8. Hemolytic assay

Cm-p1 hemolytic activity was evaluated by determining the release of hemoglobin from an 8% suspension of fresh human erythrocytes at 414 nm with an ELISA plate reader [24]. The assay was performed with different concentrations of Cm-p1 (0–400 μg ml^{-1}). The percentage of hemolysis was calculated using the following equation: % hemolysis = [($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)] × 100. PBS was used as the negative control, and Triton X-100 was used as the positive control. All assays were performed in triplicate.

2.9. Cytotoxicity assay

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; Sigma–Aldrich, USA) assay was performed according to [25]. RAW 264.7 murine macrophage-like cells (Rio de Janeiro Cell Bank, Brazil) were plated at a concentration of 1×10^5 cells per well in supplemented DMEM medium (4 mM glutamine, 10% FCS and 100 units/ml penicillin/streptomycin) and incubated with different concentrations of peptides (0–400 μg ml^{-1}). After overnight incubation, 10 μl of the MTT solution (5 mg mL^{-1} in PBS) was added to each well. Plates were incubated for 4 h in 5% CO_2 at 37 °C. The generated blue formazan product was dissolved by the addition of 100 μl of 100% DMSO (Mallinckrodt Chemical, USA) per well. The absorbance was monitored at 575 nm in an ELISA plate reader (Bio-Tek, USA).

2.10. In silico analyses and molecular modeling

Initially, the physiochemical parameters were calculated using the software ProtParam from the ExPASy server [26]. ClustalW was used for aligning multiple sequences [27]. Helical wheel projection was performed using the tool wheel from Gromacs software [28]. PSI-BLAST was used to find the best templates for homology modeling, but no results were returned. Alternatively, data mining in a non-redundant database (APD2) highlighted reliable templates [29]. The Protein Data Bank (PDB) structure 2RMI showed 22% of identical residues and was thus used as the template. Template utilized was astressin, which is able to binds to CRF-R1, a G-protein coupled receptor that specifically binds corticotropin-releasing hormone, at low nanomolar affinity [30]. Fifty theoretical three-dimensional peptide models were constructed with Modeller v.9.8 [31] using the selected template. The final models were evaluated according to geometry, stereochemistry, and energy distributions. PROSA II was utilized to analyze packing and solvent exposure characteristics, and PROCHECK was used for additional stereochemical quality checks [32,33]. Additionally, the root mean square deviation (RMSD) was calculated from the overlap of Cα traces and backbones onto the template structure using the program 3DSS [34]. In order to confirm model reliability, I-Tasser and HHPred were also utilized for additional tridimensional model construction according multiple-threading alignments and iterative structural assembly simulations [35,55]. The RMSD among model construct by MODELLER, I-Tasser and HHPred server were calculated from the overlap of Cα traces and backbones onto the template structure using the program 3DSS. Electrostatic surfaces were calculated with the ABPS tool [36]. The protein structure was visualized and analyzed with PyMOL (Delano Scientific) [37].

3. Results

3.1. Identification of antimicrobial peptides

First, the low-molecular-weight fraction (<10 kDa) of the 100% ammonium sulfate precipitation pellet was applied onto a reversed-phase HPLC C-18 column. Proteins and peptides were eluted using a linear methanol gradient (0–100%), which yielded 14 fractions (Fig. 1A). The antimicrobial activities of all peaks were evaluated against diverse pathogens. The second fraction was eluted with 49.4% methanol and showed the highest antimicrobial activity (data not shown).

3.2. Amino acid sequencing and bioinformatics

To identify the fraction responsible for the higher activity, fraction 2 was analyzed by MALDI-TOF/TOF. Fraction 2 consisted of a single peptide that exhibited an *m*/*z* value of 1485 (Fig. 1B). It was not possible to determine the sequence of this peptide by MS/MS; therefore, the peptide was digested with trypsin. The digestion yielded various peptide fragments that exhibited a more intense ion at an *m*/*z* value of 1224 (data not shown). This ion was then sequenced by MS/MS and yielded the 10-amino acid peptide SRSELIVHQR. This peptide fragment was named Cm-p1 and was compared to the AMP database [29]. The comparison of the primary structure of Cm-p1 showed clear identity with other antimicrobial peptides. Cm-p1 shared 30% and 40% identity with urechistachykinin I and hyposin-H5, respectively. Moreover, we also observed a sequence relationship between Cm-p1 and different members of the hyposin family (Fig. 2). Additionally, similarity was observed with Vesp-VB1 from the invertebrate *V. bicolor*, which exhibits both antifungal and bactericidal activities (data not shown) [38]. In all cases, we noted a conserved hydrophobic core (italicized

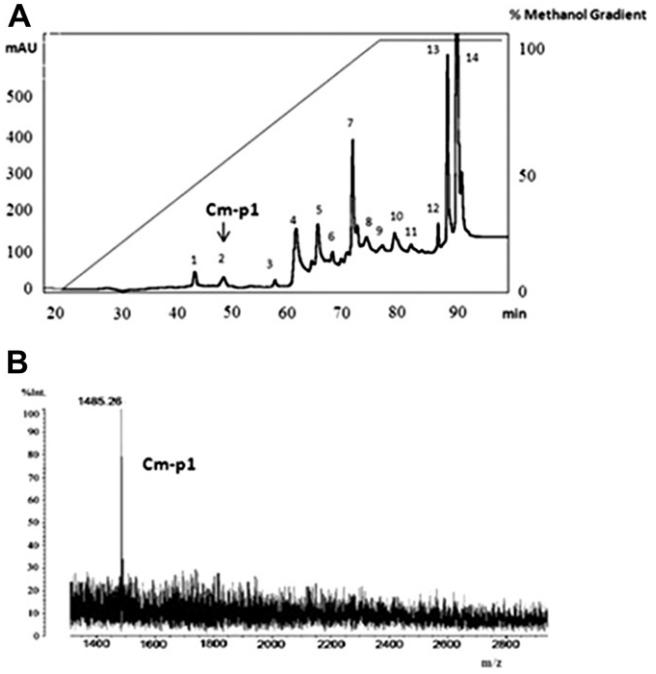

Fig. 1. A: HPLC reversed-phase chromatogram profile (Vydac C18-TP) of the low-molecular-weight fraction from *Cenchritis muricatus* snails. The diagonal line indicates a linear methanol gradient. B: Mass spectrum of Cm-p1 isolated by HPLC in a MALDI-ToF/ToF mass spectrometer operating in reflector mode and using a matrix of α-cyano-4-hydroxycinnamic acid.

residues, Fig. 2). Furthermore, cationic residue number 10 (bold type, Fig. 2), which ended the hydrophobic region, was also conserved in all the antimicrobial peptides we analyzed. In parallel, a similar purification procedure was performed using the same crude extract, and a peptide with an *m*/*z* value of 5282 was also isolated (data not shown). This peptide, named Cm-p2, was partially sequenced by automated Edman degradation and yielded the sequence SESILIVHQQQSSRSSGS. Cm-p1 and Cm-p2 share 70% identity between them, suggesting that they could be two members of a single family of antimicrobial peptides or could be significantly correlated.

3.3. Antimicrobial activity

To demonstrate the antimicrobial activity of Cm-p1, the peptide was synthesized in solid-phase. Despite the high bactericidal activity of the *C. muricatus* crude extract, synthesized Cm-p1 was incapable of inhibiting either the growth of Gram-negative bacteria (*E. coli* and *Klebsiella pneumoniae*) or the development of Gram-positive bacteria (*S. aureus*) (data not shown). Nevertheless, a broad spectrum of antifungal activity was observed for the synthetic peptide (Table 1). Cm-p1 was active against filamentous fungi and yeasts of both medical and agricultural interest. As Table 1 shows, a lower MIC was obtained against *T. rubrum* (4 μg ml^{-1}), whereas a higher MIC was obtained against different clinical isolates of *C. neoformans* (256 μg ml^{-1}).

3.4. Toxicity against mammalian cells

To evaluate the toxicity of Cm-p1 against mammalian cells, different doses of the peptide were incubated in the presence of human erythrocytes. No concentrations of Cm-p1 were capable of provoking hemoglobin release (data not shown). Additionally, the viability and proliferation of cultured RAW 264.7 cells incubated with different doses of Cm-p1 were evaluated using the MTT reagent. The viability and proliferation of RAW 264.7 cells were unaffected by Cm-p1 (Fig. 3).

3.5. Theoretical structural analysis

Initial analyses show that Cm-p1 is a hydrophilic molecule scoring a grand average of hydropathicity (GRAVY) of −0.830 and exhibits a small central hydrophobic region flanked by basic amino acids at the extremes (Fig. 4). Furthermore, Cm-p1 is not an amphipathic molecule, as was demonstrated by helix-wheel projection using the tool wheel of Gromacs software (data not shown). To improve peptide structure analyses, a three-dimensional model of the hydrophilic Cm-p1 peptide was constructed (Fig. 4). The model exhibited a α-helix conformation when generated by three different methodologies as previously described before in session 2.11. The model constructed present cationic arginines (Arg^2 and Arg^{10}) and histidine (His^8) residues and also an exposed hydrophobic region (Leu^5–Val^7) that favors a membrane–peptide interaction (Figs. 2 and 4). The model shared 22% identity with the neuropeptide PDB structure 2RMI [30]. A validation of the three-dimensional model of Cm-p1 by Ramachandran plot showed that 100% of the modeled amino acid residues were in physically acceptable regions for secondary structure formation in relation to the torsion angles phi and psi. The z-score value given in PROSA II was used to verify whether the input structure was within the range of scores typically found for native proteins of similar size. The z-score value was −1.36 and is comparable to NMR structures of antimicrobial peptides of similar length, which have z-scores of 1 to −1.5 [39–41]. The RMSD value obtained for Cm-p1 model acquired by using Modeller strategy was 1.7 Å. Moreover when this helical model was compared with models constructed by I-Tasser and HHPred servers, 0.8 Å RMSD values were obtained. These values clear demonstrate the similar structural conformation among all models proposed and strongly suggests a helical conformation.

Table 1
Antifungal activity of Cm-p1 against different yeasts and mycelial fungi. The minimum inhibitory concentration (MIC) [13] of the peptide is represented for each fungus.

Microorganism	Minimum Inhibitory Concentration (μM)
Botrytis cinerea	20
Fusaryum oxysporum	20
Aspergillus niger	41
Candida albicans 01U	13
Candida albicans 38U	7
Candida parapsilosis	105
Cryptococcus neoformans L26	209
Cryptococcus neoformans L30	209
Trichophyton mentagrophytes	–
Trichophyton rubrum	3

4. Discussion

Functional studies have determined the residues or domains that could be responsible for antimicrobial properties. In many cases, the N-terminal region has been hypothesized to act as an initial membrane anchor [42]. Although structural and functional differences can be observed among the different antimicrobial peptides, some properties could be very common, such as hydrophobicity and amphipathicity [16]. In this regard, Cm-p1 is not a classical antimicrobial peptide. In fact, it is a small, hydrophilic peptide; there is no clear evidence of the amphipathicity properties that are commonly found in other antimicrobial peptides. Indeed, of the 1755 peptides in the AMP database, only three have features similar to Cm-p1. One of these, urechistachykinin I, is a neuropeptide derived from the invertebrate *Urechis unicinctus* that shares 30% identity with Cm-p1 [43]. This peptide exhibits a broad spectrum of antibacterial activity against the Gram-positive bacteria *Streptococcus mutans*, *S. aureus* and *Enterococcus faecium* and the Gram-negative bacteria *E. coli* O-157, *Pseudomonas aeruginosa* and *Vibrio vulnificus* with MIC values in the 10.6–42.5 μM range [43]. Despite the similarities between these peptides, Cm-p1 is not an

Name	Sequence	Species	Activity	Reference
Cm-p1	SRSE***LIV***H**Q**R--------	*C. muricatus*	Antifungal	This report
Cm-p2	SESI***LIV***H**QQQ**SSRSSGS	*C. muricatus*	Not determined	This report
Hyposin-H5	FRPA***LIV***R**T**KGTRL----	*P. hypochondrialis*	Putative antibacterial	[53]
Hyposin-H4	LRPA***VIV***R**T**KGK------	*P. hypochondrialis*	Putative antibacterial	[53]
Hyposin-H2	LRPA***FI***-R**P**KGK------	*P. hypochondrialis*	Putative antibacterial	[53]

Fig. 2. Primary structure alignment of Cm-p1 and Cm-p2 against other antimicrobial peptides from different animal sources. Italicized residues correspond to the conserved hydrophobic region, and bold residues correspond to the conserved cationic residues.

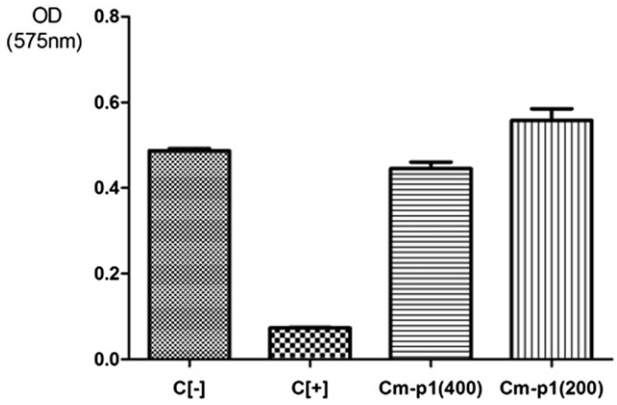

Fig. 3. Effects of Cm-p1 on RAW 264.7 murine macrophage-like cells viability. Different concentrations of peptide were added to 1000 cells per well and incubated for 72 h at 37 °C in 5% CO_2. Triton X-100 was used as a positive control for toxicity, and PBS was used as a negative control. Cm-p1 were assayed at 200 μg ml^{-1} (Cm-p1 200) and at 400 μg ml^{-1} (Cm-p1 400).

antibacterial peptide, possibly due to its lower molecular charge (+1) in comparison to urechistachykinin I (+2). Moreover, hyposin-H5, an antimicrobial peptide identified from the skin secretions of the frog *Phyllomedusa hypochondrialis azurea* [44], shares 40% identity with Cm-p1. Despite the clear identity shared with hyposins (Fig. 2), Cm-p1 exhibits different physiochemical properties,

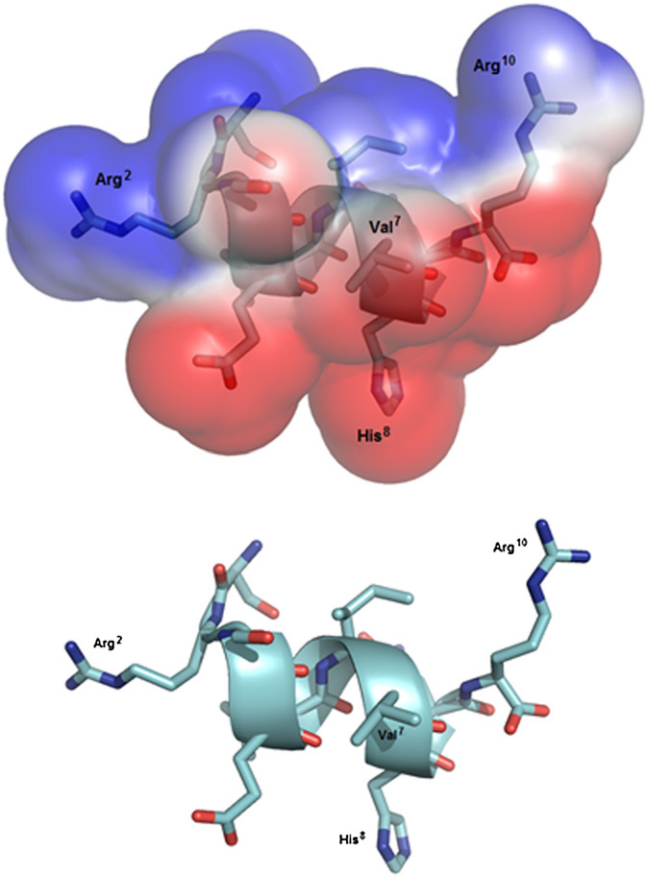

Fig. 4. Three-dimensional structural modeling of Cm-p1. Blue and red regions correspond to the cationic and anionic areas, respectively. Labeled residues may be related to the peptide's possible mechanism of action. The structure was visualized using PyMOL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

such as hydrophobicity (GRAVY: 0.007) and net charge (+4). Another antimicrobial peptide that shares sequence similarity with Cm-p1 is VESP-VB1. This peptide was isolated from the venom of the wasp *Vespa bicolor Fabricius*. Interestingly, despite the low cationicity of VESP-VB1 (+1), this peptide exhibits antimicrobial activity against different isolates of *E. coli* and *S. aureus*, possibly due to the molecule's hydrophobicity (GRAVY: 1.131) [38].

To confirm that the Cm-p1 sequence was derived from *C. muricatus*, we identified a parallel sequence (Cm-p2) that was part of a larger peptide (5282 Da). Cm-p1 shared 70% identity with Cm-p2. The divergence between these sequences could be attributed to the existence of isoforms, which is relatively probable for AMPs, or simply the fragmentation of similar peptides. Even with this divergence, Cm-p1 could be interpreted as a closely related protein sequence from *C. muricatus*. Taking into account that Cm-p1 is more positively charged and less hydrophilic than Cm-p2, we synthesized Cm-p1 to explore its antimicrobial potential.

Although Cm-p1 was not capable of inhibiting bacterial growth, even at high doses, it exhibited a broad spectrum antimicrobial activity against fungi. The MIC values of Cm-p1 for yeast and filamentous fungi were in the 4–256 μg ml^{-1} range. The peptide was less effective against the yeasts *C. neoformans* and *Candida parapsilosis*. Interestingly, some echinocandins/pneumocandins are also less effective against these fungi [45]. Furthermore, it has been demonstrated that *C. parapsilosis* has developed a major resistance to amphotericin-B compared to other *Candida* species [46]. Taking into account the other evaluated yeast and filamentous fungi, the range of MIC values was 4–50 μg ml^{-1}. This range of effectiveness is similar to the antifungal activities reported for other peptides [47,48]. Antimicrobial peptides that primarily or exclusively have antifungal properties are less ubiquitous than those with broad antimicrobial actions. Nevertheless, both natural and synthetic peptides do exist that exhibit primarily antifungal activity [48]. The plant defensins Hs-AFP1, Dm-AMP1 and Rs-ARF2 and the insect defensins heliomycin and drosomycin belong to this group of peptides [49,50]. Furthermore, urechistachykinin I is also capable of inhibiting fungal strains at concentrations of 21.3–42.5 μM [43].

In addition to functional studies, theoretical modeling shows that Cm-p1 could assume an α-helix conformation with a distribution of net charge (+2) caused by exposed cationic arginine (Arg^2 and Arg^{10}) and histidine (His^8) residues, which probably favor a membrane–peptide interaction. Additionally, despite the hydrophilicity of Cm-p1, the hydrophobic conserved region Leu^5–Val^7 seems to play a critical role in the peptide's antifungal activity, as has been previously demonstrated [51]. Moreover, the presence of the amino acid residue Val^7 might also be important in fungal interaction. Several studies have demonstrated that an amidated valine residue at the C-terminus had lethal effects against fungi and a broad spectrum of pathogenic microorganisms [52].

One of the current antifungal therapeutic problems is the toxicity of many approved drugs, such as amphotericin-B, echinocandins and pneumocandins. For this reason, it is essential to search for new antifungal molecules without toxicity against mammalian cells. At the highest evaluated dose (256 μg ml^{-1}), Cm-p1 showed no signs of toxicity against human red blood cells and RAW 264.7 cells. This effect could be expected due to the low hydrophobicity of Cm-p1. In fact, a correlation between hydrophobicity and hemolysis has been previously established. The selectivity of antimicrobial peptides for microbial cells can be significantly increased by simply reducing the hydrophobicity of the helix hydrophobic face [53,54].

The potential of peptide therapeutics has improved each year; however, the production process appears to be a substantial challenge for the pharmaceutical industry, especially in the case of

peptides, which are expensive compounds to produce. In this regard, the physiochemical properties of this peptide permit high recovery yields after solid-phase synthesis, which makes it possible to obtain large quantities of Cm-p1 by a procedure that is suitable for large-scale downstream experiments. In summary, Cm-p1, the peptide fragment identified in this study, features physiochemical properties that suggest that it could be utilized in the near future as a biotechnological tool. Cm-p1 has strong potential for the development of an antifungal drug and clearly possesses the potential to aid in the solution of both health and agribusiness problems.

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References

[1] M.E. Kruijshaar, J.M. Watson, F. Drobniewski, C. Anderson, T.J. Brown, J.G. Magee, E.G. Smith, A. Story, I. Abubakar, Increasing antituberculosis drug resistance in the United Kingdom: analysis of National Surveillance Data, BMJ 336 (2008) 1231–1234.
[2] N.J. Snell, Examining unmet needs in infectious disease, Drug Discov. Today 8 (2003) 22–30.
[3] C.A. Arias, B.E. Murray, Antibiotic-resistant bugs in the 21st century – a clinical super-challenge, N. Engl. J. Med. 360 (2009) 439–443.
[4] J.L. Blanco, M.E. Garcia, Immune response to fungal infections, Vet. Immunol. Immunopathol 125 (2008) 47–70.
[5] M. Gupte, P. Kulkarni, B.N. Ganguli, Antifungal antibiotics, Appl. Microbiol. Biotechnol. 58 (2002) 46–57.
[6] D.P. Kontoyiannis, R.E. Lewis, Antifungal drug resistance of pathogenic fungi, Lancet 359 (2002) 1135–1144.
[7] I.H. Lee, C. Zhao, Y. Cho, S.S. Harwig, E.L. Cooper, R.I. Lehrer, Clavanins, alpha-helical antimicrobial peptides from tunicate hemocytes, FEBS Lett. 400 (1997) 158–162.
[8] S.F. Ribeiro, A.O. Carvalho, C.M. Da, R. Rodrigues, L.P. Cruz, V.M. Melo, I.M. Vasconcelos, E.J. Melo, V.M. Gomes, Isolation and characterization of novel peptides from chilli pepper seeds: antimicrobial activities against pathogenic yeasts, Toxicon 50 (2007) 600–611.
[9] X.Y. Ye, T.B. Ng, Hypogin, a novel antifungal peptide from peanuts with sequence similarity to peanut allergen, J. Pept. Res. 57 (2001) 330–336.
[10] X.Y. Ye, T.B. Ng, Isolation of a new cyclophilin-like protein from chickpeas with mitogenic, antifungal and anti-HIV-1 reverse transcriptase activities, Life Sci. 70 (2002) 1129–1138.
[11] S.E. Lofgren, L.C. Miletti, M. Steindel, E. Bachere, M.A. Barracco, Trypanocidal and leishmanicidal activities of different antimicrobial peptides (AMPs) isolated from aquatic animals, Exp. Parasitol. 118 (2008) 197–202.
[12] Y. Park, S.H. Jang, D.G. Lee, K.S. Hahm, Antinematodal effect of antimicrobial peptide, PMAP-23, isolated from porcine myeloid against *Caenorhabditis elegans*, J. Pept. Sci. 10 (2004) 304–311.
[13] J.D. Hale, R.E. Hancock, Alternative mechanisms of action of cationic antimicrobial peptides on bacteria, Expert. Rev. Anti. Infect. Ther. 5 (2007) 951–959.
[14] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.
[15] S.M. Mandal, L. Migliolo, O.L. Franco, A.K. Ghosh, Identification of an antifungal peptide from *Trapa natans* fruits with inhibitory effects on *Candida tropicalis* biofilm formation, Peptides 32 (2011) 1741–1747.
[16] A.J. Otero-Gonzalez, B.S. Magalhaes, M. Garcia-Villarino, C. Lopez-Abarrategui, D.A. Sousa, S.C. Dias, O.L. Franco, Antimicrobial peptides from marine invertebrates as a new frontier for microbial infection control, FASEB J. 24 (2010) 1320–1334.
[17] Y. Gonzalez, A.S. Tanaka, I.Y. Hirata, M.A. del Rivero, M.L. Oliva, M.S. Araujo, M.A. Chavez, Purification and partial characterization of human neutrophil elastase inhibitors from the marine snail Cenchritis muricatus (Mollusca), Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146 (2007) 506–513.
[18] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat. Protoc. 1 (2006) 2856–2860.
[19] B. Merrifield, Solid phase synthesis, Science 232 (1986) 341–347.
[20] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[21] CLSI, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, eighth ed. Clinical and Laboratory Standards Institute, Wayne, PA, 2009.
[22] C. Hetru, P. Bulet, Strategies for the isolation and characterization of antimicrobial peptides of invertebrates, Methods Mol. Biol. 78 (1997) 35–49.
[23] CLSI, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard, eighth ed. Clinical and Laboratory Standards Institute, Wayne, PA, 2008.
[24] W.S. Sung, I.S. Lee, D.G. Lee, Damage to the cytoplasmic membrane and cell death caused by lycopene in *Candida albicans*, J. Microbiol. Biotechnol 17 (2007) 1797–1804.
[25] M. Pasupuleti, A. Schmidtchen, A. Chalupka, L. Ringstad, M. Malmsten, End-tagging of ultra-short antimicrobial peptides by W/F stretches to facilitate bacterial killing, PLoS. One 4 (2009) e5285.
[26] E. Gasteiger, A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel, A. Bairoch, ExPASy: the proteomics server for in-depth protein knowledge and analysis, Nucleic Acids Res. 31 (2003) 3784–3788.
[27] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[28] D. Van Der Spoel, E. Lindahl, B. Hess, G. Groenhof, A.E. Mark, H.J. Berendsen, GROMACS: fast, flexible, and free, J. Comput.Chem. 26 (2005) 1701–1718.
[29] G. Wang, X. Li, Z. Wang, APD2: the updated antimicrobial peptide database and its application in peptide design, Nucleic Acids Res. 37 (2009) D933–D937.
[30] C.R. Grace, L. Cervini, J. Gulyas, J. Rivier, R. Riek, Astressin-amide and astressin-acid are structurally different in dimethylsulfoxide, Biopolymers 87 (2007) 196–205.
[31] N. Eswar, D. Eramian, B. Webb, M.Y. Shen, A. Sali, Protein structure modeling with MODELLER, Methods Mol. Biol. 426 (2008) 145–159.
[32] R.A. Laskowski, J.A. Rullmannn, M.W. MacArthur, R. Kaptein, J.M. Thornton, AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR, J. Biomol. NMR 8 (1996) 477–486.
[33] A.L. Morris, M.W. MacArthur, E.G. Hutchinson, J.M. Thornton, Stereochemical quality of protein structure coordinates, Proteins 12 (1992) 345–364.
[34] K. Sumathi, P. Ananthalakshmi, M.N. Roshan, K. Sekar, 3dSS: 3D structural superposition, Nucleic Acids Res. 34 (2006) W128–W132.
[35] A. Roy, A. Kucukural, Y. Zhang, I-TASSER: a unified platform for automated protein structure and function prediction, Nat. Protoc. 5 (2010) 725–738.
[36] S. Unni, Y. Huang, R.M. Hanson, M. Tobias, S. Krishnan, W.W. Li, J.E. Nielsen, N.A. Baker, Web servers and services for electrostatics calculations with APBS and PDB2PQR, J. Comput. Chem. 32 (2011) 1488–1491.
[37] W.L. DeLano, The PyMOL Molecular Graphics System (2002).
[38] W. Chen, X. Yang, L. Zhai, Z. Lu, J. Liu, H. Yu, Antimicrobial peptides from the venoms of *Vespa bicolor Fabricius*, Peptides 29 (2008) 1887–1892.
[39] P.M. Hwang, N. Zhou, X. Shan, C.H. Arrowsmith, H.J. Vogel, Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin, Biochemistry 37 (1998) 4288–4298.
[40] P.E. Kristiansen, G. Fimland, D. Mantzilas, J. Nissen-Meyer, Structure and mode of action of the membrane-permeabilizing antimicrobial peptide pheromone plantaricin A, J. Biol. Chem. 280 (2005) 22945–22950.
[41] G. Wang, Y. Li, X. Li, Correlation of three-dimensional structures with the antibacterial activity of a group of peptides designed based on a nontoxic bacterial membrane anchor, J. Biol. Chem. 280 (2005) 5803–5811.
[42] R.E. Hancock, H.G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24 (2006) 1551–1557.
[43] W.S. Sung, S.H. Park, D.G. Lee, Antimicrobial effect and membrane-active mechanism of Urechistachykinins, neuropeptides derived from *Urechis unicinctus*, FEBS Lett. 582 (2008) 2463–2466.
[44] A.H. Thompson, A.J. Bjourson, D.F. Orr, C. Shaw, S. McClean, Amphibian skin secretomics: application of parallel quadrupole time-of-flight mass spectrometry and peptide precursor cDNA cloning to rapidly characterize the skin secretory peptidome of *Phyllomedusa hypochondrialis* azurea: discovery of a novel peptide family, the hyposins, J. Proteome. Res. 6 (2007) 3604–3613.
[45] S. Tawara, F. Ikeda, K. Maki, Y. Morishita, K. Otomo, N. Teratani, T. Goto, M. Tomishima, H. Ohki, A. Yamada, K. Kawabata, H. Takasugi, K. Sakane, H. Tanaka, F. Matsumoto, S. Kuwahara, In vitro activities of a new lipopeptide antifungal agent, FK463, against a variety of clinically important fungi, Antimicrob. Agents Chemother. 44 (2000) 57–62.
[46] S.M. Seidenfeld, B.H. Cooper, J.W. Smith, J.P. Luby, P.A. Mackowiak, Amphotericin B tolerance: a characteristic of *Candida parapsilosis* not shared by other Candida species, J. Infect. Dis. 147 (1983) 116–119.
[47] D. Mania, K. Hilpert, S. Ruden, R. Fischer, N. Takeshita, Screening for antifungal peptides and their modes of action in *Aspergillus nidulans*, Appl. Environ. Microbiol. 76 (2010) 7102–7108.
[48] A. Matejuk, Q. Leng, M.D. Begum, M.C. Woodle, P. Scaria, S.T. Chou, A.J. Mixson, Peptide-based antifungal therapies against emerging infections, Drugs Future 35 (2010) 197.
[49] K. Thevissen, H.H. Kristensen, B.P. Thomma, B.P. Cammue, I.E. Francois, Therapeutic potential of antifungal plant and insect defensins, Drug Discov. Today 12 (2007) 966–971.
[50] P. Fehlbaum, P. Bulet, L. Michaut, M. Lagueux, W.F. Broekaert, C. Hetru, J.A. Hoffmann, Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides, J. Biol. Chem. 269 (1994) 33159–33163.
[51] J. Lee, D.G. Lee, Influence of the hydrophobic amino acids in the N- and C-terminal regions of pleurocidin on antifungal activity, J. Microbiol. Biotechnol. 20 (2010) 1192–1195.

[52] W. Cao, Y. Zhou, Y. Ma, Q. Luo, D. Wei, Expression and purification of antimicrobial peptide adenoregulin with C-amidated terminus in *Escherichia coli*, Protein Expr. Purif. 40 (2005) 404–410.
[53] Y. Chen, M.T. Guarnieri, A.I. Vasil, M.L. Vasil, C.T. Mant, R.S. Hodges, Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides, Antimicrob. Agents Chemother. 51 (2007) 1398–1406.
[54] D.L. Lee, J.P. Powers, K. Pflegerl, M.L. Vasil, R.E. Hancock, R.S. Hodges, Effects of single D-amino acid substitutions on disruption of beta-sheet structure and hydrophobicity in cyclic 14-residue antimicrobial peptide analogs related to gramicidin S, J. Pept. Res. 63 (2004) 69–84.
[55] J. Söding, Protein homology detection by HMM-HMM comparison, Bioinformatics 21 (2005) 951–960.