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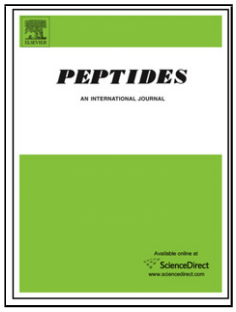

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PII: S0196-9781(18)30257-2
DOI: https://doi.org/10.1016/j.peptides.2018.12.007
Reference: PEP 70053

To appear in: *Peptides*

Received date: 30 August 2018
Revised date: 19 December 2018
Accepted date: 21 December 2018

Please cite this article as: Popov CSFC, Magalhães BS, Goodfellow BJ, Bocca AL, Pereira DM, Andrade PB, Valentão P, Pereira PJB, Rodrigues JE, de Holanda Veloso Júnior PH, Rezende TMB, Host-defense peptides AC12, DK16 and RC11 with immunomodulatory activity isolated from Hypsiboas raniceps skin secretion, *Peptides* (2019), https://doi.org/10.1016/j.peptides.2018.12.007

Host-defense peptides AC12, DK16 and RC11 with immunomodulatory activity isolated from *Hypsiboas raniceps* skin secretion

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Conflict of interest statement: The authors declare that they have no conflict of interest.

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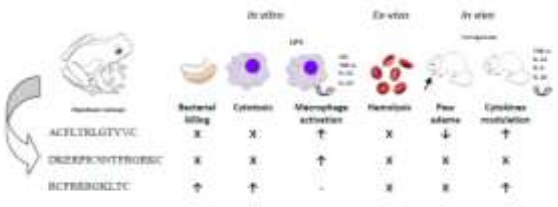

Highlights

1. Peptides AC12 and DK16 showed low toxicity in eukaryotic cells, both *in vitro* and *in vivo.*
2. The peptides effectively suppressed the secretion of TNF-α and IL-12.
3. Peptides AC12 and DK16 decreased the secretion of IL-12 and the NO production in LPS-stimulated culture.
4. Peptide AC12 inhibited carrageenan induced edema by 48.7 % in Balb/c mice.
5. A carrageenan-induced peritonitis model showed that AC12 and RC11 reduced TNF-α levels.

Abstract

Inflammation is a natural defense mechanism of the immune system; however, when unregulated, it can lead to chronic illness. Glucocorticoids are the most commonly used agents to effectively treat inflammatory conditions, including autoimmune diseases, however these substances can trigger a number of side effects. Thus, viable alternatives to the use of these drugs would be advantageous. In this study, we have analyzed the anti-inflammatory profile of three synthetic peptides first identified in skin secretion of the tree frog *Hypsiboas raniceps*. Structural characterization was performed using NMR spectroscopy and Mass Spectrometry, and the peptides were tested *in vitro* in RAW 264.7 cells

and *in vivo* in Balb/c mice for their functional properties. The samples did not show a significant antimicrobial profile. NMR spectroscopy indicated that AC12 (ACFLTRLGTYVC) has a disulfide bond between C2 and C11 and a β-sheet-turn-β-sheet conformation in aqueous solution. This peptide showed no cytotoxic effect in mammalian cells and it was the most effective in reducing anti-inflammatory markers such as NO, TNF-α and IL-12. Peptide DK16 (DKERPICSNTFRGRKC) demonstrated anti-inflammatory properties *in vitro,* while RC11 (RCFRRRGKLTC) significantly altered the cell viability in RAW 264.7 but was shown to be safe in Balb/c erythrocytes. Our results indicate that, of the three peptides studied, AC12 is the most efficient in reducing anti-inflammatory markers, and it could be a potential agent for the treatment of inflammatory diseases.

Abbreviations

Trifluoroethanol (TFE); 2,2,2-Trifluoroethanol-1,1-d2 (TFE-d_2); Trifluoracetic acid (TFA); Sodium Dodecyl Sulfate (SDS); Sodium Sodecyl-d25 Sulfate (SDS-d_{25}); Type 1 T helper cells (Th1); Acetonitrile (ACN); Matrix-Assisted Laser Desorption/Ionization (MALDI); Time Of Flight (TOF); Mass Spectrometry (MS); Nuclear Magnetic Resonance (NMR); Total Correlation Spectroscopy (TOCSY); Rotating-frame Overhauser Effect Spectroscopy (ROESY).

Keywords: Peptide; Anura; Immunomodulatory; Anti-inflammatory; NMR.

1. Introduction

Dermal granular glands of anuran amphibians are responsible for the synthesis, storage and secretion of a wide range of peptide based biomolecules [1,2]. They are involved in a wide range of biological functions, including antimicrobial, vasodilator, analgesic, inflammatory and neuronal activity [3]. The presence of these peptides on frog skin plays a primary role in a primitive but elaborate innate host defense mechanism in response to stimulation of the sympathetic nervous system, tissue damage, or systemic stress contact [4, 5].

Antimicrobial peptides (AMPs) were first recognized for their cytolytic activity against microorganisms. However, many of them are now known to have multifunctional activity in the innate and adaptive immune response and are best known as host defense peptides (HDP) [6]. The transition from the aquatic to the terrestrial environment, along with so many different environmental variables, has forced the anurans to develop a series of evolutionary adaptations resulting in their ability to synthesize, secrete and expose an array of defense molecules [7]. Furthermore, due to the equally immense pathogen diversity, this defense mechanism must be quick and broad acting. Before activating the whole adaptive immune system of slow-growing B and T cells, a first line of host defense is immediately mobilized, in the form of gene-encoded peptides, composing the innate immune system [8]. Since many of these peptides function by binding or inhibiting target proteins and receptors with incredible specificity, it is not hard to imagine the potential they may have as therapeutic agents [9,10]. Peptides secreted by anurans have already demonstrated effective antibiotic action [2]. They have also been found to act indirectly in the inflammatory reaction, inhibiting the recruitment of macrophages and pro-inflammatory cytokines, restoring

homeostasis [9]. Since an exacerbated immune response is characterized by the increased production of cytokines, these peptides may have potential therapeutic uses in regulating the pro-inflammatory response. Recent studies have demonstrated that anuran-derived peptides, have activities that include cytokine-mediated immunomodulatory properties. As a result, new therapeutic applications of these molecules are being studied [10].

In this work we have investigated the properties of three synthetic peptides analogous to skin secretion peptides from *Hypsiboas raniceps* (Cope, 1862), a species mainly found in the Chaco, Pantanal and Amazon regions. Previous studies have identified a new family of antimicrobial peptides, the raniceptins, isolated from this species [11] and now we describe the functional and structural characterization of these three cysteine peptides. To start to understand the structure-function relationship for these molecules and therefore to design cheaper and more efficient analogues [12], we also performed NMR spectroscopy in aqueous solution.

Apart from the physiological and biochemical conclusions that this study of *H. raniceps* skin secretion peptides could provide, a biotechnological application can be envisaged where an understanding of the mechanism of action could allow us to synthesize new peptides with enhanced anti-inflammatory effects for therapeutic use, since the anurans' innate immune system shares several characteristics with the mammalian immune system [13].

2. Materials and Methods

2.1. Peptide synthesis and oxidation

Synthetic peptides AC12, DK16 and RC11 represent the mature peptides of prepropeptides dissulfin-01 (GenBank KY748206), dissulfin-02 (GenBank KY748205) and Hr2 [14], respectively. The peptides were synthetized at Aminotech (São Paulo, SP, Brazil), according to the data given in Table 1. Since the native peptides isolated from skin secretion are in an oxidation state [11], the synthetized peptides were kept in water for two weeks at room temperature, at a concentration of less than 3 $mg.mL^{-1}$ for complete oxidation to occur [15]. Total oxidation of the peptides was monitored by MALDI-TOF/TOF mass spectrometry (Ultraflex III, Bruker Daltonics, Billerica, MA, EUA). Subsequently, the samples were mixed with a saturated matrix solution consisting of 1:3 (v/v) alpha-cyano-4-hydroxycinnamic acid. Samples were applied in duplicate on an MTP Anchor Chip TM 600/384 TF steel plate (Bruker Daltonics) and crystallized at room temperature. The molecular masses of the peptides were determined in the positive reflected mode with a detection range between 600 and 4000 m/z. Mass calibration was according to the manufacturer using Peptide Calibration Standard I (Bruker Daltonics).

2.2. Quantification

Peptides were quantified by determining the UV absorption at 205, 215 and 225 nm in a spectrophotometer [16].

2.3. NMR spectroscopy

Samples for NMR contained 1 mM of peptide dissolved in 550 µL of solvent (7 % v/v D_2O in water). The pH of each solution was adjusted to 4 with dilute (0.1 M) HCl. All NMR spectra were recorded on a Bruker Avance 700 MHz

spectrometer (Bruker BioSpin) at 298 K. The 2D total correlation spectroscopy (TOCSY) spectra were recorded using the Bruker program dipsi2etgpsi19, with 90 ms mixing time and a recycle delay of 1 s. The 2D rotating-frame Overhauser spectroscopy (ROESY) spectra were recorded with a 200 ms mixing time and 1 s recycle using the Bruker program roesygpph19. The TOCSY and NOESY spectra were recorded with 1k x 512 data points and were multiplied by a shifted sine-squared window and zero filled to a final size of 1k x 1k. Spectral processing was carried out using TopSpin, and peak picking, assignment and integration were carried out using NMRFAM-SPARKY [17]. The assigned ROESY peak list was used as input into CYANA [18], where upper limit distance restraint files were produced and used as input to produce 200 structures, with a final low target function ensemble of 20 conformers being selected. The structures were visualised using PyMOL (Molecular Graphics System, Version 1.8 Schrödinger, LLC) and MOLMOL [19]. The NMR structure family has been deposited in the PDB with the accession code 6FGM.

2.4. Antimicrobial tests

The antimicrobial assays were performed using *Escherichia coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 25923), according to *Clinical and Laboratory Standards Institute* protocol for bacteria [20]. The bacteria were inoculated at log phase ($5x10^5$ $CFU.mL^{-1}$) in Mueller Hinton medium (Sigma-Aldrich, St. Louis, MO, USA) with the peptides and the controls. Peptides were tested at concentrations between 4 and 256 $\mu g.mL^{-1}$. The commercial antibiotics, ampicillin and chloramphenicol, were used as positive controls, at the same molar

concentrations as the peptides tested. The Rsp-1 peptide, also used as a positive control, was isolated from the amphibian *Hypsiboas raniceps* and chosen because of its known antimicrobial activity [11]. Bacteria were incubated at 37 °C for 12 h, with medium agitation in a microplate reader (Bio-Tek PowerWave HT, Biotek Instruments, Inc., Winooski, VT, USA) in 96-well plates, in triplicate, with readings at 600 nm taken every hour. Subsequently, the minimum inhibitory concentration (MIC) of the peptides was determined, considering 100% inhibition.

2.5. Cell Culture

The RAW 264.7 cell line was obtained from Laboratório de Farmacognosia, Faculdade de Farmácia – UP (Porto, Portugal) and grown in Dulbecco's Modified Eagle's Medium (Gibco, Waltham, MA, USA) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen, Grand Island, NY, USA), 1 % (w/v) non-essential amino acids (Invitrogen), 1 % (w/v) L-glutamine and 1 % (v/v) penicillin/streptomycin (1000 $U.mL^{-1}$) (Invitrogen), in an incubator at 37 °C and 5 % (v/v) CO_2 [21].

2.6. Cell Viability Assay

Cells were cultured in 96-well plates ($3.5x10^4$ cells/well) and allowed to attach for 24 hours. After this period, the cells were treated with 1 $\mu g.mL^{-1}$ lipopolysaccharide (LPS). After 2 hours, the controls or the peptides were added. Samples were tested in six different concentrations from 8 to 256 $\mu g.mL^{-1}$. After 24 hours of incubation, MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added to each well to a final concentration of 0.5 $mg.mL^{-1}$ and the plate was incubated for 2 hours at 37 °C. Formazan was dissolved by addition of

a DMSO:isopropanol mixture (3:1) and quantified at 560 nm [22]. Cell viability results correspond to the mean of three independent experiments performed in triplicate and expressed as a percentage of the untreated control cells. Only non-toxic concentrations of the peptides were used in subsequent experiments.

2.7. Assay of production of inflammatory mediators

The production of nitric oxide was assessed using the Griess reaction [23]. Briefly, 50 µL of either extract solution or culture media with an equal volume of Griess reagent (1 % sulfanilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2 % H_3PO_4) was incubated for 10 minutes in the dark, after which the absorbance (540 nm) was read in a multiplate reader set (Bio-Tek PowerWave HT). The amount of NO released by the cells stimulated by the samples was plotted relative to the positive control. Thus, NO production following LPS incubation was considered 100 %. TNF-α, IL-12 and IL-10 levels were determined by *Enzyme-Linked Immunoabsorbent Assay* (ELISA). Here, the cell culture supernatant was removed after an incubation time of 24 h. Murine ELISA development kits (Peprotech, Rocky Hill, NJ, USA) were used, following the manufacturer's specifications. Concentration of the cytokines was expressed in picograms per milliliter ($pg.mL^{-1}$), after comparison with the standard curve from the ELISA kit according to the manufacturer's specifications [24].

2.8. Animals

Experiments were conducted using male Balb/c mice (8-10 weeks of age) and weighing 20–30 g. Animals were housed at animal facilities of Universidade Católica de Brasília (Brazil) with 12:12 h light/dark cycle, free access to water and

food, and controlled temperature (21 ± 1 °C). The number of animals in each group is as depicted in Section 2.10. All experimental protocols were reviewed and approved by the Ethics Committee on Animal Use at Universidade Católica de Brasília (CEUA/UCB n. 15/16), and all procedures followed the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.9. Hemolysis Assay

A solution of 1 % erythrocytes was prepared using 1 mL of Balb/c mouse blood. The hemolysis percentage of the peptides was calculated for three concentrations (128, 256 and 512 $\mu g.mL^{-1}$) using 1 % (v/v) of 20 %Triton X-100 as positive control and PBS as negative control. The supernatant from each solution was transferred into a flat-bottomed 96-well plate and the absorbance was measured in a multiplate reader set (Bio-Tek PowerWave) at 450 nm [25]. The following formula was used to calculate the % of hemolysis:

$$\% \text{ Hemolysis} = \frac{Abs_{540} \text{ of RBC treated sample} - Abs_{540} \text{ PBS}}{A_{540} \text{ Triton X -100 1\%} - A_{540} \text{ PBS}}$$

2.10. Carrageenan-induced paw edema

Animals were divided into 6 groups of 5 animals each and were inoculated with the following formulations: i) PBS 0.1 M; ii) dexamethasone (1 $mg.kg^{-1}$); iii) AC12 (7.5 $mg.kg^{-1}$); iv) Hr6b (7.5 $mg.kg^{-1}$); v) DK16 (7.5 $mg.kg^{-1}$) and vi) RC11 (7.5 $mg.kg^{-1}$). Acute inflammation was induced by intraplantar injection of 12 $mg.kg^{-1}$ carrageenan in 20 µL of 1% PBS into the left hind paws of mice 1 hour after treatment (peptides or controls). Paw thickness (distance between the plantar surface and the external surface) was measured 2, 4, 6, 8, 12 and 24 hours after the carrageenan injection using a digital caliper (resolution 0.01 mm).

Paw edema thickness was calculated by subtracting the basal volume from the paw to the final volume after edema induction [26].

2.11. Histopathology analysis

Mice footpads were fixed in 10 % phosphate-buffered formalin overnight (room temperature), transferred to 70 % (v/v) ethanol, included in paraffin using an automatic tissue processor (OMA® DM-40, São Paulo, SP, Brazil), cut to 5 μm thickness in a manual microtome and stained with hematoxilin-eosin (HE) for histological analyses (light microscopy). Histological sections were examined to analyze the inflammatory infiltrate [27,28].

2.12. Induction of acute carrageenan-induced peritonitis and peritoneal lavage

Acute peritonitis was induced by an intraperitoneal injection of 1 % (w/v) carrageenan in 300 μL of PBS 0.9 % one hour after the treatment (peptides or controls) [23]. For this, mice were divided into 6 groups of 5 animals each and were inoculated with the following formulations: i) 300 μL of PBS 0.9 %; ii) dexamethasone (1 $mg.kg^{-1}$); iii) AC12 (7.5 $mg.kg^{-1}$); iv) Hr6b (7.5 $mg.kg^{-1}$); v) DK16 (7.5 $mg.kg^{-1}$) and vi) RC11 (7.5 $mg.kg^{-1}$). Four hours after the carrageenan injection, animals were euthanized and their peritoneal cavities were rinsed with 2 mL cold sterile PBS (pH 7.4) (Sigma-Aldrich), to analyze IL-10, IL-6 and TNF-α cytokines production. Murine ELISA development kits (Peprotech) were used following the manufacturer's specifications [24].

2.13. Statistical analysis

Data were presented as mean ± SD. Results were analyzed with Prism 6 (GraphPad Software) using one-way ANOVA test and Tukey´s multiple comparisons test. A p value of <0.05 was considered statistically significant. All experiments were performed in triplicate with three independent assays.

3. Results

3.1. Peptide synthesis and oxidation

Theoretical and experimental monoisotopic molecular mass values [M + H^+] were obtained by MALDI/TOF-MS. Table 1 summarizes all the peptide amino acid sequences obtained and their molecular masses. For verification of complete peptide oxidation, these were analyzed by MALDI-TOF/TOF mass spectrometry (Ultraflex III, Bruker Daltonics), as detailed in Figure S1 (A, B and C). The complete oxidation of the peptides can be verified by comparing the mass (z) of the peptides in reduced state and oxidized state (Table 1).

3.2. NMR Spectroscopy

All protons for the peptide AC12 were assigned using the TOCSY and ROESY spectra and the assignments are shown in Table S2. Analysis of the cross peaks present in the ROESY spectra identified some long-range peaks that were indicative of the structure present in solution, these included C2(Hβ)-Y10(Hδ,Hε), F3(Hδ,Hε)-C12(Hβ), F3(Hδ,Hε)-V11(Hγ) and L4(Hδ)-Y10(Hδ,Hε). There was also a cross peak between C2(Hα) and C12(Hα) and also C2(Hβ) and C12(Hβ), indicating that the expected disulfide bridge, C2-C12, was present in solution (Figure 1A). The pattern of ROESY peaks (Figure 1B) also indicated that the peptide has a sheet-loop-sheet conformation running from C2 to C12, with

the loop region occurring at R6-G8 (Figure 1C). By integrating the ROESY cross peaks and using the program CYANA, we were able to calculate an ensemble of solution structures for this peptide. Of the 78 upper distance limits used, 49 were short (1-2), 2 were medium (2-5) and 27 were long range (above 5). Figure 1D shows the 20 structures (backbone RMSD 0.3 ± 0.09 Å) with the lowest target function (0.55-0.72 $Å^2$) obtained from 400 initial random structures, where it was possible to identify (using the program MOLMOL) backbone NH-O H-bonds for F3-V11, T5-T9, G8-T5, V11-F3 and V11-T9 (magenta lines).

For the peptide RC11, the 1H assignments obtained from the TOCSY/ROESY spectra are shown in Table S2 and S3. The TOCSY (and ROESY) spectrum showed precisely twice the number of expected spin systems (20 rather than 10), suggesting that there were 2 distinct forms of the peptide present in solution at 298K (Figure 2A). The peak volumes for the 2 forms indicated that form A (sequence numbering 1-11) was 1.5 times more abundant than form B (sequence numbering 101-111). Both forms have cross peaks indicating that residues 3 (or 103) and 11 (or 111) are in proximity. This may suggest that a disulfide bridge is present for both forms. However, a C2(Hα)-C11(Hα) cross peak was identified for form A, but not for form B. The pattern of NH-NH cross peaks is also slightly different for the 2 forms: in form A it is 6-7, 7-8 and 9-10, while for form B it is 107-108, 108-109 and 109-110 (Figure 2B). By integrating the ROESY cross peaks and using the program CYANA, we were able to calculate an ensemble of solution structure for one of the forms of this peptide in solution, form A. Figure 2C shows the 20 conformers (backbone RMSD for residues 4-10, 0.74 ± 0.22 Å) with the lowest target function (2.51-3.14 $Å^2$) for RC11(A) obtained using the integrated ROESY peaks as input for the program

CYANA. No H-bonds were detected for this ensemble using the program MOLMOL. It can also be seen that there are 2 distinct conformations present in the NMR ensemble, mainly due to a different conformation adopted for the backbone near Arg4. This, however, may be a result of the fact that RC11 has fewer long-range NMR restraints (11 long range, 2 medium and 51 short range) compared to AC12.

The TOCSY and ROESY spectra for peptide DK16 did not show as many cross peaks as AC12 and RC11. This may be related to the S/N for the ROESY spectrum, which was 30 % of that for RC11 and 50 % of that for AC12. No cross peaks indicative of a disulfide bridge between C7 and C16 were seen in the ROESY spectrum, and only 2 NH-NH cross peaks could be identified, K2-E3 and T10-R12. In a similar manner to RC11, the NMR spectra for DK16 have more signals than expected. It was possible to identify a second set of spin systems (4.5 times less abundant), although sequential identification was not possible for many of the systems, due to weak or absent ROESY cross peaks.

3.3. Antimicrobial assays

Peptides were tested for their antimicrobial activity against Gram-positive and Gram-negative bacteria using the microdilution method. Peptides AC12 and DK16 had no detectable effect on any of the reference strains (*E. coli* ATCC 8739 and *S. aureus* ATCC 25923) up to the maximum tested concentration (256 $\mu g.mL^{-1}$) (Figure 3). However, RC11 was able to inhibit *E. coli* at 32 $\mu g.mL^{-1}$. Peptide Rsp-1, isolated from *H. raniceps*, was used as positive control and inhibited the two bacterial strains at 4 $\mu g.mL^{-1}$.

3.4. Cell viability assay

Peptide toxicity was evaluated as RAW 264.7 cell viability and as Balb/c mice erythrocyte hemolytic activity. As demonstrated by the cell viability assay, cell survival was not affected at AC12 concentrations ranging from 8 to 256 $\mu g.mL^{-1}$ (Figure 4A). On the other hand, the addition of 8 and 256 $\mu g.mL^{-1}$ of the DK16 peptide resulted in an evident decrease in cell viability (28 and 19 %, respectively), relative to the control (Figure 4B). Peptide RC11 had moderate cytotoxic activity (reduction of 20 % in cell viability) for two tested concentrations, 32 and 64 $\mu g.mL^{-1}$. When tested in higher concentrations, 128 and 256 $\mu g.mL^{-1}$, RC11 reduced the culture cell viability by 43 and 77 % ($p<0.0001$), respectively (Figure 4C). For this reason, peptide RC11 was excluded from subsequent RAW 264.7 assays.

For the hemolysis assay, all peptides were tested at three different concentrations: 128, 256 and 512 $\mu g.mL^{-1}$. The samples showed low cytotoxicity (3.8 %) when added at 128 and 256 $\mu g.mL^{-1}$. At the highest tested concentration (512 $\mu g.mL^{-1}$), AC12 and RC11 showed 10.6 and 73.5 % cytotoxicity against Balb/c blood cells with DK16 being the most toxic (73.5 %) (Figure 5).

3.5. Assay of production of inflammatory mediators

It was observed that AC12 and DK16 decreased the level of TNF-α by 14 % ($p=0.017$) and 70 % ($p<0.0001$) respectively, when compared to LPS-stimulated groups (Figure 6A). At the same concentration of AC12 and DK16, IL-12 production was also significantly reduced by about 46 % ($p<0.0001$) and 21 % ($p=0.0001$), respectively, compared to the positive control (Figure 6B). AC12 and DK16 also decreased the level of IL-10, an anti-inflammatory cytokine, by 24

% (p=0.001) and 18 % (p=0.01), respectively, compared to that obtained in the presence of LPS (Figure 6C). Regarding the production of the inflammatory mediator NO, initially, all the peptides were tested at concentrations ranging from 8 to 256 $\mu g.mL^{-1}$ (data not shown). AC12 and DK16 reduced NO levels in a dose-dependent manner. This occurred significantly for the two highest tested concentrations. Thus, AC12 inhibited NO production by 38 % ($p<0.0001$) when added to the cell culture at concentrations of 128 $\mu g.mL^{-1}$ and 256 $\mu g.mL^{-1}$. DK16 inhibited NO release by 50 % ($p<0.0008$) when added to the cell culture at a concentration of 128 $\mu g.mL^{-1}$ and by 63 % at a concentration of 256 $\mu g.mL^{-1}$ (Figure 6D).

3.6. In vivo carrageenan-induced paw edema

As expected, in the negative control group (PBS), edema formation was higher compared to the treated groups. In the positive control group (dexamethasone) the edema formation was reduced by 59.3 % ($p<0.05$) in comparison to the non-treated group. The peak edema volume was at 4 h. At this point, treatment with AC12 decreased the edema by 48.7 % in relation to the untreated-animals ($p<0.05$) and displayed no statistical difference in relation to the dexamethasone treatment. Treatment with DK16 resulted in a 22.3 % reduction in edema volume, while peptide RC11 decreased the edema volume by 46.2 % ($p<0.05$) (Figure 7A).

The histopathology of the PBS group (control) paw tissue presented normal histological features, with intact paw, dispersed cell infiltration and preserved membranes (Figure 7B). However, 4 h after carrageenan administration, the paw tissue showed a pronounced inflammatory response, with

cell inflammatory infiltrates accompanied by intense edema (Fig 7C). However, the treatment with peptide AC12 decreased cell migration and edema at a concentration of 7.5 $mg.kg^{-1}$ (Fig 7D). The peptides DK16 and RC11 did not decrease the inflammatory response (Figures 7D and 7F), although peptide DK16 decreased the edema significantly at a concentration of 15 $mg.kg^{-1}$ (data not shown). Figure 7G shows a representative structure of the plantar paw tissue analyzed in the histopathology assay.

3.7. In vivo acute carrageenan-induced peritonitis

The *in vivo* carrageenan-induced peritonitis assays were performed according to a common temporal line (Figure 8A). In this assay, AC12 and RC11 were able to decrease TNF-α production by 41 and 45 %, respectively, in relation to the non-treated group ($p<0.02$) (Figure 8B). DK16 showed no statistical difference in relation to the PBS-treated group. Finally, dexamethasone, used as positive control, decreased TNF-α production by 32 % ($p<0.05$) (Figure 8B). The release of IL-12 was not affected by the addition of AC12 and DK16 (Figure 8C), but RC11 decreased it by 28 % ($p<0.05$). The peptides had no effect on IL-6 production (Figure 8D). However, the positive control, dexamethasone, decreased the production of this cytokine by 27 % ($p<0.05$). Regarding the anti-inflammatory cytokine IL-10, it was observed that dexamethasone treatment increased its production by 55 % compared to the PBS-treated group ($p<0.0005$) (Figure 8E), while only the AC12-treated group displayed a 39 % increase in IL-10 production, when compared to the untreated group ($p<0.05$).

4. Discussion

The structural analysis, performed by NMR, showed that in AC12 a disulfide bond is present in solution, allowing the polypeptide chain to adopt a stable tertiary structure. The pattern of ROESY peaks indicated that AC12 has a βsheet-turn-βsheet conformation running from C2 to C12, with the turn occurring at R6-G8. Interestingly we saw 2 different sets of peaks in solution for the other two peptides, RC11 and DK16, but only RC11 presented ROESY cross peaks that could confirm the presence of a disulfide bridge. With four arginine and one lysine residue, RC11 has a high concentration of positive charges that has always been a hallmark of antimicrobial peptides [8]. However, the NMR structure for RC11 has much poorer definition than that obtained for AC12 due to the presence of 2 forms and a lower number of assigned ROESY peaks. As all samples were prepared in an identical manner, these differences may be significant when attempting to explain the inflammatory effects of the peptides, although further work would be required to confirm this hypothesis.

For the functional analysis, *in vitro* antimicrobial assays were performed against human pathogenic bacterial strains. The results showed that AC12 and DK16 have no significant antimicrobial action against *E. coli* (ATCC8739) and *S. aureus* (ATCC25923). The MIC value determined for RC11 was 4 $\mu g.mL^{-1}$ for both strains. This value is in the same range as MIC values commonly determined for *Hypsiboas* species [29]. This antimicrobial activity of RC11 is in agreement with the structural analysis of the peptide, regarding the high concentration of positive charges.

Samples were tested for their cytotoxic potential in RAW 264.7 cell cultures: AC12 and DK16 did not cause significant changes in cell viability. However, the MTT assay revealed that RC11 was toxic in concentrations up to

32 μg.mL^{-1} and, for this reason, it was not used in subsequent assays using these cells. The three peptides were also tested for their toxicity in Balb/c erythrocytes. The peptides showed low hemolytic activity (<1.3 % for a concentration of 128 μg.mL^{-1}), which is a significant result when considering that some isolated anuran peptides are cytotoxic in mammalian cells when tested at lower concentrations [30, 31]. Based on these results, AC12 and DK16 showed themselves to be safe in eukaryotic cells.

As peptides AC12 and DK16 were not toxic, their immunomodulatory properties were also tested. Classically activated macrophages can release several inflammatory modulators, such as NO and cytokines [32, 33]. Therefore, LPS was used as a pro-inflammatory stimulus in RAW 264.7 cells, resulting in the production of TNF-α, lL-12, IL-10 and NO [33].

This *in vitro* model demonstrated that AC12 and DK16 effectively suppressed the secretion of two pro-inflammatory cytokines, TNF-α and lL-12. DK16 down-regulated TNF-α production by 70 %. This is relevant since numerous clinical studies have linked high levels of TNF-α to central nervous system disorders and chronic neurodegenerative diseases, such as ischemia, Parkinson's disease, Alzheimer´s, epilepsy and traumatic brain injury [34, 35]. Classical chronic diseases, including rheumatoid arthritis and Crohn's disease, have also been well studied and are associated with high levels of TNF-α, [36]. IL-12 is responsible for the generation of Th1 T cells and is capable of inducing the production of IFN-γ [37]. The production of IL-12 is required for acute infection involving activation of the phagocytic cells [38]. Despite being important for host defense, exacerbated levels of IL-12 can cause persistent inflammation, leading to auto-immune diseases, such as multiple sclerosis and psoriasis [39]. In this

context, AC12 and DK16 decreased the secretion of IL-12 by 46 % and 21 %, respectively, hinting at their anti-inflammatory properties. Another important inflammatory mediator is NO, synthesized via the conversion of arginine to citrulline by the inducible isoform of nitric oxide synthase (iNOS) [40]. Apart from its beneficial activity against microorganisms and parasitic infections, dysfunctional NO levels seem to be involved in the pathophysiology of several disorders, such as asthma, rheumatoid arthritis and systemic sclerosis [41]. The peptides AC12 and DK16 decreased NO production in LPS-stimulated culture supernatant by 38 and 50 %, respectively, displaying an anti-inflammatory capacity, as already demonstrated by other anuran peptides [9]. The peptides also decreased the levels of the anti-inflammatory cytokine IL-10. The same pattern was observed in Pseudhymenochirins (Ps-1Pb and Ps-2Pa), isolated from the skin secretion of the frog *Pseudhymenochirus merlini* (Pipidae) [42]. Agents that inhibit IL-10 production have been shown to be useful as coadjuvants in the treatment of cancerous diseases, since IL-10 is spontaneously produced and secreted by a wide range of cancer cells [43]. Thus, as AC12 and DK16 reduced the production of IL-10 by 24 % and 18 %, respectively, they may have potential use as coadjuvant agents in the treatment of neoplasia.

The anti-inflammatory activity of the peptide was also assessed in a carrageenan-induced paw edema assay in Balb/c mice [44]. Dexamethasone was able to decrease the edema volume by 59 %. Similarly, peptide AC12 inhibited the edema by 48.7 %. This result is corroborated by the histopathology and immunohistochemistry analyses, in which AC12 showed the most significant effect decreasing the cell migration and edema in the mouse paw tissue samples. However, although DK16 and RC11 use reduced the edema, they were not as

effective as dexamethasone. Temporins, isolated from the European red frog (*Rana temporaria*) have shown *in vivo* anti-inflammatory effect when tested in mice intravenously infected with bacteria [45]. However, there is a lack of studies that characterize the anti-inflammatory activity of these peptides in models of aseptic inflammation.

A second systemic inflammation mouse model was used to test the anti-inflammatory potential of the peptides. Peritonitis was induced by intraperitoneal injection of carrageenan, followed by the evaluation of cytokine production in the peritoneal lavage. AC12 and RC11 reduced TNF-α levels by 41 and 45 %, respectively. These values are higher than those obtained with the control drug, dexamethasone, which reduced the production of TNF-α by 32 %. These results are significant, since agents that decrease TNF-α levels can be used to treat disease conditions, such as sepsis [46]. Previous studies have already shown the connection between high TNF-α levels and carrageenan-induced paw edema [47, 48]. Since TNF-α is major pro-inflammatory mediator, it promotes the induction and release of kinins, such as bradykinin. In turn, bradykinin leads to vasodilation and edema [49]. Serum TNF-α levels were higher in the carrageenan-injected mice than in treated groups [48]. Thus, the fact that AC12 and RC11 were the most efficient peptides to decrease paw edema correlates well with the significant decrease in TNF-α levels caused by these peptides. It was also observed that RC11 decreased the production of IL-12, another pro-inflammatory cytokine. As the peptides decreased IL-10 production *in vitro*, we decided to test their effect on IL-6, another cytokine frequently released by several types of tumors, and which can act as a growth factor for neoplasms [50]. However, none of the peptides affected IL-6 production in the peritonitis model.

Previous studies have already shown that frog skin peptides can inhibit the release of IL-6. Cathelicidin-PP, isolated from the tree frog *Polypedates puerensis* (Rhacophoridae) [51], and pseudhymenochirin-1Pb and 2Pa, isolated from *Pseudhymenochirus merlini* (Pipidae) [42], decreased IL-6 levels in mouse peritoneal macrophages activated with LPS. However, such an effect was not observed in the present study. The last cytokine analyzed in the peritoneal lavage was IL-10, an anti-inflammatory mediator. Results showed that only AC12 increased the production of IL-10 (by 39 %), confirming its anti-inflammatory profile in this systemic inflammatory model, since this peptide also reduced the levels of TNF-α by 41 %. These results are corroborated by the fact that IL-10 strongly inhibits the production of TNF-α [52]. Auto-regulation of IL-10 is essential for homeostasis of the immune system [53] and previous studies in mice have concluded that during acute infection, the suppression of IL-10 signaling can lead to host fatality [54]. Also, reduced hippocampal IL-10 expression is linked with anxiety and depression and high levels of IL-10 are associated with chronic infections [55].

Glucocorticoids (GC's), such as dexamethasone, are a highly efficient treatment for several inflammatory conditions [55]. However, GC therapy can induce numerous side effects that include skin atrophy, growth failure, delayed puberty, osteoporosis, myopathy, cataracts, glaucoma, psychoses, diabetes mellitus, among others [57]. Therefore, the search for new drug candidates with effective inflammatory action but without the characteristic side effects associated with GC's, is vital.

5. Conclusion

In conclusion, the functional characterization of AC12 showed this peptide to be the most efficient, of the three studied here, in reducing anti-inflammatory markers, and could be a potential new agent for the treatment of inflammatory diseases. Our results indicate that for *Hypsibias raniceps*, there may be many new diverse secreted peptides that are waiting to be discovered. All our sequenced peptides, AC12, DK16 and RC11, still have no defined family and probably perform an immunomodulatory function in *H. raniceps*, modulating NO and cytokine production in the presence of skin pathogens. However, further testing will be required to establish their classification as a novel immunomodulatory peptide family, to clarify their mechanism of action, as well as to develop their potential as anti-inflammatory agents.

Author Contributions

Conceived and designed the experiments: C.S.F.C.P., B.S.M. and T.M.B.R.; Performed the experiments: C.S.F.C.P., B.S.M., J.E.R., B.J.G., A.L.B. and P.H.H.V.J.; Interpretation/Acquisition/Analysis: D.M.P., B.J.G., A.L.B.; Contributed reagents, materials, analysis tools: D.M.P., P.J.B.P., P.B.A., P.V.; Wrote the paper: C.S.F.C.P., B.S.M., T.M.B.R.; Revision and final approval: T.M.B.R.

Conflict of interest

The authors report that there is no conflict of interest associated with this publication.

Acknowledgments

This study was supported by CAPES, CNPq, FAPDF and Universidade Católica de Brasília. We thank i3S, Faculdade de Farmácia da Universidade do Porto, Departamento de Química da Universidade de Aveiro and Universidade de Brasília for collaboration. This work received financial support from National Funds (FCT/MEC, Fundação para a Ciência e a Tecnologia/Ministério da Educação e Ciência) through project UID/QUI/50006/2013, co-financed by the European Union (FEDER under the Partnership Agreement PT2020). This work was also developed within the scope of the project CICECO-Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID/CTM/50011/2013), financed by national funds through the FCT/MEC and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement. The NMR spectrometers are part of the National NMR Network (PTNMR) and are partially supported by Infrastructure Project Nº 022161 (co-financed by FEDER through COMPETE 2020, POCI and PORL and FCT through PIDDAC).

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7. Table Legends

Table 1. (A) Amino acid sequence and molecular weight $[M + H]^+$ of the peptides in their reduced ([a]) and oxidized states ([b]). *represents the amidated C-terminal. MMe - experimental molecular weight.

Peptide	Sequence	MMe[a]	MMe[b]
AC12	H-ACFLTRLGTYVC*	1345.64	1343.65
DK16	H-DKERPICSNTFRGRKC*	1909.22	1906.95
RC11	H-RCFRRRGKLTC*	1394.73	1392.73

8. Figure Legends

Figure 1. Structural characterization of AC12 using NMR spectroscopy. A - The region of the ROESY spectra for AC12 in solution where the Hα-Hα cross peak between C2 and C12 can be seen. This peak is indicative of the presence of an expected disulfide bond. B - The NH-NH region of the ROESY spectrum for AC12 in solution where the NH-NH cross peaks that indicate a β-turn can be identified. C - The ROESY patterns for peptide AC12 that indicate an extended-loop-extended conformation for the peptide. The strong Hα-N(i,i+1) cross peaks are indicative of extended (beta-sheet) conformations while N-N(i,i+1) cross peaks indicate the presence of a loop (beta-turn). D - The 20 lowest target function conformers for AC12 obtained using the integrated ROESY peaks as input for the program CYANA. The backbone and side chain carbons and the disulfide bridge (yellow) are shown along with the H-bonds identified using MOLMOL (magenta lines). The backbone rmsd for the ensemble to the mean structure is 0.3 ± 0.09 Å.

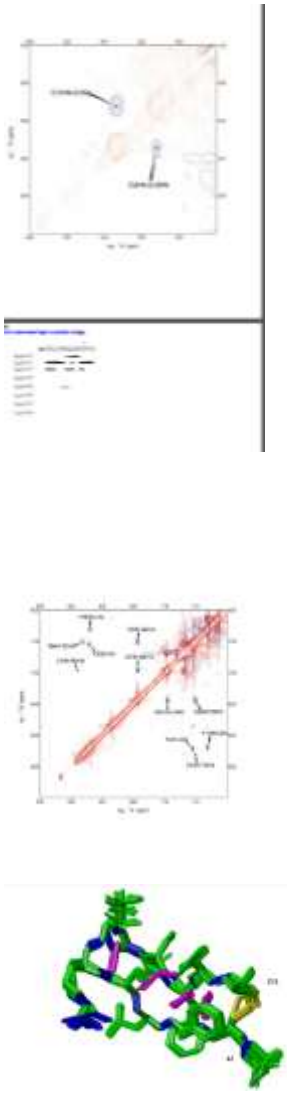
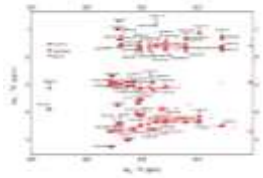
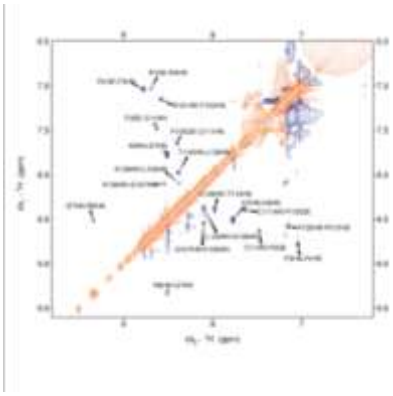


Figure 2. Structural characterization of RC11 using NMR spectroscopy. A - The NH-Hα region of the TOCSY spectrum for RC11 in solution where the resonance assignments for forms A and B are indicated. B - The NH-NH region of the ROESY spectrum for RC11 in solution where the NH-NH cross peaks that indicate a β-turn for both forms can be identified. C - The 20 lowest target function conformers for form A of RC11 obtained using the integrated ROESY peaks as input for the program CYANA. The backbone carbons and the disulfide bridge (yellow) are shown. The backbone rmsd for the ensemble to the mean structure is 0.74 ± 0.22 Å.





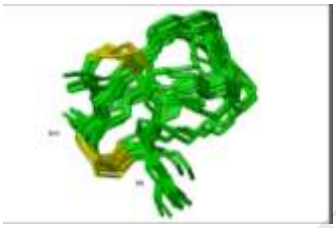


Figure 3. Minimum inhibitory concentrations of AC12, DK16 and RC11. Minimum inhibitory concentrations (µg.mL^{-1}) for the peptides against the reference strains *E. coli* (ATCC 8739) and *S. aureus* (ATCC 25923) in comparison with the positive controls: raniseptin-1 (Rsp-1), ampicillin (Amp) and chloramphenicol (Clor). Peptides were tested at concentrations between 4 and 256 µg.mL^{-1}. The results are represented as mean of three independent experiments performed in triplicate.

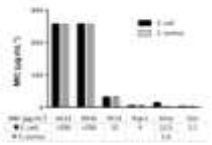


Figure 4. RAW 264.7 cell viability. Percentage of cell viability in RAW 264.7 cell culture stimulated with or without the peptides after 24 h of incubation. The peptides (A) AC12, (B) DK16 and (C) RC11 were tested at different concentrations (8 to 256 µg.mL^{-1}). Data represent the mean of three independent experiments performed in triplicate and are expressed as mean ± SD of absorbance at 560 nm. Statistical analyses were performed by one-way ANOVA and Tukey´s multiple test. Letters shared between or among the groups indicate no significant difference. Means with different letters are significantly different ($p<0.05$).

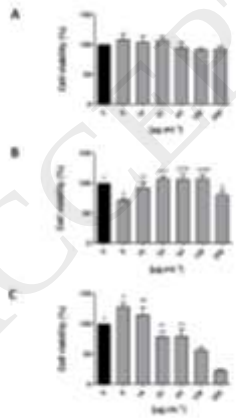


Figure 5. Percentage of hemolysis in an erythrocyte solution (1 %) treated with the peptides. The percentage of hemolysis in the erythrocyte solution of

Balb/c mouse after treatment with the peptides at different concentrations (128, 256 and 512 µg.mL^{-1}). 1% Triton X-100 was used as a positive control. Results are represented as mean of three independent experiments performed in triplicate.

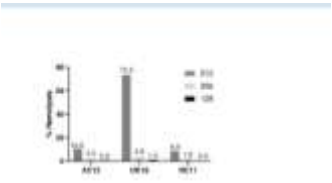


Figure 6. Effect of peptides AC12 and DK16 on the production of inflammatory mediators. Effect of peptides on TNF-α (A), IL-12 (B), IL-10 (C) and NO (D) production in RAW 264.7 cells stimulated with LPS (1 µg.mL^{-1}), after 24 hours of incubation with the peptides. Results correspond to the mean ± SD of three independent experiments performed in triplicate. Statistical analyses were performed by one-way ANOVA and Tukey´s multiple comparison test. Letters shared between or among the groups indicate no significant difference. Means with different letters are significantly different ($p<0.05$).

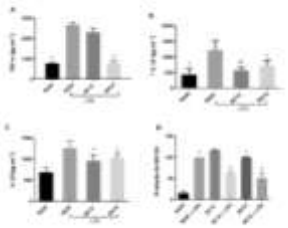


Figure 7. Effect of the peptides on carrageenan-induced paw edema. A - Acute anti-inflammatory activity of the peptides (7.5 mg.kg^{-1}) and dexamethasone (1 mg.kg^{-1}) on carrageenan-induced edema in Balb/c mice at different time points. One hour after treatment with peptides or controls, the animals (n=5) received an intraplantar injection of carrageenan (300 µg in 20 µL of 0.01 M PBS) and the edema thickness was measured at 2, 4, 6 and 8 hours with a digital caliper (0.01 mm resolution). Data correspond to the difference between the paw edema thickness and its basal thickness, prior to the induction of local inflammation (mm). PBS (0.01 M, pH 7.4) was used as negative control. Values represent the mean ± SD of the paw edema. *p<0.05 (vs LPS). Statistical analyses were performed by multiple t tests and Sidak-Bonferroni multiple comparison. B - F - Photomicrographs from mice paw stained with hematoxylin and eosin (20 x magnification). (B) PBS group, (C) carrageenan group, (D) peptide AC12 treatment in carrageenan group, (E) peptide DK16 treatment in carrageenan group, (F) peptide RC11 treatment in carrageenan group. All groups received peptides at 7.5 mg.kg^{-1}, (G) representative structure of the plantar paw tissue of Balb/c mice analyzed in the histology assay.

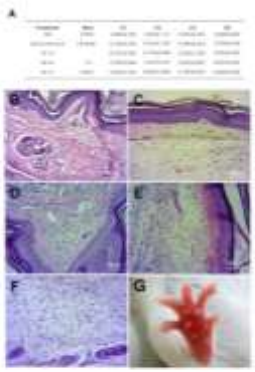


Figure 8. Effect of the peptides on carrageenan-induced peritonitis. A – Assay scheme used for carrageenan-induced peritonitis. B – E - Effect of AC12, DK16 and RC11 on TNF-α (B), IL-12 (C), IL-6 (D) and (E) IL-12 production from the peritoneal lavage after induction of peritonitis with 1 % carrageenan in Balb/c mice (n=5). Peptides (7.5 $mg.kg^{-1}$) or controls were administered intraperitoneally 1 hour prior to induction of peritonitis. Negative control corresponds to the PBS solution (0.01 M), and positive control corresponds to dexamethasone (1 $mg.kg^{-1}$). Results presented are the mean ± SD of three independent experiments performed in triplicate. *p<0.05, **p<0.005; ***p<0.0006; analyses were performed by one-way ANOVA and Tukey´s multiple comparison test using GraphPad Prism software (version 6.01, 2012).