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Short communication

In vivo antimicrobial evaluation of an alanine-rich peptide derived from *Pleuronectes americanus*

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ABSTRACT

In several organisms, the first barrier against microbial infections consists of antimicrobial peptides (AMPs) which are molecules that act as components of the innate immune system. Recent studies have demonstrated that AMPs can perform various functions in different tissues or physiological conditions. In this view, this study was carried out in order to evaluate the multifunctional activity *in vivo* of an alanine-rich peptide, known as *Pa*-MAP, derived from the polar fish *Pleuronectes americanus*. *Pa*-MAP was evaluated in intraperitoneally infected mice with a sub-lethal concentration of *Escherichia coli* at standard concentrations of 1 and 5 mg kg^{-1}. At both concentrations, *Pa*-MAPs exhibited an ability to prevent *E. coli* infection and increase mice survival, similar to the result observed in mice treated with ampicillin at 2 mg kg^{-1}. In addition, mice were monitored for weight loss. The results showed that mice treated with *Pa*-MAPs at 1 mg kg^{-1} gained 0.8% of body weight during the 72 h of experiment. The same was observed with *Pa*-MAP at 5 mg kg^{-1}, which had a gain of 0.5% in body weight during the treatment. Mice treated with ampicillin at 2 mg kg^{-1} show a significant weight loss of 5.6% of body weight. The untreated group exhibited a 5.5% loss of body weight. The immunomodulatory effects were also evaluated by the quantification of IL-10, IL-12, TNF-α, IFN-γ and nitric oxide cytokines in serum, but no immunomodulatory activity was observed. Data presented here suggest that *Pa*-MAP should be used as a novel antibiotic against infection control.

1. Introduction

Bacterial infection control in hospitalized patients is an enormous challenge due to numerous contamination sources including invasive procedures and devices such as mechanical ventilators [10], ultrasound probes [50] and catheters [58]. Aiming to control such microorganisms, permanent surveillance protocols are adopted in hospitals informing about preventive strategies to reduce infection [9,52]. According to the World Health Organization (WHO), 8.7% of hospitalized patients of 55 hospitals in 14 countries in 4 WHO regions (Europe, Eastern Mediterranean, South-East Asia and Western Pacific) and 1.4 million people world-wide suffer from nosocomial infections [53]. Moreover, nosocomial infections have a direct impact on country costs due to increases in length of hospitalization, number of physician visits and deaths [15,33].

Enterobacteriacea is one of the most prevalent bacterial families in nosocomial infections mainly represented by *Pseudomonas aeuruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* [10,28]. *E. coli* is a facultative anaerobe able to colonize the human large intestine and can be divided in virulent and avirulent strains. Virulence factors that differentiate these strains are commonly acquired on mobile genetic elements by horizontal gene transference. Furthermore, these virulence factors confer upon *E. coli* strains the ability to resist to human host defenses [20,39]. *E. coli* strains are attributed to cause nosocomial infections and a wide number of human diseases, such as sepsis, meningitis, and diarrhea [30,35,36,47].

Otherwise, the application of novel antimicrobials seems to be an alternative for infectious disease treatment including the development of antimicrobial peptides (AMPs) [7,23]. AMPs consist of peptides from 12 to 100 amino acid residues length, which exert activity against Gram-positive and -negative bacteria, fungi and viruses through multiple mechanisms [12,26,43]. These peptides can be isolated from various organisms such as plants [48], insects [45], amphibians [57], fishes [1] and mammals [18]. Despite their different origins, AMPs may show some common properties including cationic surfaces and amphipathic structures [49]. Furthermore, some peptides also show promiscuity as they attach to different targets such as membranes, cell walls, cytosolic proteins and nucleic acids [7,27,49]. This property could lead to multifunctionality derived from a single protein molecule. This process could also occur due to a specific stimulus, such as pH or protein concentrations. This property is commonly found in plant and animal

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defense peptides, in which a wide number of different functions must be generated by several structural homologs with identical structures [16]. Moreover, cationic AMPs conformation seems to interact with anionic microorganism membranes by electrostatic interactions in a first step. AMPs inset into membrane bilayers and aggregate, forming pores and leading to an efflux of intracellular ions [40,64].

Additionally, some studies have shown the relation between resistance to certain infectious diseases and AMPs secretion. Cipriano et al. [8] showed that AMPs secreted in fish external mucus may confer resistance to *Aeromonas salmonicida* in salmonids. Likewise, in Teleostei marine polar fish, some peptides are commonly secreted into the blood and tissues depending on sub-zero temperature [13,31]. These peptides are known as antifreeze peptides (AFP), and the type I AFP family is commonly found in winter flounder (*Pleuronectes americanus*), named HPLC-6 and HPLC-8 [18]. Comparing AMPs and AFPs, similar structural and physical–chemical properties have been found, such as the hydrophobic ratio, hydrophobic moment and specific amino acid composition [61]. Migliolo et al. [34] studied a synthetic peptide named *Pa*-MAP, a derivate of the HPLC-8 peptide [25]. Additionally, *Pa*-MAP primary sequence was selected from the AFP HPLC-8 produced by the polar fish *P. americanus* with length (decreased from 37 residues to 26) and residue modifications, such as lysine 7 and 18 substituted by alanine, valine 2 and 13 by treonine, and glutamic acid 11 by alanine. The first amino acid residue in HPLC-8 is aspartic acid, also substituted by histidine [34]. Surprisingly, *Pa*-MAP is devoid of arginine and lysine cationic residues, which seems to be important for antimicrobial activity [19,41]. Indeed, the peptide has mostly hydrophobic amino acid residues suggesting that that *Pa*-MAP antimicrobial activity could be attributed mostly to hydrophobic interaction. Furthermore, it shows the ability of inhibiting the HSV virus, the development of mycellar fungi *T. mentagrophytes* and *T. rubrum*, and deleterious activity against *E. coli*, besides cytotoxic effects in tumor cells. Moreover, *Pa*-MAP did not show any cytotoxic effects against human red blood cells, enabling this peptide for *in vivo* assays. An additional activity was the antitumoral effects against Caco-2 (human epithelial colorectal adenocarcinoma cells), HCT-116 (human colorectal carcinoma cell lines) and MCF-7 (human breast cancer cells) [34].

One of the main challenges of AMP utilization has been related to peptide stability in such models. Several studies have demonstrated that the activity of AMPs *in vitro* was not the same as *in vivo* models, and these controversial results may be attributed to certain proteases present in serum [22]. Another cause of *in vivo* inactivity is the high polar property of some AMPs, resulting in a reduction in membrane crossing or in an irregular distribution into mammalian cells, losing activity against intracellular microorganisms [59]. Moreover, as revised by Brinch et al. [3], *in vivo* AMP activity may also be impeded by poor drug distribution and AMP degradation by increased metabolism inside the cell. AMPs also can induce the immune system to produce anti-AMP antibodies [2], reducing their effectiveness

In this view, this study evaluated the *in vivo* antimicrobial activity of the synthetic multifunctional peptide *Pa*-MAP. Mice infected with *E. coli* strains were used as experimental models. Moreover, the serum was obtained and cytokines were evaluated in order to determine a possible immunomodulatory effect.

2. Materials and methods

2.1. Peptide synthesis and mass spectrometry analyses

The *Pa*-MAP peptide was synthesized by China Peptides (Shanghai, China) based on two 11-residue repeating segments from HPLC-8 with the following sequence: H-His-Thr-Ala-Ser-Asp-Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asn-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Ser-Met-Ala-NH_2, with the stepwise solid-phase method using the N-9-fluorenylmethyloxycarbonyl (Fmoc) strategy with a Rink amine resin (0.52 mmol g^{-1}), and purified by reversed-phase high-performance liquid chromatography (HPLC) with purity degree >95% [6,34]. *Pa*-MAP molecular mass was determined using matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS/MS) analysis on UltraFlex III, Bruker Daltonics, Billerica, MA. Purified peptides were dissolved in a minimum volume of water that was mixed with an α-cyano-4-hydroxycinnamic acid saturated matrix solution (1:3, v:v), spotted onto a MALDI target plate and dried at room temperature for 5 min. The α-cyano-4-hydroxycinnamic acid matrix solution was prepared at 50 mM in H_2O:ACN:TFA (50:50:0.3, v:v:v). Peptide monoisotopic mass was obtained in the reflector mode with external calibration using the Peptide Calibration Standard II for mass spectrometry (up to 4000 Da mass range, Bruker Daltonics, Billerica, MA).

2.2. Bacterial strains and growth conditions

Escherichia coli (ATCC 8739) strains were cultivated in solid Muller–Hinton medium. An isolated colony was transferred to 5 mL of liquid Luria–Bertani (LB) medium and grown in a rotating drum at 37 °C with aeration during 24 h. Posteriorly, 100 μL of this pre inoculum was transferred to 4.9 mL of LB medium and grown at the same conditions for 2 h. The absorbance was evaluated to identify the late log phase, when the OD at 600 nm was 1.0, corresponding a concentration of 1×10^8 UFC mL^{-1} (5×10^8 UFC at final volume). These cells were centrifuged for 6 min at 1200 rpm and the sediment was resuspended in 5 mL of phosphate buffered saline (PBS) and equalized to a concentration of 1×10^6 UFC at final volume for virulence and immunomodulatory assays [63].

2.3. Escherichia coli in vivo bioassays

In vivo experiments were performed with 6–10 weeks old female BALB/c mice from University of Campinas (Campinas/SP). Mice were housed and used in accordance with guidelines established by the Ethical Committee of Animal Use of University of Brasília (Brasilia/DF), registered under protocol number UnBDOC:83931/2011, and all efforts were made to minimize animal suffering. Mice were divided into 5 groups of 5 animals each (Table 1). As described above, groups were infected *via* intraperitoneal (IP) injection with *E. coli* suspension equalized and diluted in cold PBS to a sub lethal concentration of 1×10^5 UFC (50 μL in each animal) [54]. Treatments of infected mice were performed with *Pa*-MAP at 1 and 5 mg kg^{-1}, both dissolved in 100 μL of PBS, respectively. PBS was utilized as the negative control, and ampicillin at 2 mg kg^{-1} dissolved in 100 μL of PBS was utilized as the positive control. Moreover, an uninfected control was also performed. All mice were housed with constant water and food in an air-filtered environment maintained at 20 ± 2 °C during 72 h and

Table 1
Experimental groups distribution.

Experimental groups ($n = 5$)	*E. coli* infection	Treatments
1 – Infected	Yes	Ampicillin 2 mg kg^{-1}
2 – Infected and untreated	Yes	No
3 – Non infected	No	No
4 – Infected and treated group 1	Yes	*Pa*-MAP 1 mg kg^{-1}
5 – Infected and treated group 2	Yes	*Pa*-MAP 5 mg kg^{-1}

Groups 1, 2, 4 and 5 were infected with *E. coli* at 1×10^5 UFC sublethal concentration. Drug treatments were injected 24 and 48 h after infection.

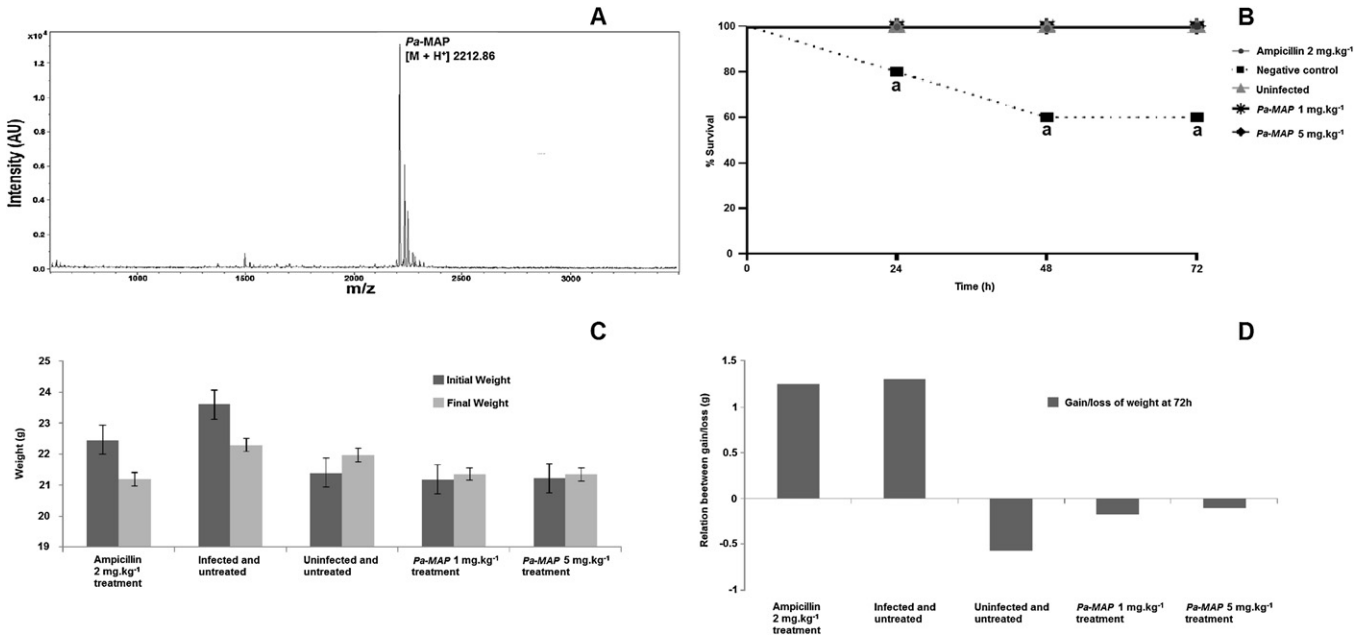

Fig. 1. (A) MALDI-TOF mass spectrometry analysis of *Pa*-MAP monoisotropic mass [M+H$^+$] = 2212.86. (B) After *E. coli* sub lethal infection, mice were treated with ampicillin at 2 mg kg^{-1} (—●—), *Pa*-MAP at 1 (—✱—) and 5 mg kg^{-1} (—◆—) at 24 h and 48 h. At 24 h, 20% of mice of infected and untreated group (·■·) died and another 20% had died at 48 h. (—▲—) represent the Untreated group. (a) represent Logrank test with $p < 0.05$, Chi square = 11.67, compared to negative control. (C and D) Weight loss was evaluated at the beginning and at the end of experiment. (C) Mice lost weight in infected and untreated group and mice treated with ampicillin at 2 mg kg^{-1}. Mice treated with *Pa*-MAP gained weight until the end of the experiment, as well as mice that were uninfected and untreated. (D) Difference of weight from each group at 72 h after procedures. $P > 0.05$.

further treated as described above (Table 1). Treatments occurred 24 h and 48 h after infection. Moreover, all mice were weighed at the beginning and at the end of the experiment.

2.4. Cytokines evaluation

Mice were anesthetized by xilazine and ketamine at 10 mg kg^{-1} and 50 mg kg^{-1}, respectively, after 72 h. Blood collection was performed by decapitation and serum obtained by centrifugation and stored at −20 °C. The cytokines interleukin-10 (IL-10), IL-12, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and nitric oxide (NO) were measured in serum by enzyme-linked immunosorbent assays (ELISA) using ELISA kit (Peprotech) according to the manufacturer's instructions.

2.5. Statistical Analysis

The statistical significance of the experimental results was determined by one-way Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. Values of $P < 0.05$ were considered statistically significant. Graphpad Prism version 6.0 was used for all statistical analyses.

3. Results

3.1. Mass spectrometry analysis

MALDI-ToF evaluation showed an ion with an m/z of 2212.86, corresponding to the calculated value for the peptide sequence, above 95% in purity. All further bioassays were performed using purified *Pa*-MAP (Fig. 1A).

3.2. Antimicrobial activities

In order to confirm the *in vitro* protective effects of *Pa*-MAP against *E. coli*, *in vivo* antibacterial activity was evaluated by a sub-lethal *E. coli* mice IP infection. Two concentrations of *Pa*-MAP (1 mg kg^{-1} and 5 mg kg^{-1}) treatment were tested. Ampicillin at 2 mg kg^{-1} was used as a positive control. During the first 24 h after infection, the infected and untreated group decreased 20%, further decreasing to 60% 48 h after infection. In contrast, all mice treated with *Pa*-MAP in both concentrations survived at the end of experiment. The same pattern was observed in mice treated with ampicillin (Fig. 1B).

Mice weights were further evaluated in the beginning and at the end of experiment. Infected and untreated mice lost 5.5% of their body weight after 72 h of experiment. In contrast, mice treated with *Pa*-MAP at 1 mg kg^{-1} gained 0.8% of their body weight, similar to *Pa*-MAP at 5 mg kg^{-1}, which gained 0.5% of their body weight during the same period. Non-infected mice gained slightly more body weight (2.7%) compared to the *Pa*-MAP treatment groups. Infected mice treated with ampicillin at 2 mg kg^{-1} also had lost weight, equivalent to 5.6% of their initial body weight (Fig. 1C and D).

3.3. Immunomodulatory activity

Some cytokines were evaluated in attempt to identify an immunomodulatory effect of *Pa*-MAP in the mice immunologic system. This evaluation of immunomodulatory activity *in vivo* was investigated by quantification of IL-10, IL-12, TNF-α and NO in serum. *Pa*-MAP used as treatment was evaluated at 1 mg kg^{-1}, corresponding to a concentration of twice the minimum inhibitory concentration (MIC) of 512 μg mL^{-1} [34], and 5 mg kg^{-1}, corresponding 10 times the MIC encountered in early study with *Pa*-MAP. Ampicillin at 2 mg kg^{-1} was used as a positive control. These concentrations of *Pa*-MAP were unable to modify IL-10 release when compared to the non-infected and untreated mice group. Similar data were observed for IL-12 and TNF-α production in all treatments groups (Supplementary Fig. 1).

See Supp Figure S1 as supplementary file. Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2013.02.001.

4. Discussion

Antimicrobial resistance mechanisms developed by bacteria is a serious worldwide threat to public health, particularly for immunocompromised patients and those under immunosuppression therapy, e.g. patients after organ transplant [29]. Moreover, infections caused by antibiotic-resistant microorganisms have contributed to increases in patient mortality, especially for those whose treatment with currently available drugs has become less efficient [14]. Due to these facts, peptides with antimicrobial activities have become extremely attractive for microorganisms control, mainly due their low toxicity effects into mammalian cells [24]. In our study, an alanine-rich peptide designed from a polar fish, *P. americanus*, with two repeat antifreeze motifs and clear *in vitro* deleterious activity against *E. coli*, with identical purification degree (see Fig. 1A) previously reported by Migliolo et al. [34] was evaluated *in vivo*.

Some peptides were designed to develop a multifunctional product, able to eliminate microbes and increase the immune response, involving systematic variations in the structure of a base molecule, i.e. cationic charge, hydrophobicity and hydrophobic moment [21]. Moreover, some cationic peptides are known to be able to induce some immunomodulatory effect [37,62]. Some studies demonstrated that only a fraction of the peptide was presented to the immune system, and these peptides should be able to overcome some "bottlenecks" and only those with specific characteristics would be able to induce the immune system [32,46]. Nevertheless, *Pa*-MAP seems to be unable to interact with immune system cells to induce cytokine production. Data presented here shows that *Pa*-MAP neither significantly stimulates nor inhibits some cytokine production, despite of others could be modified by the presence of peptide. This result is similar to the Fritsche et al. studies [17]. In their studies, it was demonstrated that a short, proline-rich antimicrobial peptide has direct antibacterial action *in vivo*, but was unable to stimulate cytokine production.

Despite the absence of immunomodulatory activity, data reported here shows strong evidence that the peptide *Pa*-MAP could be useful for pharmaceutical design once it shows the ability to perform *E. coli* inhibition *in vivo*. *Pa*-MAP demonstrated *in vivo* activity against *E. coli* at low concentrations when compared to other antimicrobial peptides. Schaal et al. [51] demonstrated similar effect with rhesus θ-defensin (RTD), a macrocyclic antimicrobial peptide expressed in leukocytes of Old World monkeys. This RTD peptide was administrated at a single subcutaneous dose at 5 mg kg^{-1} in mice previously intraperitoneally infected with *E. coli* and resulted in an increase in mice survival. Vingsbo et al. [60] demonstrated that the novel synthetic polymyxin derivatives NAB737 and NAB739 are as effective as polymyxin B, an effective antibiotic against Gram-negative bacterial infections, in effectively treating *E. coli* peritoneal infection in mice at 1, 2 and 4 mg kg^{-1}. In another study, a non-natural AMP named M33 (with 9 amino acid residues long) showed the ability at 12.5 and 25.0 mg kg^{-1} to protect 100% of mice infected with lethal doses of *E. coli* and *P. aeruginosa*. Lower concentrations were unable to protect mice [42].

Although antimicrobial activity, the mechanism of action has been unclear until now. Some researchers have suggested that AMPs can cause bacterial membrane disruption, leading to intracellular leakage and later microorganism death [4]. In addition, AMPs can interact with immune cells and increase immune response in the face of injury or inflammation, modulating the innate immune response, for example, through chemotactic activity, stimulation of cytokine release, neutralization of LPS-induced septic effects, wound healing and tissue repair [11]. Nevertheless, *Pa*-MAP did not exhibit the ability to stimulate cytokine release from immune cells as previously described, suggesting that direct microorganism control could be related to the *Pa*-MAP mechanism of action.

One of the most documented effects of *E. coli* infection is progressive weight loss, mainly due to water loss during infection [44]. This symptom is also caused by heat-labile toxin (LT) and heat-stable toxin (ST) presented in enterotoxigenic *E. coli* infection [55]. In the current study, 5.5% of mice body weight loss was observed in the infected and untreated group. A study developed in murine model, mice infected with non-pathogenic and enterohemorrhagic *E. coli* (NPEC and EHEC) demonstrated a clear weight loss of about 6% [5]. Furthermore, mice treated with ampicillin at 2 mg kg^{-1} also showed 5.6% weight loss, demonstrating that ampicillin can eliminate bacterial infection, but did not exhibit ability to inhibit weight loss. In contrast, *Pa*-MAP exhibited protective effects against *E. coli* and body weight loss in both concentrations, preventing this pathological effect. Similar data was observed in a study with the AMP IB-367, a protegrin peptide, evaluated to prevent oral mucositis in hamsters. In this study, animals treated with IB-367 at 0.12–2.0 mg mL^{-1} showed body weight gain in comparison with mice treated with placebo, and became significantly greater during the passing days [38]. Soni et al. [56] evaluated *in vivo* the efficacy of two combined antibiotics, ceftriaxone and vancomycin, against *E. coli* intra-abdominally infected mice. Infected mice showed significant weight loss during infection and became normalized after vancomycin and ceftriaxone treatment.

5. Conclusions

Due to increasing number of cases of multi-resistant bacterial disease against a variety of antimicrobial drugs, antimicrobial peptides have a great and considerable potential to become the new generation of bioactive products. Here, a peptide with an antimicrobial novel effect *in vivo* was confirmed but any immunomodulatory activity was observed indicting that action mechanism is only related to a direct antimicrobial activity. This peptide demonstrated a protective effect against *E. coli* at lower concentrations in comparison to other antimicrobial peptides and synthetic pharmacological antibiotics [42,60]. Moreover, weight loss in mice was prevented during treatment with *Pa*-MAP, in contrast with other treatments, i.e. ampicillin and other AMPs. In the future, *Pa*-MAP could be used in the development of a novel biopharmaceutical against microorganisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2013.02.001.

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