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Biochemical and biophysical combined study of bicarinalin, an ant venom antimicrobial peptide

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ABSTRACT

We have recently characterized bicarinalin as the most abundant peptide from the venom of the ant *Tetramorium bicarinatum*. This antimicrobial peptide is active against *Staphylococcus* and Enterobacteriaceae. To further investigate the antimicrobial properties of this cationic and cysteine-free peptide, we have studied its antibacterial, antifungal and antiparasitic activities on a large array of microorganisms. Bicarinalin was active against fifteen microorganisms with minimal inhibitory concentrations ranging from 2 and 25 μmol L^{-1}. *Cronobacter sakazakii*, *Salmonella enterica*, *Candida albicans*, *Aspergilus niger* and *Saccharomyces cerevisiae* were particularly susceptible to this novel antimicrobial peptide. Resistant strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *C. albicans* were as susceptible as the canonical strains. Interestingly, bicarinalin was also active against the parasite *Leishmania infantum* with a minimal inhibitory concentrations of 2 μmol L^{-1}. The bicarinalin pre-propeptide cDNA sequence has been determined using a combination of degenerated primers with RACE PCR strategy. Interestingly, the N-terminal domain of bicarinalin pre-propeptide exhibited sequence similarity with the pilosulin antimicrobial peptide family previously described in the *Myrmecia* venoms. Moreover, using SYTOX green uptake assay, we showed that, for all the tested microorganisms, bicarinalin acted through a membrane permeabilization mechanism. Two dimensional-NMR experiments showed that bicarinalin displayed a 10 residue-long α-helical structure flanked by two N- and C-terminal disordered regions. This partially amphipathic helix may explain the membrane permeabilization mechanism of bicarinalin observed in this study. Finally, therapeutic value of bicarinalin was highlighted by its low cytotoxicity against human lymphocytes at bactericidal concentrations and its long half-life in human serum which was around 15 h.

1. Introduction

Classical antibiotics are under intense pressure from emerging resistance and antimicrobial peptides (AMPs) are now considered as credible alternatives in the development of novel biocidal agents [1,2]. AMPs play important roles in preventing infections by providing an effective and fast acting defense against harmful microorganisms [3–5]. Furthermore, they display an ability to efficiently destroy a broad spectrum of microorganisms and particularly, multi-drug-resistant (MDR) bacteria. AMPs are generally small amphipathic cationic peptides of variable length (12–30 amino acids), active against bacteria, parasites, yeasts, fungi, viruses and tumour cells [6–8]. They usually act through relatively non-specific processes resulting in a membranolytic activity based on pore formations by barrel-stave, carpet or toroidal-pore mechanisms [3,9,10]. Physical interactions between AMP and microorganisms such as charge–charge and hydrophobic contacts might explain why development of resistance against cationic peptides is difficult. When these natural AMPs exhibit substantial cytotoxicity towards eukaryotic cells, a decrease in their hydrophobicity, helicity and amphipathicity, by replacing specific

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amino-acids by cationic residues, reduces hemolytic activity and concomitantly promotes antibacterial potency, turning them into therapeutically valuable anti-infective agents [11].

Venoms of arthropods are a rich source of biologically active compounds [12–15]. With 13,161 known species, ants constitute a highly diversified group of arthropods [16]. AMPs have been identified in several species, such as pilosulins from the Australian *Myrmecia pilosula* [17,18] or ponericins from the neotropical ant *Pachycondyla goeldii* [19]. Unlike stingless species which typically spray or deposit small molecules such as formic acid, most stinging ants have evolved complex venoms particularly rich in peptides and enzymes [20]. This is the case for the *Tetramorium* genus for which the venoms are predominantly proteinaceous [21]. The discovery of novel AMPs is scientifically challenging and their development as drugs is often limited due to the lack of detailed data on their physicochemical characteristics [2,5]. Especially, pharmacological and pharmacokinetic data describing their mechanism of action and their physicochemical properties, respectively, are generally missing.

Bicarinalin is a cystein-free polycationic linear and amidated peptide, active against Staphylococcus and Enterobacteriaceae strains. It exhibits a very low hemolytic activity against human red blood cells [21].

The first aim of the present study was to further investigate the biological activities of bicarinalin against a large collection of microorganisms such as bacteria, fungi, yeasts and a parasite, to determine its cytotoxicity on human lymphocytes and its stability in human serum. Then, in order to gain further insight into the structure of ant toxin precursors, we also investigated the pre-probicarinalin cDNA organisation using a RACE PCR strategy. Finally, we explored its mechanism of action and determined its solution structure using two dimensional (2D) ^{1}H NMR and molecular dynamics.

2. Materials and methods

2.1. Peptide synthesis

Bicarinalin (KIKIPWGKVKDFLVGGMKAV-NH_2), its randomly-designed amidated scrambled counterpart (VVMKLGKAFVPIGKWKKDGI-NH_2) and bicarinalin$_{(4–20)}$ (IPWGKVKDFLVGGMKAV-NH_2) were synthesized on a Liberty microwave assisted automated peptide synthesizer (CEM, Saclay, France) at a purity grade higher than 99% as previously described [21]. The scrambled counterpart was randomly designed from bicarinalin amino-acids ensuring a total disrupting of the native helix as predicted by Agadir program and was used as negative control. The authenticity and the molecular identity of the synthetic peptides were controlled by MALDI-TOF-MS.

2.1.1. Reagents and microorganisms

All chemical reagents (melittin, ampicillin, tetracycline, SYTOX green, fluconazole and methicillin) were obtained from Sigma-Aldrich (Saint-Quentin-Falavier, France). Trifluoroethanol-d_2 (TFE-d_2) was from SDS (Peypin, France). Referenced bacterial strains were purchased from Institut Pasteur (CIP) and/or AES Biomerieux (ATCC). All microorganisms were grown in broth medium with continuous shaking at 150 rpm. The Gram negative strains used were *Pseudomonas aeruginosa* (CIP 82118 and multiresistant ATCC 15442), *Cronobacter sakazakii* (ATCC 29544), *Escherichia coli* (CIP 7624) and *Salmonella enterica* (ATCC 29934). *Enterococcus hirae* (CIP 5855), *Bacillus subtilis* (CIP 5262), *Staphylococcus aureus* (CIP 53156) and MRSA resistant to methicillin and oxacillin (ATCC 43300), and *Staphylococcus xylosus* (ATCC 35033) were used as Gram positive strains. Fungal and yeast strains used were *Geotrichum candidum* (ATCC 204307), *Aspergillus niger* (CIP 143183), *Saccharomyces cerevisiae sp*. and *Candida albicans* (CIP 4872 and wild multiresistant (CAAL 117; Supplementary Table 1). Bacteria were grown in tryptic soy broth at 37 °C (except for *S. aureus* and *C. sakazakii*, 40 °C) while fungi and yeast were grown in Sabouraud broth at 30 °C. After incubation, 100 μL of bacteria inoculum were suspended in 5 mL fresh broth medium for 4 h to obtain a mid-log-phase culture which was diluted in the same medium, to an absorbance of 0.002 at λ_{max} = 600 nm.

2.1.2. Antimicrobial activity

Minimal inhibitory concentrations (MIC) of native and scrambled bicarinalin were determined by a standard two-fold dilution method as previously described [22]. Bacteria were incubated with different concentrations of each compound (from 0.05 to 97.5 μmol L^{-1} for bicarinalin and melittin, and from 0.049 to 100 μmol L^{-1} for other antibiotics) in 100 μL final volume of soy broth medium (10^4 to 10^5 CFU). The microplates were incubated 24 h at specific temperature with continuous shaking. Absorbance was measured at the wavelength of 600 nm using a spectrophotometric microplate reader (Infinite 200, Tecan, Lyon, France). MICs were expressed as the lowest concentration demonstrating no visible growth. Phosphate buffered saline (PBS) and scrambled bicarinalin were used as a negative control while conventional antibiotics were used as positive controls. The minimal bactericidal concentration (MBC) value was established by counting the colony growth on an inoculated agar plate after 24 h of incubation with different concentrations (≥MIC) as previously described [22]. They were expressed as the lowest concentration that caused a 99.9% reduction of the initial inoculum.

2.1.3. Antiparasitic activity

The proliferation of axenic *Leishmania infantum* (MHOM/MA/67/ITMAP-263) amastigotes expressing luciferase activity was evaluated in Rosswell Park Memorial Institute medium containing 10% fetal calf serum for 48 h at 25 °C in presence of native or scrambled bicarinalin. Their leishmanicidal activity was measured for concentrations ranging from 0.05 to 97.5 μmol L^{-1}. The luciferase substrate (Promega, Lyon, France) was added after incubation and its activity was measured using a luminometer following the conditions described by Sereno et al. [23].

2.1.4. Membrane permeabilization assay

The membrane permeabilization of each strain by bicarinalin was evaluated using SYTOX green uptake assay as previously described [20]. Briefly, SYTOX green was added to these bacteria suspensions (optical density adjusted to 0.6 at the wavelength of 600 nm) at a final concentration of 0.5 μmol L^{-1}. After distribution into a micro-well plate with 100 μL of each bacterial suspension, bicarinalin was added to perform a serial two-fold dilution (same range of concentrations as above). After 30 min of incubation, SYTOX green uptake was recorded by using an Infinite 200 spectrophotometric microplate reader (λ_{ex} 488 nm, λ_{em} 540 nm).

Lymphocyte isolation and cell cytotoxicity assay

Lymphocytes were isolated from total heparinized blood samples obtained from one healthy donor using Ficoll-PlaqueTM PLUS kit (GE healthcare, Toulouse, France). After two washes in PBS, lymphocytes were suspended in PB-MaxTM medium supplemented with 1% of bovine serum albumin, 100 μg.mL^{-1} of streptomycine, 100 U/mL of penicillin and 2 mmol L^{-1} of glutamine. Viability and numbering of the cells were determined using trypan blue stain 0.4% and Malassez hemocytometer.

The lactate deshydrogenase (LDH) cytotoxicity assay kit (Life Technologies) was used to determine the cytotoxic effect of bicarinalin. Lymphocytes were incubated in triplicate into a 96-well plate overnight with various concentrations of bicarinalin in PB-MaxTM