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A family of antimicrobial and immunomodulatory peptides related to the frenatins from skin secretions of the Orinoco lime frog *Sphaenorhynchus lacteus* (Hylidae)

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ABSTRACT

Peptidomic analysis of norepinephrine-stimulated skin secretions of the Orinoco lime tree frog *Sphaenorhynchus lacteus* (Hylidae, Hylinae) revealed the presence of three structurally related host-defense peptides with limited sequence similarity to frenatin 2 from *Litoria infrafrenata* (Hylidae, Pelodryadinae) and frenatin 2D from *Discoglossus sardus* (Alytidae). Frenatin 2.1S (GLVGTLLGHIGKAILG.NH_2) and frenatin 2.2S (GLVGTLLGHIGKAILS.NH_2) are C-terminally α-amidated but frenatin 2.3S (GLVGTLLGHIGKAILG) is not. Frenatin 2.1S and 2.2S show potent bactericidal activity against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MIC ≤16 μM) but are less active against a range of Gram-negative bacteria. Frenatin 2.1S (LC_{50} = 80 ± 6 μM) and 2.2S (LC_{50} = 75 ± 5 μM) are cytotoxic against non-small cell lung adenocarcinoma A549 cells but are less hemolytic against human erythrocytes (LC_{50} = 167 ± 8 μM for frenatin 2.1S and 169 ± 7 μM for 2.2S). Weak antimicrobial and cytotoxic potencies of frenatin 2.3S demonstrate the importance of C-terminal α-amidation for activity. Frenatin 2.1S and 2.2S significantly ($P < 0.05$) increased production of proinflammatory cytokines IL-1β and IL-23 by lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages and frenatin 2.1S also enhanced production of TNF-α. Effects on IL-6 production were not significant. Frenatin 2.2S significantly downregulated production of the anti-inflammatory cytokine IL-10 by LPS-stimulated cells. The data support speculation that frenatins act on skin macrophages to produce a cytokine-mediated stimulation of the adaptive immune system in response to invasion by microorganisms. They may represent a template for the design of peptides with therapeutic applications as immunostimulatory agents.

1. Introduction

Skin secretions of frogs belonging to certain families contain high concentrations of cytotoxic host-defense peptides with the ability to inhibit the growth of bacteria and fungi that may also show anti-tumor and anti-viral activity [12,14]. With few exceptions, these peptides are cationic (charge between +1 and +6 at pH 7), contain between 40 and 70% hydrophobic amino acids, and adopt an amphipathic α-helical conformation in a membrane-mimetic environment [40]. At the time of writing, peptides with antimicrobial activity have been identified in the skins of frogs from species belonging to the Alytidae, Bombinatoridae, Hylidae, Hyperoliidae, Leiopelmatidae, Leptodactylidae, Myobatrachidae, Pipidae, and Ranidae families [12] and the Antimicrobial Peptide Database (http://aps.unmc.edu/AP) lists 929 amphibian host-defense peptides [40]. Although considered to be a component of the animal's system of innate immunity, the sporadic species distribution of these peptides suggests that their production in the skin may confer some evolutionary advantage to the organism but is not necessary for survival. It has been suggested that cutaneous symbiotic

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bacteria may provide the major system of defense against pathogenic microorganisms in the environment with antimicrobial peptides assuming a supplementary role in some species [11].

According to currently accepted taxonomic classifications, the extensive and widely distributed family Hylidae is divided into the subfamilies Phyllomedusinae (currently 59 species in 5 genera), Pelodryadinae (currently 203 species in the single genus *Litoria*), and Hylinae (currently 674 species in 43 genera) [18]. Skin secretions from the Central and South American tree frogs, particularly species in the genera *Agalychnis*, *Hylomantis*, *Pachymedusa*, and *Phyllomedusa* in the subfamily Phyllomedusinae, have proved to be rich source of host-defense peptides with antimicrobial activity (reviewed in [30]). These include the dermaseptins, dermatoxins, hyposins, phylloseptins, phylloxins, plasticins, and a number of "orphan" peptides not included in these groups. Similarly, the skin secretions from the tree frogs of the Australo-Papuan region belonging to the genus *Litoria* in the subfamily Pelodryadinae have provided multiple antimicrobial peptides that may be arranged in at least six groups on the basis of structural similarity. These are the aureins, caerins, citropins, dahleins, frenatins, and maculatins (reviewed in [5]).

Synthesis of dermal host-defense peptides among species in the subfamily Hylinae is more restricted. Four structurally related peptides, termed pseudin 1–4, were isolated from an extract of the skin of the South American paradoxical frog *Pseudis paradoxa*, although these peptides were not detected in norepinephrine-stimulated skin secretions [31]. Skin secretions from several North American species belonging to the genera *Hyla*, *Hypsiboas*, *Osteopilus*, and *Pseudacris* in the sub-family Hylinae have been shown not to contain host-defence peptides [12] but peptides with broad-spectrum antimicrobial activity and low hemolytic activity have been identified in skin secretions from the tree frogs *Hyla punctata* [34], *Hypsiboas albopunctatus* [7], and *Hypsiboas raniceps* [25] from Brazil and *Hyla eximia* from Mexico [39].

The Orinoco lime tree frog *Sphaenorhynchus lacteus* (Daudin, 1802) in the subfamily Hylinae is a moderately sized (snout-vent length 2.6–4.2 cm in males, 3.8–4.6 cm in females) frog that is widely distributed in the Amazon basin of Columbia, Venezuela, Peru, Brazil and Ecuador and also in the Guianas and on the islands of Trinidad and Tobago [18]. Its preferred habitat is wetland, such as flooded plains, ponds and lagoons with floating vegetation, and seasonally flooded agricultural land and it is common throughout its range. It is listed as a Species of Least Concern by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species in view of its relatively wide distribution and tolerance of a broad range of habitats but numbers have declined in some areas due to deforestation and water pollution [23].

The present study describes the purification of three structurally related peptides from norepinephrine-stimulated skin secretions from *S. lacteus* and presents their antimicrobial potencies against a range of reference strains and clinical isolates of Gram-positive and Gram-negative bacteria and cytotoxic activities against human erythrocytes and non-small cell lung adenocarcinoma A549 cells. Several frog peptides that were first identified on the basis of their abilities to inhibit growth of bacteria have been shown to possess cytokine-mediated immunomodulatory properties and effects upon production of both pro- and anti-inflammatory cytokines have been described [1,16,27,32,33,37]. Consequently, the action of the synthetic *S. lacteus* peptides on the production of proinflammatory tumor necrosis factor-α (TNF-α), interleukin IL-1β (IL-1β), interleukin-6 (IL-6), and interleukin-23 (IL-23) and immunosuppresive interleukin-10 (IL-10) by mouse peritoneal macrophages was investigated. The peptides show limited structural similarity to previously isolated frenatin 2 from *Litoria infrafrenata* (Hylidae, Pelodryadinae) [35] and frenatin 2D from *Discoglossus sardus* (Alytidae) [16] and so are referred to as frenatin 2.1S, 2.2S, and 2.3S.

2. Experimental

2.1. Collection of skin secretions

All experiments with live animals were approved by the Animal Research Ethics committees of U.A.E. University (Protocol No. A21-09) and the University of Kragujevac Animal Ethics Committee and were carried out by authorized investigators. Adult specimens ($n = 2$; weights 7.0 g and 8.1 g; sex not determined) of *S. lacteus* were collected in north central Guyana near the Essequibo river (6°28′ N, 58°35′ W). Each animal was injected at two sites into the dorsal lymphatic sac with norepinephrine bitartrate (40 nmol per g body weight) and immersed in water (50 ml) for 15 min. The solution containing the secretions was acidified with trifluoroacetic acid (TFA) (final concentration 1%, v/v) and stored at −20 °C. After collection of the secretions, the animals were released into their environment unharmed.

2.2. Peptide purification

The pooled skin secretions were passed at a flow rate of 2 ml/min through 6 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/TFA (70.0/29.9/0.1, v/v/v) and freeze-dried. The lyophilized skin secretions were redissolved in 0.1% (v/v) TFA/water (4 ml) and injected onto a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL,USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 63% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 nm and 280 nm and peaks were collected by hand. Aliquots (50 μl) of each major peak were analysed by MALDI-TOF mass spectrometry. The major peaks in the chromatogram were sequentially injected onto (1 cm x 25 cm) Vydac 214TP510 (C-4) and (1 cm × 25 cm) Vydac 208TP510 (C-8) columns. The concentration of acetonitrile in the eluting solvent was raised from 21% to 56% over 50 min and the flow rate was 2.0 ml/min.

2.3. Structural characterization

MALDI-TOF mass spectrometry was carried out using a Voyager DE-PRO instrument (Applied Biosystems, Foster City, CA, USA) that was operated in reflector mode as previously described [16]. The instrument was calibrated with peptides of known molecular mass in the 1–4 kDa range. The accuracy of mass determinations was ±0.02%. The primary structures of the peptides were determined by automated Edman degradation using a model 491 Procise sequenator (Applied Biosystems). MS/MS sequencing was performed using a Q-ToF Ultima API mass spectrometer (Micromass, Manchester, UK) using collision-induced dissociation (CID)-based fragmentation. Ions are fragmented by subjecting them to collisions with the background gas, argon as previously described [38].

2.4. Peptide synthesis

Frenatin 2.1S (GLVGTLLGHIGKAILG.NH_2), frenatin 2.2S (GLVGTLLGHIGKAILS.NH_2), and frenatin 2.3S (GLVGTLLGHIGKAILG) were supplied in crude form by GL Biochem Ltd (Shanghai, China). The peptides were purified to near homogeneity by reversed-phase HPLC on a (2.2 cm × 25 cm) Vydac 218TP1022 (C-18) column equilibrated with acetonitrile/water/TFA (21.0/78.9/0.1, v/v/v) at a flow rate of 6 ml/min. The concentration of acetonitrile was raised to 56% (v/v) over 60 min using a linear gradient. Absorbance was measured at 214 nm and 280 nm and the major peak in the chromatogram