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Anti-cancer, immunoregulatory, and antimicrobial activities of the frog skin host-defense peptides pseudhymenochirin-1Pb and pseudhymenochirin-2Pa

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ABSTRACT

Pseudhymenochirin-1Pb (Ps-1Pb) and pseudhymenochirin-2Pa (Ps-2Pa) are host-defense peptides, first isolated from skin secretions of the frog *Pseudhymenochirus merlini* (Pipidae). Ps-1Pb and Ps-2Pa are highly cytotoxic (LC_{50} < 12 μM) against non-small cell lung adenocarcinoma A549 cells, breast adenocarcinoma MDA-MB-231 cells, and colorectal adenocarcinoma HT-29 cells but are also hemolytic against human erythrocytes (LC_{50} = 28 ± 2 μM for Ps-1Pb and LC_{50} = 6 ± 1 μM for Ps-2Pa). Ps-2Pa shows selective cytotoxicity for tumor cells (LC_{50} against non-neoplastic human umbilical vein (HUVEC) cells = 68 ± 2 μM). Ps-1Pb and Ps-2Pa (5 μg/mL) significantly inhibit production of the anti-inflammatory cytokine IL-10 and the multifunctional cytokine IL-6 from lipopolysaccharide (LPS)-stimulated peritoneal macrophages from C57BL/6 mice and enhance the production of the pro-inflammatory cytokine IL-23 from both unstimulated and LPS-stimulated macrophages. Ps-1Pb potently (MIC ≤ 10 μM) inhibits growth of multidrug-resistant clinical isolates of the Gram-positive bacteria methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, and the Gram-negative bacteria *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*.
Ps-2Pa shows the same high potency (MIC ≤ 10 μM) against the Gram-positive bacteria but is 2–4 fold less potent against the Gram-negative isolates. Ps-1Pb at 4 × MIC kills 99.9% of *Escherichia coli* within 30 min and 99.9% of *S. aureus* within 180 min. In conclusion, cytotoxicity against tumor cells, cytokine-mediated immunomodulatory properties, and broad-spectrum antimicrobial activity suggest that the Ps-1Pb and Ps-2Pa represent templates for design of non-hemolytic analogs for tumor therapy and for treatment of infections in cancer patients produced by multidrug-resistant pathogens.

1. Introduction

Skin secretions from many, but by no means all, species of Anura (frogs and toads) contain a wide range of compounds with biological activity, often in very high concentrations, that have excited interest because of their potential for drug development [1]. These include peptides with potent antibacterial and antifungal activity that play an important role in the system of innate immunity that predates adaptive immunity and constitutes the first-line defense against invading pathogens for a wide range of vertebrate and invertebrate species [2]. Although usually referred to as antimicrobial peptides, these components are multifunctional, displaying cytokine-mediated immunomodulatory properties as well as anti-cancer, anti-viral, chemoattractive, and insulin-releasing activities [3,4]. Consequently, it is more informative, therefore, to refer to them as host-defense peptides rather than as exclusively antimicrobial peptides.

The development of resistance to commonly used anticancer agents poses serious problems in cancer chemotherapy. Naturally occurring host-defense peptides, and analogs that show selective cytotoxicity against tumor cells, have potential for development into anti-cancer agents in cases in which the tumor is not responsive to conventional pharmaceutical therapy [5,6]. As well as producing tumor cell death by disruption of the integrity of the plasma membrane, certain cationic antimicrobial peptides can instigate apoptosis via the mitochondrial pathway and act as anti-angiogenic factors [7,8]. Several frog skin peptides that were first identified as a result of their cytotoxic properties have subsequently been shown to possess complex cytokine-mediated immunoregulatory activities. Effects on the production of both pro-inflammatory and

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anti-inflammatory cytokines by peritoneal macrophages and peripheral blood mononuclear cells have been observed (reviewed in [4,9]) so that clinical applications in cancer immunotherapy are a possibility.

The emergence in all regions of the world of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics constitutes a serious threat to public health. Although effective new types of antibiotics against multidrug-resistant Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) have been introduced or are in clinical trials, the situation regarding new treatment options for infections produced by other multidrug-resistant Gram-negative pathogens is less encouraging [10–12]. Infections are an important cause of morbidity and mortality in patients with aggressive malignancies and a shift towards multidrug-resistant pathogens as causative agents has been noted [13]. Thus, there is a constant need for new types of agents with appropriate pharmacokinetic and toxicological profiles that are active against these antibiotic-resistant microorganisms.

Skin secretions of frogs belonging to the family Pipidae have proved to be a rich source of host-defense peptides, some of which show promise for development into therapeutic agents (reviewed in [9]). The Pipidae are the only principally aquatic group of frogs and, at this time, the taxon comprises 33 well characterized species distributed in five genera: *Hymenochirus*, *Pipa*, *Pseudhymenochirus*, *Silurana*, and *Xenopus* [14]. Following the discovery of magainins in the South African clawed frog, *Xenopus laevis* [15,16], numerous host-defense peptides belonging to different families have been purified from other species in the genera *Xenopus* and *Silurana* (reviewed in [9]). More recently, peptidomic analysis of norepinephrine-stimulated skin secretions from the Congo dwarf clawed frog *Hymenochirus boettgeri* led to identification of five structurally-related host-defense peptides termed the hymenochirins that show little structural similarity to peptides isolated from *Xenopus*/*Silurana* species [17]. Hymenochirin-1B shows potent broad-spectrum antimicrobial activity against a range of clinically relevant bacteria [18] and shows relatively potent in vitro cytotoxicity against a range of human tumor cells [19].

A similar peptidomic analysis of skin secretions from Merlin's clawed frog *Pseudhymenochirus merlini*, the single species within the genus *Pseudhymenochirus*, led to purification and characterization of 13 host-defense peptides with antimicrobial activity [20]. While the majority of the peptides show moderate sequence identity with peptides from *H. boettgeri*, two peptides differing by a single amino acid, termed pseudhymenochirin-1Pa and pseudhymenochirin-1Pb (Ps-1Pb), and pseudhymenochirin-2Pa (Ps-2Pa) do not resemble host-defense peptides previously isolated from frogs of the family Pipidae. Preliminary data indicated that endogenous Ps-1Pb and Ps-2Pa inhibited the growth of reference strains of the bacteria *Escherichia coli* and *S. aureus* [20] but otherwise their biological properties have not been investigated.

The aim of the present study is to assess the therapeutic potential of synthetic replicates of Ps-1Pb and Ps-2Pa. Their potential as anticancer agents is assessed by comparing effects on the viability of three well-characterized human tumor cell lines: non-small cell lung adenocarcinoma A549 cells, breast adenocarcinoma MDA-MB-231 cells, and colorectal adenocarcinoma HT-29 cells with effects on human umbilical vein HUVEC cells and erythrocytes. Immunomodulatory activities are determined by measuring effects on the production of selected anti-inflammatory and pro-inflammatory cytokines by peritoneal macrophages from C57BL/6 mice. Their therapeutic potential as anti-infective agents is assessed by measuring growth-inhibitory activity against a range of clinical isolates of MRSA and multidrug-resistant strains of *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*.

2. Materials and methods

2.1. Peptide synthesis

Ps-1Pb (IKIPSFFRNILKKVGKEAVSLIAGALKQS) and Ps-2Pa (GIFPIFAKLLGKVIKVASSLISKGRTE) 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 (Grace, Deerfield, IL, USA) equilibrated with acetonitrile/water/trifluoroacetic acid (35.0/64.9/0.1, v/v/v) at a flow rate of 6 mL/min. The concentration of acetonitrile was raised to 63% (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 was collected manually. The final purity of the peptides was >98% as determined by symmetrical peak shape and electrospray mass spectrometry. Both peptides showed high solubility in physiological buffers.

2.2. Cytotoxicity assays

Human non-small cell lung adenocarcinoma A549 cells, human breast adenocarcinoma MDA-MB-231 cells, and human colorectal adenocarcinoma HT-29 cells were maintained at 37 °C in culture medium supplemented with antibiotics as previously described [19]. EndoGRO human umbilical vein endothelial HUVEC cells were maintained in EndoGRO MV-VEGF Complete Media Kit (Millipore, Temecula, CA, USA) as previously described [21]. In all experiments, cell viability was higher than 99% using trypan blue dye exclusion.

Cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h, all cells were treated for 24 h with increasing concentrations of Ps-1Pb and Ps-2Pa (1–100 μM) in triplicate. The effect of the peptides on cell viability was determined by measurement of ATP concentrations using a CellTiter-Glo Luminescent Cell Viability assay (Promega Corporation, Madison, WI, USA). Luminescent signals were measured using a GLOMAX Luminometer system. The LC_{50} value was taken as the mean concentration of peptide producing 50% cell death in a minimum of three independent experiments.

2.3. Hemolysis assay

Ps-1Pb and Ps-2Pa in the concentration range 1.6–100 μM were incubated with washed human erythrocytes (2×10^7 cells) taken from a healthy donor as previously described [19]. Parallel incubations of erythrocytes in the presence of DPBS and 1% v/v Tween-20 were carried out to determine the absorbance associated with 0% and a 100% hemolysis respectively. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

2.4. Measurement of cytokine production

All experiments involving live animals were approved by the University of Kragujevac Animal Ethics Committee. Effects of Ps-1Pb and Ps-2Pa on cytokine production by peritoneal macrophages from C57BL/6 mice were determined using previously described methodologies [18,22]. A population of cells containing more than 95% of F4/80 positive macrophages was used in all experiments. Cell number and viability were determined using trypan blue exclusion (>95% viable). The peptides (5, 10 and 20 μg/mL) were incubated with peritoneal macrophages (2×10^5 cells/well), with and without 0.5 μg/mL lipopolysaccharide (LPS) from *E. coli* 055:B5 (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured for 24 h at 37 °C in supplemented culture medium in an humidified atmosphere of 5% CO_2–95% air as previously described [18,22]. After incubation, cell-free supernatants were collected and kept at −20 °C until time of analysis. Concentrations of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-23 (IL-23), and tumor necrosis factor-α (TNF-α) were determined in triplicate using ELISA assay kits from R & D Systems (Minneapolis, MN, USA) according to manufacturer's recommended protocols. Data are presented as mean ± SEM (n = 5).