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Purification of nasulysin-1: A new toxin from *Porthidium nasutum* snake venom that specifically induces apoptosis in leukemia cell model through caspase-3 and apoptosis-inducing factor activation

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ABSTRACT

Nasulysin-1, a new zinc-metalloproteinase from the snake venom of the hognose pit viper *Porthidium nasutum*, was purified to homogeneity using molecular exclusion chromatography and high performance liquid chromatography on a reverse phase column. The molecular mass of the purified enzyme was 25,900 kDa and pI 4.1, as determined by 1D and 2D polyacrylamide gel electrophoresis. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis of the N-terminal amino acid sequence ($_1$FSPRYIELVVVADHGMFKKYNSNLNTIR$_{28}$; $_1$TASLANLEVWSK$_{12}$; $_1$DLLPR$_6$) of the purified nasulysin-1, shows close structural homology with other snake venom metalloproteinases isolated from different snake venoms. The purified nasulysin-1 showed specific apoptosis-inducing activity in Jurkat and K562 cells, a T-cell acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (AML) cell model, respectively, without affecting the viability of human lymphocyte cells. After 48 h treatment, nasulysin-1 (20 μg/mL) induced loss of the mitochondrial membrane potential ($\Delta\Psi_m$), activated the apoptosis-inducing factor (AIF), activated the protease caspase-3, and induced chromatin condensation and DNA fragmentation, all hallmarks of apoptosis. These results strongly suggest that nasulysin-1 selectively induces apoptosis to eliminate leukemia cells. Thus, these data warrant further investigation into the use of the metalloproteinase protein, nasulysin-1 as a potential therapeutic agent for treating leukemia.

1. Introduction

Acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (CML) are hematologic disorders characterized by uncontrolled cell production of lymphoblast and myeloblast cells, respectively, in the bone marrow. At present, however, little is known about its causes. According to recent data by the American Cancer Society, about 12,910 new cases and about 2590 deaths occurred in the United States during 2015 from ALL/CML (http://www.cancer.org/cancer/leukemia). Despite progressive improvements in the efficacy of treatment (Ju et al., 2014; Jamison et al., 2016) and increased knowledge about the biologic features of leukemia cells (Durinck et al., 2015), a subset of patients relapse or remain refractory to chemotherapy and anti-kinase treatments (Locatelli et al., 2012; Frey and Luger, 2015; Lim et al., 2015; Muraoka et al., 2016). Therefore, ALL and CML are incurable diseases in an important fraction of pediatric (~10%) and adult (~30%) patients (August et al., 2013). One of the major reasons for this outcome might be cellular evasion of apoptosis (Hanahan and Weinberg, 2011) -a regulated form of cell death. Apoptosis is characterized by morphological and biochemical changes such as the reduction of cellular and nuclear volume, loss of plasma membrane, dissipation of the mitochondrial membrane potential ($\Delta\Psi$m), loss of the mitochondrial membrane potential (MMP), massive activation of caspases (e.g., caspase-3), activation of the apoptosis-inducing factor (AIF), chromatin condensation, and DNA fragmentation (Kroemer et al., 2009; Galluzzi et al., 2012). Therefore, a rational strategy would be to stimulate ALL/CML cells to trigger apoptosis.

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Porthidium nasutum (*P. nasutum*, hognose pit vipers) are found from northwestern Mexico throughout Central America to Panama and the Caribbean lowlands, and further from northern Colombia to Ecuador (http://www.iucnredlist.org/details/64344/0). Interestingly, snake venoms are complex mixtures of proteins, mainly with enzymatic activities capable to kill different tumor and leukemia cell lines by apoptosis (Vyas et al., 2013; Calderon et al., 2014). Snake venoms therefore represent a potential anticancer source (Utkin, 2015). Recent proteomic analysis of the *P. nasutum* venom from Costa Rica has shown SVMPs as its major component (52.1%, expressed as percentages content) followed by moderate amounts of phospholipases A2 (PLA2, 11.6%), C-type lectin/lectin-like (CTL, 10.4%), disintegrins (DIS, 9.9%), serine proteinases (SP, 9.6%), and low/scant amounts of L-amino acid oxidases (LAO, 3%), bradykinin-potentiating peptides (BPP, 1.9%), cystein-rich secretory proteins (CRISP, 1.3%), and nucleotidases (NUCL, 0.2%) (Lomonte et al., 2012). Recently, an acidic phospholipase A_2 has shown antibacterial activity from Colombian *P. nasutum* snake venom (Vargas et al., 2012). However, whether *P. nasutum* venom is capable to induce cell death in ALL/CML cells is not yet established.

Snake venom metalloproteinases (SVMPs) are monozinc endopeptidases classified according to their functional domain organization as P-I class metalloproteinases (20–30 kDa), composed of one metalloproteinase domain; P-II class metalloproteinases (30–60 kDa), composed of a metalloproteinase and a disintegrin domain; and P-III class metalloproteinases (60–100 kDa), composed of a metalloproteinase, a disintegrin, two lectin-like and cysteine-rich domains (Escalante et al., 2011). Although the functional activities of SVMPs are mainly associated with hemorrhage or the disruption of the homeostatic system (i.e., proteolysis of fibrinogen, fibrin, and capillary basement), SVMPs are also able to provoke apoptosis in cell lines, such as human umbilical-vein endothelial cells, endothelial cells, and rat smooth muscle cells (Takeda et al., 2012). However, no data are available to establish whether SVMPs can specifically induce apoptosis in leukemia cells.

To gain insight into the biological activities of Colombian *P. nasutum* venom, the first aim of this study was to determine the *in vitro* effect of various concentrations of crude *P. nasutum* snake venom and isolated venom fractions on Jurkat (clone E6-1) and K562 cell lines, as models of human T cell ALL and human CML, respectively. Analysis of cell nuclear morphology by fluorescence microscopy and evaluation of $\Delta\Psi m$ by flow cytometry revealed that crude *P. nasutum* snake venom induces both apoptosis and necrosis –characterized by cytoplasmic swelling, rupture of the plasma membrane, and mild clumping of nuclear chromatin, while the fractions F I-VI and sub-fractions SF IV.1–9 induced only apoptosis in Jurkat and K562 cells. Thus, the second aim of this study was to further isolate a small SVMP (MW ~26 kDa), named hereafter nasulysin-1, from *P. nasutum* venom, and evaluate its capacity to provoke apoptotic cell death in Jurkat/K562 cells and normal lymphocytes, as control non-leukemic cells. To further characterize the molecular mechanism of apoptotic cell death in the cells treated with nasulysin-1, AIF and Casp3 activation were quantified by immunocytochemistry. Our findings suggest that nasulysin-1 specifically induces apoptosis in leukemia cell lines. Accordingly, the current effort is directed towards biologically driven therapies (Saedi et al., 2014) and nasulysin-1 might be a useful metalloproteinase protein to investigate as a potential therapy to treat leukemia.

2. Materials and methods

2.1. Reagents

3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, cat # D-273) was obtained from Invitrogen Molecular Probes (Eugene, OR, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Venom

Venom was obtained by manual extraction of 45 *P. nasutum* specimens from the state of Antioquia located in northwest Colombia. The vipers are maintained in captivity at the Universidad de Antioquia's Serpentarium (Medellin, Colombia). Venoms were centrifuged at 800 *g* for 15 min, and supernatants lyophilized and stored at −20 °C.

2.3. Purification of the venom of P. nasutum by molecular exclusion chromatography and HPLC

The crude venom from *P. nasutum* (150 mg) was fractionated by molecular exclusion chromatography using a HiPrep 26/60 Sephacryl S-200 HR column (16 mm, Pharmacia Biotech, cat# 17-9511-01). The sample was separated using 0.5 mM sodium phosphate buffer (pH 7.2) at a flow rate of 1 mL/min. The fractions obtained were dialyzed, lyophilized and stored at −20 °C. The fraction IV (2 mg) dissolved in 200 μL 0.1% trifluoroacetic acid (TFA) was further fractionated by reverse-phase high performance liquid chromatography (RP-HPLC) using a Pinacle DB C18 column (250 mm × 4.6 mm, 5 μm particle size, 140 Å pore size, RESTEK Corporation, cat·# 9414575) eluted with a 0–66% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) and developed on a Shimadzu Prominence system at a flow rate of 0.7 mL/min. Absorbance was monitored at 215 nm. The fractions were manually collected and dried under vacuum in an Eppendorf vacufuge concentrator until total evaporation of the eluents. Samples were then stored at 4 °C to evaluate their pro-apoptotic activity.

2.4. One-dimensional (1D) and two-dimensional (2D) electrophoresis

The crude venom, the fractions (F I-VI) and sub-fraction IV.5 (SF IV.5) obtained from the venom of *P. nasutum* were analyzed by denaturing (1D) SDS-PAGE electrophoresis (5 min at 90 °C) in reducing conditions (2 mL of 1 mM DTT). Samples (20 μg) were separated on a 12% polyacrylamide gel at 120 V for 1 h and 30 min. For 2D electrophoresis, a total of 250 μg of protein was loaded onto 7-cm DryStrip strips with a non-linear pH of 4–7 (GE Healthcare Life Sciences, cat # 17-6001-14) and allowed to hydrate passively for 12 h. The first dimension (isoelectric focusing) was conducted at 20 °C with a 50 mA current per strip, using the Ettan IPGphor 3 system (GE Healthcare Life Sciences, cat # 11003364) until 12,000 V/h was reached. The strips were equilibrated by incubating them in buffer I (7 mM Urea, 4 mM de thiourea, 4% CHAPS, 1 mM DTT and 1% ampholytes) for 20 min, followed by incubation in buffer II (6 M urea, 2% SDS, 375 mM Tris–HCl pH 8.8, 20% glycerol, 25 mg/mL of iodoacetamide) for 20 min. After equilibration, the strips were placed on 12.5% acrylamide/bis-acrylamide gels, and the second dimension was conducted using the MiniPROTEAN system (Biorad, cat # 1658025, Hercules, CA) at 100 V for 120 min. The gels obtained from 1D and 2D were fixed for 1 h in a solution of 50% methanol and 10% acetic acid, and subsequently stained with CBB R250 and/or silver nitrate.

2.5. Protein preparation and analysis by LC-MS/MS

The protein spot of interest from sub-fraction IV.5 (SF IV.5), visualized by CBB R250 staining, was excised from blue native gel and in-gel digested with trypsin was performed according to laboratory standardized procedures. The peptides were taken up in