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Characterization of dual effects induced by antimicrobial peptides: Regulated cell death or membrane disruption

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ABSTRACT

Background: Some reports describe lysis mechanisms by antimicrobial peptides (AMPs), while others describe the activation of regulated cell death. In this study, we compare the cell death-inducing activities of four β-hairpin AMPs (gomesin, protegrin, tachyplesin and polyphemusin II) along with their linear analogs in the human erythroleukemia K562 cell line to investigate the relationship between their structure and activity.
Methods: K562 cells were exposed to AMPs. Morphological and biochemistry alterations were evaluated using light microscopy, confocal microscopy and flow cytometry.
Results: Gomesin and protegrin displayed cytotoxic properties that their linear counterparts did not. Tachyplesin and polyphemusin II and also their linear analogs induced cell death. We were able to distinguish two ways in which these AMPs induced cell death. Lower concentrations of AMPs induced controlled cell death mechanisms. Gomesin, tachyplesin and linear-tachyplesin promoted apoptosis that was characterized by annexin labeling, sensitivity to Z-VAD, and caspase-3 activation, but was also inhibited by necrostatin-1. Gomesin and protegrin induced cell death was dependent on intracellular Ca^{2+} mechanisms and the participation of free radicals was observed in protegrin induced cell death. Polyphemusin II and its linear analog mainly induced necrosis. Conversely, treatment with higher concentrations of AMPs primarily resulted in cell membrane disruption, but with clearly different patterns of action for each AMP tested.
Conclusion: Different actions by β-hairpin AMPs were observed at low concentrations and at higher concentrations despite the structure similarity.
General significance: Controlled intracellular mechanism and direct membrane disruption were clearly distinguished helping to understand the real action of AMPs in mammalian cells.

1. Introduction

The limited number of chemotherapeutic drugs available on the market for use against cancer cells led to a frantic search for new compounds. In recent years, several antimicrobial peptides (AMPs) have been described as molecules with potential anti-cancer activity [1–6]. AMPs are present in nearly all organisms displaying diverse tridimensional structures. In general, AMPs are cationic, amphipathic and with molecular weights of less than 10 kDa [7].

Studies performed in bacteria indicate that the main action of AMPs involves the formation of membrane pores [8–11]. Conversely, studies in mammalian cells have shown that AMPs may promote cell death by apoptosis, autophagy or necrosis. For instance, magainin, a cationic and amphipathic α-helix peptide, induces cell death by apoptosis in HL-60 cells [12]. Tachyplesin, a peptide with a disulfide bridge that forms a stabilized amphipathic β-hairpin structure [4], induces apoptosis in HL-60 cells [13] but promotes membrane permeabilization in a prostate carcinoma cell line [4]. Gomesin, another β-hairpin AMP, presents high cytotoxic activity against cancer and normal cells by regulating membrane permeabilization [1], although it also triggers a complex intracellular Ca^{2+} signaling pathway prior to membrane permeabilization [14]. In a hepatocellular carcinoma, peptaibols, a family of antibiotic peptides from fungi, have been demonstrated to suppress tumor growth by inducing Ca^{2+} influx. This effect on Ca^{2+} influx in turn leads to the activation of μ-calpain and promotion of Bax translocation to the mitochondria, triggering apoptosis and autophagy, suggesting less evidence for a mode of action via membrane permeabilization [2]. There are few reports comparing how the structures and activities of AMPs induce cell death in mammalian cells.

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Herein, we compare the ability of four β-hairpin AMPs (tachyplesin, gomesin, polyphemusin II and protegrin) to induce cell death, distinguishing whether cellular actions are different among them. We also tested their corresponding linear (Lin) analogs and also magainin II, a linear AMP. β-Hairpin AMPs such as tachyplesin and polyphemusin II display antitumoral ability against leukemia cells [13,15]. Therefore, in this study the human erythroleukemia K562 cell line was used as cellular model to investigate the effects of these AMPs. Herein, we were able to distinguish diverse effects among β-hairpin AMPs. We observed that at concentrations below the EC_{50} AMPs promoted cell death by different intracellular mechanisms depending on the AMP used, and at concentrations above the EC_{50}, AMPs induced diverse types of membrane disruptions.

2. Experimental procedures

2.1. Peptide synthesis

Peptides were synthesized manually by the solid-phase method on a 4-methylbenzhydrylamine-resin (MBHAR) (0.8 mM/g) using the *t*-Boc strategy [16]. Full deprotection and cleavage of the peptide from the resin were carried out using anhydrous hydrogen fluoride (HF) treatment with anisole and dimethyl sulfide (DMS) as scavengers at 0 °C for 1.5 h. Formation of disulfide bridges was achieved immediately after the HF cleavage and extraction of the crude peptide. The resulting peptide solution was kept at pH 6.8–7.0 and 5 °C for 72 h. Cyclization reactions were monitored by reverse-phase liquid chromatography coupled to an electrospray ionization mass spectrometer (LC/ESI-MS). Lyophilized crude peptides were purified by preparative RP-HPLC on a Vydac C_{18} column (25 × 250 mm, 300 Å pore size, and 15 μm particle size) in two steps. The first was performed by using triethylammonium phosphate (TEAP) pH 2.25 as solvent A and 60% acetonitrile (ACN) in A as solvent B. The second step was carried out using 0.1% trifluoroacetic acid (TFA) H_2O as solvent A and 60% ACN in A as solvent B. Pure peptides were characterized by amino acid analysis and by LC/ESI-MS. The sequences of the AMPs are shown in Table 1.

2.2. Cell line and culture conditions

A K562 erythroleukemia cell line obtained from the American Type Culture Collection (ATCC, USA), was cultured in suspension in RPMI 1640 medium (Cultilab, Brazil) supplemented with 10% fetal calf serum (FBS, Cultilab, Brazil), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere at 37 °C in 5% CO_2.

2.3. Cell viability assay

Cells were seeded in 96-well plates (10^5 cells/ml) and cultured in medium containing 10% FBS in the presence or absence of AMPs for 24 h. After this period, K562 cells were washed with PBS and resuspended in binding buffer (0.01 M Hepes, pH 7.4, 0.14 M NaCl and 2.5 mM $CaCl_2$). The suspensions were labeled with annexin-FITC and propidium iodide (PI) (Becton Dickinson, USA) according to the manufacturer's instructions. The cells were incubated at room temperature for 20 min. 10,000 events were collected per sample. The analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson, USA) using the CellQuest software (Becton Dickinson, USA).

2.4. Caspase-3 activity

K562 cells were treated with the AMPs as indicated, and activation of caspase-3 was evaluated by flow cytometry according to the manufacturer's instructions (Cell Signaling, USA). After treatment, K562 cells were centrifuged, washed and fixed in 2% paraformaldehyde in PBS for 30 min. Cells were then permeabilized in PBS containing 0.01% saponin for 15 min and blocked in PBS containing 1% BSA for 30 min at room temperature. Afterwards, 10 μl of cleaved caspase-3 (Asp175) Alexa Fluor 488-conjugated antibody was added, and cells were incubated in the dark at room temperature for 1 h. After washing, cells were resuspended in PBS and analyzed (10,000 events collected per sample) in a FACSCalibur flow cytometer (Becton Dickinson, USA) using the CellQuest software.

Table 1
Sequence and cytotoxicity activities of the peptides.

Peptides	Sequences	MW	EC_{50}[a] (μM)
Magainin	**G-I-G-K-F-L-H-S-A-K-K-F-G-K-A-F-V-G-E-I-M-N-S-NH$_2$**	2465.95	>40
Gomesin	**Z-C-R-R-L-C-Y-K-Q-R-C-V-T-Y-C-R-G-R-NH$_2$**[b]	2270.72	4.5
[Trp^1,$Ser^{2,6,11,15}$]-Gomesin or Lin-gomesin	**W-S-R-R-L-S-Y-K-Q-R-S-V-T-Y-S-R-G-R-NH$_2$**	2285.62	>40
Tachyplesin	**K-W-C-F-R-V-C-Y-R-G-I-C-Y-R-R-C-R-NH$_2$**	2263.78	20
[Trp^0,$Ser^{3,7,12,16}$]-Tachyplesin or Lin-tachyplesin	**W-K-W-S-F-R-V-S-Y-R-G-I-S-Y-R-R-S-R-NH$_2$**	2389.76	21
Polyphemusin II	**R-R-W-C-F-R-V-C-Y-K-G-F-C-Y-R-K-C-R-NH$_2$**	2425.97	22
[Trp^0,$Ser^{4,8,13,17}$]-Polyphemusin II or polyphemusin II	**W-R-R-W-S-F-R-V-S-Y-K-G-F-S-Y-R-K-S-R-NH$_2$**	2551.95	22
Protegrin	**R-G-G-R-L-C-Y-C-R-R-R-F-C-V-C-V-G-R-NH$_2$**	2155.64	12
[Trp^0,$Ser^{6,8,13,15}$]-Protegrin or Lin-protegrin	**W-R-G-G-R-L-S-Y-S-R-R-R-F-S-V-S-V-G-R-NH$_2$**	2281.64	>40

[a] Cytotoxicity activities of the AMPs were evaluated against K562 erythroleukemia cell line.
[b] Z = pyroglutamic acid residue.