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Viola plant cyclotide vigno 5 induces mitochondria-mediated apoptosis via cytochrome C release and caspases activation in cervical cancer cells

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ABSTRACT

Cyclotides describe a unique cyclic peptide family that displays a broad range of biological activities including uterotonic, anti-bacteria, anti-cancer and anti-HIV. The vigno cyclotides consist of vigno 1–10 were reported recently from *Viola ignobilis*. In the present study, we examined the effects of vigno 5, a natural cyclopeptide from *V. ignobilis*, on cervical cancer cells and the underlying mechanisms. We found that vigno 5-treated Hela cells were killed off by apoptosis in a dose-dependent manner within 24 h, and were characterized by the appearance of nuclear shrinkage, cleavage of poly (ADP-ribose) polymerase (PARP) and DNA fragmentation. The mitochondrial pathway of apoptosis revealed that cytochrome C is released from mitochondria to cytosol, associated with the activation of caspase-9 and -3, and the cleavage of poly (ADP-ribose) polymerase (PARP). Overall, the results indicate that vigno 5 induces apoptosis in part via the mitochondrial pathway, which is associated with a release of cytochrome C and elevated activity of caspase-9 and -3 in Hela cells.

1. Introduction

Cyclotides are a group of natural plant cyclopeptides that have sparked great research interest due to their unusual structure and varied biological activities [1]. Their unique structure (Fig. 1) created by a head-to-tail cyclic backbone comprising 27–32 residues in size, six conserved cysteine residue and three disulfide bonds [2], give them extraordinary stability against thermal and enzymatic degradation [3], also give them a broad variety of biologically activities including uterotonic [4,5], anti-bacterial [6], anti-cancer [7–9], anti-HIV [10–12] etc.

Continued discovery of cyclotides from uncharacterized plants increases the chance of obtaining new sequence with useful activities [13]. In a recent study, we reported the discovery of vigno 1–10 from *Viola ignobilis* [14,15]. In this present study, the biological activity of vigno 5 (Fig. 1) has been investigated in the context on human cervical cancer. Apoptosis, or programmed cell death has been described as a mechanism by which cells undergo a natural dying process to control cell proliferation or in response to DNA damage [16,17]. This apoptotic and cell death program is used to develop novel therapies against cancer cells using agents with cytotoxic properties in chemotherapy and/or radiation therapy [18–20]. Two main core pathways that established in apoptosis are: (a) the cell death receptor mediated extrinsic pathway; and (b) the mitochondrial mediated intrinsic pathway. This extrinsic pathway is triggered by extracellular membrane anchored death receptors binding such as Fas (and other similar receptors such as tumor necrosis receptor 1 and its relatives) with its extracellular ligand, Fas-L and sequential caspase activations. The intrinsic pathway leads to apoptosis under the control of mitochondria and its associated molecules. In both cases, these pathways merge and result in cell death. Extracellular or intracellular signals alter mitochondrial membrane permeability and cytochrome C releases into the cytosol which is an apoptotic signal. Cytochrome C recruits Apaf-1, pro-caspase-9 and forms the apoptosome, which is a downstream trigger of the caspase 9/3 signaling cascade, that is established as the main process of cell death by apoptosis [21,22]. Natural compounds have been a fertile source of potential chemotherapeutic and chemoprevention agents, and they have received great attention because they are considered to be safer due to lower risk of mutagenicity in non-cancerous cells [23–25]. Molecules such as taxol, vincristine, vinblastine, camptothecin derivatives, and epipodophyllotoxin are invaluable contributions from natural sources in modern medicine. However, the quest to uncover novel therapeutic compounds for cancer treatment and management is a never-ending venture. Recently, the anti-tumor activity of cyclic peptides has attracted much attention [7].

In the present study, for the first time, it was found that cyclotide vigno 5 significantly reduces Hela cell survival and induces cell death. It was also demonstrated that cyclotide vigno 5 induces apoptosis in

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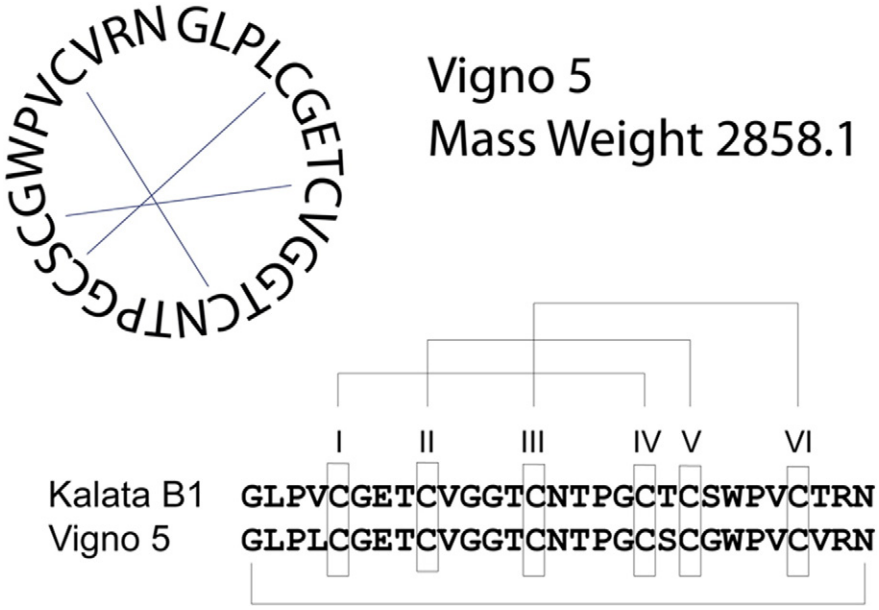

Fig. 1. Primary structure of vigno 5 and Kalata B1 (a representative member of cyclotide family).

Hela cells mainly via the mitochondrial pathway including the release of cytochrome C to the cytosol and the activation of caspases.

2. Materials and methods

2.1. Cell lines, chemicals and biochemicals

MTT (3-[4,5-dimehyl-2-thiazolyl]-2,5-diphenyl-2 H-tetrazolium bromide), dimethylsulfoximine (DMSO), paclitaxel, and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). Anti-Bcl-, anti-Bax, anti-pro-caspase 3, anti-pro-caspase 9, anti-cytochrome C, and anti-poly ADP ribose polymerase (PARP), antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). DMEM cell cultures and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). All other reagents were commercial products of the highest available purity grade.

2.2. Plant material collection

Plant material of *V. ignobilis* was collected in the mountain area at an altitude of 1500–1700 m around the region of Gara Dagh near to Negarestan village in East Azerbaijan of Iran in spring 2010. A voucher specimen was identified and deposited in Medicinal Plants Herbarium at Medicinal Plants and Drug Research Institute, Iran (MPH-1917).

2.3. Extraction and clean-up process

The dried plant material (~500 g) was ground prior to solvent extraction with a mixture of MeOH/CH_2Cl_2 (1:1; *v*/*v*) overnight under continuous agitation at 20 °C. After adding of 0.5 volume water, the aqueous phase was concentrated on a rotary evaporator prior to freeze drying. The crude extract was dissolved in 0.1 M NH_4HCO_3 buffer (pH ~ 8.1) and immediately used for solid-phase extraction (SPE). The C18 solid phase extraction (SPE) cartridges (Macherey-Nagel, Chromabond; 10 g; 50 mL) were activated with 1 bed volume of MeOH and subsequently equilibrated with 1 bed volume of aqueous 1% FA. After application of the extract, the cartridges were washed with 1 bed volume of 1% FA. Putative cyclotide containing fractions of 50% and 80% EtOH were collected and freeze dried. The freeze dried samples were stored at −18 °C prior to ongoing analysis.

2.4. Semi-preparative and analytical HPLC analysis

After dissolving in 1% FA, the cyclotide mixtures were fractionated by semi-preparative RP-C18 HPLC (Knauer, Eurospher I 5 μm; 250 × 16.1 mm; 100 Å) using a Knauer 1200 series unit, with an isocratic flow of 30% ACN/(H2O, 0.1% TFA) (*v*/*v*) at a flow rate of 8 mL/min. Fractions were collected manually by UV detection at 210 nm. The collected samples were concentrated on a rotary evaporator prior to freeze dried. After dissolving in 5% ACN, the purity of collected samples were analyzed by analytical RP-C8 HPLC (Knauer, Eurospher I 5 μm; 250 × 4.6 mm; 100 Å) using a Knauer 1200 series unit, with a 1 mL/min gradient flow of solvent B (acetonitrile, 0.08% TFA), 0–5 min 5%, 5–10 min 20%, 10–40 min 60%, 40–45 min 100%, 45–50 min 100%, 50–50.1 min 5% and 50.1–55 min 5%. And, the solvent A was 0.05% aqueous TFA.

2.5. MALDI-TOF analysis

Analysis of purified cyclotide samples were performed on a MALDI-TOF 4800 Analyzer (SCIEX, Toronto, Canada) operated in reflector positive ion mode acquiring 2000–3600 total shots per spectrum with laser intensity set between 3200 and 3800. MS experiments were carried out using α-cyano-hydroxyl-cinnamic acid matrix at a concentration of 5 mg/mL in 50% (*v*/*v*) acetonitrile. 0.5 μL of each sample was mixed with 3 μL of matrix solution and the mixture was spotted onto the target plate. Tandem mass spectra were acquired using laser energy of 1 kV with and without the use of collision-induced dissociation and processed using the Data Explorer Software.

2.6. Cell culture and cyclotide treatment

The human cervical cancer cells (Hela) were purchased from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (Tehran, Iran), and maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin. These cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2. The vigno 5 was dissolved in DMSO to make a stock of 20 mM and further diluted to final concentrations of 10–100 μM with a serum free culture medium. The amount of DMSO added to the cell culture was less than 0.1% in all cases. The taxol was used as a positive control in a range of 1–10 nM.