European Journal of Pharmacology 803 (2017) 138–147

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Selective cytotoxicity of the antibacterial peptide ABP-*dHC*-Cecropin A and its analog towards leukemia cells

Ming Sang[1], Jiaxin Zhang[*,1], Qiang Zhuge[*]

Co-Innovation Center for Sustainable Forestry in Southern China, Key Laboratory of Forest Genetics & Biotechnology, Ministry of Education, Nanjing Forestry University, Nanjing 210037, China

ARTICLE INFO

Keywords:
ABP-*dHC*-Cecropin A
Antibacterial peptide
Cytotoxicity
Selectivity
Anticancer peptide

ABSTRACT

Some cationic antibacterial peptides, with typical amphiphilic α-helical conformations in a membrane-mimicking environment, exhibit anticancer properties as a result of a similar mechanism of action towards both bacteria and cancer cells. We previously reported the cDNA sequence of the antimicrobial peptide ABP-*dHC*-Cecropin A precursor cloned from drury (*Hyphantria cunea*) (*dHC*). In the present study, we synthesized and structurally characterized ABP-*dHC*-Cecropin A and its analog, ABP-*dHC*-Cecropin A-K(24). Circular dichroism spectroscopy showed that ABP-*dHC*-Cecropin A and its analog adopt a well-defined α-helical structure in a 50% trifluorethanol solution. The cytotoxicity and cell selectivity of these peptides were further examined in three leukemia cell lines and two non-cancerous cell lines. The MTT assay indicated both of these peptides have a concentration-dependent cytotoxic effect in leukemia cells, although the observed cytotoxicity was greater with ABP-*dHC*-Cecropin A-K(24) treatment, whereas they were not cytotoxic towards the non-cancerous cell lines. Moreover, ABP-*dHC*-Cecropin A and its analog had a lower hemolytic effect in human red blood cells. Together, these results suggest the peptides are selectively cytotoxic towards leukemia cells. Confocal laser scanning microscopy determined that the peptides were concentrated at the surface of the leukemia cells, and changes in the cell membrane were determined with a permeability assay, which suggested that the anticancer activity of ABP-*dHC*-Cecropin A and its analog is a result of its presence at the leukemia cell membrane. ABP-*dHC*-Cecropin A and its analog may represent a novel anticancer agent for leukemia therapy, considering its cancer cell selectivity and relatively low cytotoxicity in normal cells.

1. Introduction

Despite recent advances in treatment, cancer remains a leading cause of death around the world (Torre et al., 2015). Traditional treatment strategies are generally not cancer-cell specific (Smith and White, 2000). For instance, the use of conventional chemotherapeutic agents that typically target rapidly dividing cancer cells is often associated with deleterious side effects, caused by inadvertent drug-induced damage to healthy cells and tissues (Cassidy and Misset, 2002; Hoskin and Ramamoorthy, 2008). Moreover, conventional chemotherapies need to penetrate target cells, but cancer cells can develop resistance by multiple mechanisms, including increased expression of multi-drug resistant proteins, altered interactions between the drug and its target, an increased ability to repair DNA damage, and defects in the cellular machinery that mediate apoptosis (Gatti and Zunino, 2005). These limitations have stimulated the search for novel anticancer agents with unique mechanisms of action.

Natural cationic antibacterial peptides (ABPs) are normally characterized by their positive charges and amphipathic features (Boman, 2003; Hancock and Rozek, 2002), which enable them to bind and disrupt the negatively charged bacterial cell membranes (Chu et al., 2015; Hancock and Sahl, 2006). Some of the ABPs are toxic to bacteria but not to normal mammalian cells, and exhibit a broad range of cytotoxicity towards cancer cells (Hoskin and Ramamoorthy, 2008). The electrostatic attraction between the negatively charged components of bacteria and cancer cells and the positively charged ABPs is believed to play a major role in the binding and disruption of bacterial and cancer cell membranes (Hoskin and Ramamoorthy, 2008). While not all ABPs are lethal to cancer cells, ABPs that are highly potent against bacteria and cancer cells, but not non-cancerous mammalian cells, may provide a new class of anticancer drug (Hoskin and Ramamoorthy, 2008).

Cecropins are antimicrobial peptide found in both insects and mammals (Lee et al., 1990; Steiner et al., 1981), and derived from

* Corresponding authors.
E-mail addresses: zhangjiaxin60633@163.com (J. Zhang), qzhuge@njfu.edu.cn (Q. Zhuge).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejphar.2017.03.054
Received 10 November 2016; Received in revised form 19 March 2017; Accepted 24 March 2017
Available online 27 March 2017

insect sources consist of 34–39 amino acid residues (Boman and Dan, 1987; Chen et al., 1997). Cecropin A assumes a secondary structure that is characterized by the presence of two α helices (Holak et al., 1988; Hung et al., 1999). The NH_2-terminal α-helix is highly amphipathic while the COOH-terminal α-helix is hydrophobic (Ye et al., 2004). This structure allows targeting of non-polar lipid membranes (Zhang et al., 2015a). Once a cell membrane has been targeted, cecropins form ion-permeable channels that cause depolarization, irreversible cytolysis, and cell death (Boman, 2003).

The cDNA sequence of the antimicrobial peptide ABP-*dHC*-Cecropin A (KJ660064) was cloned from the fat body RNA of the drury (*Hyphantria cunea*) (*dHC*) (Zhang et al., 2015a). To further evaluate the therapeutic potential of ABP-*dHC*-Cecropin A, the proliferative capacity and cell selectivity of ABP-*dHC*-Cecropin A were examined in three human leukemia cell lines, human embryonic kidney cells and mononuclear cells. At the same time, ABP-*dHC*-Cecropin A-K(24), an analog of ABP-*dHC*-Cecropin A with decreased hydrophilicity, was designed by replacing the proline (Pro) residue at position 24 with a lysine (Lys).

2. Materials and methods

2.1. Peptide synthesis

The synthetic peptides ABP-*dHC*-Cecropin A (sequence: RWKIFKKIERVGQNVRDGIIKAGPAIQVLGTAKALGK) and ABP-*dHC*-Cecropin A-K(24) (sequence: RWKIFKKIERVGQNVRDGIIKAGKAIQVLGTAKALGK) were synthesized using solid-phase methodology at GL Biochemistry Corporation (Shanghai, China). Purification by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) yielded final products determined to be >95% pure by analytical RP-HPLC. Synthesis of the peptides was confirmed by analysis with an electrospray ionization (ESI)-mass spectrometer and matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrometry (MS) (Fig. S1). Selective N-terminal fluorescein labeling of the peptides was done with fluorescein isothiocyanate (FITC). The FITC-labeled peptides, which were purified by C18 reverse phase HPLC, were determined to be 95% homogeneous by MALDI MS. The peptides were dissolved in 0.01% acetic acid and stored at −20 °C until further use.

2.2. Circular dichroism (CD) spectroscopy

The CD spectrum of the peptides was obtained using a Chirascan CD spectrophotometer (Applied Photophysics, UK) at 25 °C using a fused quartz cell with a 1-mm path length over a wavelength range of 180–260 nm at 0.1-nm intervals (speed 50 nm/min; response time 0.5 s; bandwidth 1 nm). CD spectra were collected and averaged over three scans. Samples were prepared by dissolving the peptides in sodium phosphate buffer (0.3 mg/ml), in trifluorethanol (TFE, 50% v/v) or 30 mM sodium dodecyl sulfate (SDS) micelles to a final concentration of 0.2 mg/ml. All experiments were performed in triplicate. The secondary structure content was estimated from the CD spectra using the spectropolarimeter software supplied by the manufacturer and a set of standard protein spectra at room temperature. The distribution of residues around the helical axis was tested by Helical Wheel Projections (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Hydrophobicity was analyzed using ProtScale software (http://web.expasy.org/protscale/).

2.3. Cell culture

Human myelogenous leukemia (K562), human monoblastic leukemia (U937), and human acute monocytic leukemia (THP-1) cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. The human embryonic kidney cell line 293 (HEK-293) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100g/ml streptomycin. Heparinized human peripheral blood was obtained from healthy donors, and mononuclear cells (PBMCs) were isolated with the Ficoll gradient density method. Cells were pelleted by low-speed centrifugation at 1000×*g* for 5 min and suspended in phosphate-buffered saline (PBS). The PBMCs were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100g/ml streptomycin. All of the cell lines were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. All the cell line (K562, U937, THP-1 and HEK-293) stocks from our laboratory were tested on several molecular levels and compared with other cell lines throughout the experiments. All cell lines were treated with MRA (Mycoplasma Removal Agent, MP Biomedicals) to prevent infection with Mycoplasma.

2.4. Cell proliferation and viability determined by MTT assay

Anticancer activity of the ABP-*dHC*-Cecropin A and ABP-*dHC*-Cecropin A-K(24) peptides was determined by the 3-(4,5-dimethyl-2-thiazolyl)−2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983). K562, U937, THP-1, and HEK-293 cells, as well as PBMCs, were seeded at 5×10^3 cells/well in a 96-well plate 24 h before peptide treatment in medium without fetal bovine serum. Cells were treated with 0, 20, 40, 80, 160, 320, or 640 μM of the peptides. After a 24 h incubation with the different concentrations of peptides, 20 μl of 5 mg/ml MTT solution was added to each well and incubated for 4–8 h. Following removal of the supernatant, 200 μl dimethyl sulfoxide (DMSO) was added to dissolve the formazan product that remained in the wells. After 30 min the absorbance was measured with a BioTek Synergy 2 multi-mode microplate reader at a test wavelength of 490 nm. The final results were recorded by averaging at least three experiments and the viability of the control cells was set as 100% cell survival. The IC_{50} value for each cell line was defined as the dose of peptide causing a 50% decrease in absorbance compared to the control. Cell viability of the PBMCs was determined by the trypan blue dye exclusion assay after incubation for 36 h. PBMC cells were treated with 160 μM ABP-*dHC*-Cecropin A or ABP-*dHC*-Cecropin A-K(24), or without peptides. Treatment with 2 μM mellitin (purity >95%; Sigma) was used as the control.

2.5. Hemolytic activity

The hemolytic activity of ABP-*dHC*-Cecropin A and ABP-*dHC*-Cecropin A-K(24) was determined using fresh human red blood cells (hRBCs). The hRBCs were centrifuged, washed three times with PBS, dispensed into 96-well plates as 100 μl samples of 4% (v/v) hRBCs in PBS, and 100 μl of peptide solution was added to each well. Plates were incubated for 1 h at 37 °C, and centrifuged at 1000×*g* for 5 min. The supernatant (100 μl) was transferred to 96-well plates and hemoglobin release was monitored by measuring absorbance at 414 nm. Zero hemolysis was determined in PBS (A_{PBS}) and 100% hemolysis was determined in 0.1% (v/v) Triton X-100 (A_{Triton}). Percent hemolysis was calculated as: $\%\ \text{hemolysis}=100\times[(A_{sample}-A_{PBS})/(A_{Triton}-A_{PBS})]$. All hemolysis experiments were performed in triplicate.

2.6. Flow cytometry

HEK-293, K562, U937, and THP-1 cells, as well as hRBCs and PBMCs, were used to measure cellular uptake by flow cytometry. Cells at concentrations greater than 1×10^6 cells/ml were incubated with 0, 80, or 160 μM FITC-labeled peptide in medium containing 10% (v/v) bovine serum at 37 °C for 30 min, washed with PBS twice, and fixed with 4% (v/v) formaldehyde on ice for 10 min. After a second wash with ice-cold PBS, the cells were analyzed with a FACS Vantage SE flow