Regulatory Peptides 187 (2013) 51–56

Contents lists available at ScienceDirect

Regulatory Peptides

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

Anti-tumor activities of the host-defense peptide hymenochirin-1B

Samir Attoub [a], Hama Arafat [a], Milena Mechkarska [b], J. Michael Conlon [b,*]

[a] Department of Pharmacology, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates
[b] Department of Biochemistry, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates

ARTICLE INFO

Article history:
Received 8 July 2013
Received in revised form 23 October 2013
Accepted 28 October 2013
Available online 31 October 2013

Keywords:
Host-defense peptide
Hymenochirin
Frog skin
Structure–activity
Anti-cancer

ABSTRACT

The hymenochirins are a family of cationic, amphipathic, α-helical host-defense peptides, first isolated from skin secretions of the Congo clawed frog *Hymenochirus boettgeri* (Pipidae). Of the four hymenochirins tested, hymenochirin-1B (IKLSPETKDNLKKVLKGAIKGAIVAKMV.NH_2) shows the greatest cytotoxic potency against non-small cell lung adenocarcinoma A549 cells (LC_{50} = 2.5 ± 0.2 μM), breast adenocarcinoma MDA-MB-231 cells (LC_{50} = 9.0 ± 0.3 μM), colorectal adenocarcinoma HT-29 cells (LC_{50} = 9.7 ± 0.2 μM), and hepatocarcinoma HepG2 cells (LC_{50} = 22.5 ± 1.4 μM) with appreciably less hemolytic activity against human erythrocytes (LC_{50} = 213 ± 18 μM). Structure–activity relationships were investigated by synthesizing analogs of hymenochirin-1B in which Pro^5, Glu^6 and Asp^9on the hydrophilic face of the helix were replaced by one or more L-lysine or D-lysine residues. The [D9K] analog displays the greatest increase in potency against all four cell lines (up to 6 fold) but hemolytic activity also increases (LC_{50} = 174 ± 12 μM). The [D9k] and [E6k,D9k] analogs retain relatively high cytotoxic potency against the tumor cells (LC_{50} in the range 2.1–21 μM) but show reduced hemolytic activity (LC_{50} > 300 μM). The data suggest that hymenochirin-1B has therapeutic potential as a template to generate potent, non-toxic anti-cancer agents.

1. Introduction

Peptides with potent antibacterial and antifungal activity and the ability to permeabilize mammalian cells play an important role in the system of innate immunity that constitutes the first-line defense against invading pathogens for both vertebrate and invertebrate species. Such peptides are multifunctional possessing immunomodulatory and chemoattractant properties as well as cytotoxic activities so that it is more informative to refer to them as host-defense peptides rather than antimicrobial peptides [1,2]. Skin secretions from many species of Anura (frogs and toads) contain host-defense peptides that, with few exceptions, are cationic (molecular charge between +2 and +6 at pH 7), contain at least 50% hydrophobic amino acids, and have the propensity to adopt an amphipathic α-helical conformation in a membrane-mimetic environment (reviewed in [3]). Although there is no single mechanism by which the peptides produce cell death, their action generally involves a non-specific perturbation of the cell membrane and insertion into the lipid bilayer leading to membrane disruption and cell lysis rather than binding to specific receptors on the cell membrane or intracellular targets [4,5].

Host-defense peptides have excited interest because of the possibilities for development into anti-infective drugs for use against pathogenic microorganisms that have developed resistance against commonly used antibiotics (reviewed in [6]). The problem of multidrug resistance in the treatment of bacterial infections is also encountered in cancer chemotherapy [7]. Because of their non-specific and destructive mechanism of action, naturally occurring host-defense peptides show therapeutic potential for development into anti-cancer agents in cases where the tumor is not responsive to conventional pharmaceutical intervention. Several cationic host-defense peptides, first identified on the basis of their antimicrobial activity, have shown selective cytotoxic activity against a range of tumor cell lines (reviewed in [8–11]). Among the naturally occurring frog skin peptides, these include ascaphin-8 from *Ascaphus truei* [12], magainin-2 from *Xenopus laevis* [13–15], peptide XT-7 from *Silurana epitropicalis* [12], alyteserin-2a from *Alytes obstetricans* [16], dermaseptins from *Hylomantis lemur* [17] and *Phyllomedusa bicolor* [18], aureins from *Litoria aurea* and *Litoria raniformis* [19], pentadactylin from *Leptodactylus labyrinthicus* [20], brevinin-1BYa and its dicarba derivative from *Rana boylii* [21], temporin-1CEa from *Rana chensinensis* [22], and esculentin-2CHa from *Lithobates chiricahuensis* [23]. These peptides are not tumor-specific in their cytotoxic action but show >5 fold greater potency against tumor cells than against erythrocytes or fibroblasts.

The hymenochirins are a family of five host-defense peptides that were first isolated from norepinephrine-stimulated skin secretions from the Congo clawed frog *Hymenochirus boettgeri* (Pipidae) [24]. The peptides display potent, broad-spectrum growth inhibitory activity against a range of reference strains of clinically relevant bacteria and are associated with relatively weak hemolytic activity. The present study compares the effects of hymenochirin-1B, -2B, -3B, and -4B on the viability of four well-characterized human tumor cell lines: non-small cell lung

* Corresponding author at: Department of Biochemistry, College of Medicine and Health Sciences, United Arab Emirates University, 17666 Al Ain, United Arab Emirates. Tel.: +971 3 7137484; fax: +971 3 7672033.
E-mail address: jmconlon@uaeu.ac.ae (J.M. Conlon).

0167-0115/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.regpep.2013.10.006

adenocarcinoma A549 cells, breast adenocarcinoma MDA-MB-231 cells, colorectal adenocarcinoma HT-29 cells, and hepatocarcinoma HepG2cells. The very hydrophobic hymenochirin-5B has low solubility in physiological media and aggregates in solution and so was not investigated in this study.

There have been several structure–activity studies of frog skin host-defense peptides aimed at developing analogs with increased antimicrobial activity and decreased cytotoxicity to mammalian cells (reviewed in [6]) but there have been few studies that have investigated the effect of structural modifications on anti-cancer activity. Structure–activity relationships in hymenochirin-1B were investigated by synthesizing analogs containing selective substitutions of amino acids by either L-lysine or D-lysine in order to produce peptides with increased cationicity that maintain the amphipathic α-helical character of the molecule. The aim of the investigation was to develop a non-toxic peptide with high potency against tumor cells that has therapeutic potential as an anti-cancer agent.

2. Methods

2.1. Peptide synthesis

Hymenochirin-1B, -2B, -3B, and -4B and all hymenochirin-1B analogs 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 (28.0/71.9/0.1, v/v/v) at a flow rate of 6 mL/min. The concentration of acetonitrile was raised to 56% (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 all peptides tested was >98% as determined by symmetrical peak shape and electrospray mass spectrometry. All peptides had high solubility in physiological buffers.

2.2. Cytotoxicity assays

Human non-small cell lung adenocarcinoma A549 cells were maintained at 37 °C in RPMI 1640 medium containing 2 mM L-glutamine and supplemented with 10% fetal calf serum (FCS; Biowest, Nouaille, France), and antibiotics (penicillin 50 U/mL; streptomycin 50 μg/mL). Human breast adenocarcinoma MDA-MB-231 cells and human colorectal adenocarcinoma HT-29 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics (penicillin 50 U/mL; streptomycin 50 μg/mL) and 10% FCS. Human hepatocarcinoma HepG2cells were seeded into 96 well microtiter plates and grown to 90% confluence in DMEM containing 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO_2 at 37 °C. In all experiments, cell viability was higher than 99% using trypan blue dye exclusion.

Tumor 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 hymenochirin-1B and its analogs (0.1–100 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, calculated by non-linear regression analysis using commercially available software (SPSS version 17.0; SPSS Inc., Chicago, IL, USA), was taken as the mean concentration of peptide producing 50% cell death in three independent experiments.

In a second series of experiments, viabilities of A549, MDA-MB-231, HT-29 and HepG2 cells were measured after 30 min, 2 h, 6 h, and 24 h in order to determine the rate at which [E6k] hymenochirin-1B, at a concentration equal to its previously determined LC_{50} value, produced cell death.

2.3. Hemolysis assay

Peptides in the concentration range 12.5–400 μM were incubated with washed human erythrocytes (2×10^7 cells) from a healthy donor in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μL) for 1 h at 37 °C. After centrifugation (12,000 ×*g* for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in the presence of 1% v/v Tween-20 was carried out to determine the absorbance associated with 100% hemolysis. The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments.

2.4. Proliferation assay

In three independent experiments, A549 cells were incubated for 24 h at 37 °C with hymenochirin-1B (2.5 μM) in triplicate and the viability was measured as previously described. The cells were then washed twice with phosphate-buffered saline (PBS) and the viable cells incubated for a further 48 h at 37 °C in complete media. Cells were trypsinized, collected in complete media, and counted using a Scepter 2.0 Handheld Automated Cell Counter (Millipore Corporation, Billerica, MA, USA).

2.5. Secondary structure prediction

Prediction of secondary structure and determination of % helicity per residue for the peptides were performed using the AGADIR program [25]. AGADIR is a prediction algorithm based on the helix/coil transition theory which predicts the helical behavior of monomeric peptides.

Table 1
Physicochemical properties of the hymenochirin peptides used in the study.

Peptide	Amino acid sequence	Charge pH 7.0	GRAVY	Mean hydrophobic moment	Helical domain
Hymenochirin-1B	IKLSPETKDNLKKVLKGAIKGAIAVAKMV.NH_2	+6	0.169	1.44	5–27
Hymenochirin-2B	LKIPGFVKDTLKKVAKGIFSAVAGAMTPS	+4	0.466	2.33	8–16
Hymenochirin-3B	IKIPAVVKDTLKKVAKGVLSAVAGALTQ	+4	0.689	2.57	9–16
Hymenochirin-4B	IKIPAFVKDTLKKVAKGVISAVAGALTQ	+4	0.664	2.42	7–16
[P5K]-1B	IKLSKETKDNLKKVLKGAIKGAIAVAKMV.NH_2	+7	0.090	1.48	5–27
[E6K]-1B	IKLSPKTKDNLKKVLKGAIKGAIAVAKMV.NH_2	+8	0.155	1.45	5–17,22–27
[D9K]-1B	IKLSPETKKNLKKVLKGAIKGAIAVAKMV.NH_2	+8	0.155	1.45	5–17,22–27
[P5K,E6K]-1B	IKLSKKTKDNLKKVLKGAIKGAIAVAKMV.NH_2	+9	0.076	1.49	5–17,22–27
[P5K,D9K]-1B	IKLSKETKKNLKKVLKGAIKGAIAVAKMV.NH_2	+9	0.076	1.50	5–17,22–27
[E6K,D9K]-1B	IKLSPKTKKNLKKVLKGAIKGAIAVAKMV.NH_2	+10	0.141	1.46	5–16,22–27
[E6k]-1B	IKLSPkTKDNLKKVLKGAIKGAIAVAKMV.NH_2	+8	0.155	ND	ND
[D9k]-1B	IKLSPETKkNLKKVLKGAIKGAIAVAKMV.NH_2	+8	0.155	ND	ND
[E6k,D9k]-1B	IKLSPkTKkNLKKVLKGAIKGAIAVAKMV.NH_2	+10	0.141	ND	ND

Grand average of hydropathicity (GRAVY) and mean hydrophobic moment of the peptides are calculated using the hydrophobicity scales for amino acid residues of Kyte and Doolittle [37]. The symbol k represents D-Lys. ND — not determined.