

Preventing Breast Cancer Growth by Cationic Cecropin B

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Abstract

Development of treatment resistance and lack of specificity are common problems in cancer chemotherapy, resulting in diverse and negative side effects. Emerging studies have shown that certain anti-microbial peptides (AMPs) have a destructive effect on cancer cells but no effect on normal eukaryotic cells. We isolated a cationic antimicrobial peptide--Cecropin B from *silk worms* infected with *staphylococcus aureus* and investigated its effects on breast cancer growth *in vitro* and *in vivo*. Treatment with Cecropin B induced cell death of murine breast cancer cells but not of normal nonmalignant cells. Cecropin B inhibited cancer cell proliferation as compared with control. Cecropin B treatment increased gene expression of Caspase3, Fas and high mobility group box 1 (HMGB 1). Administration of Cecropin B *in vivo* reduced tumor growth. In conclusion, Cecropin B possesses specific killing ability against tumor cells. There is the potential of development of anti-microbial peptides as anti-cancer drugs.

Keywords: Cecropin B; Breast cancer; Apoptosis

Introduction

Breast cancer is the leading cause of death in western women. Chemotherapy, often combined with radiation therapy and surgery, is applied to a majority of cancer patients. However, most cytotoxic anticancer agents in current use have little or no specificity for tumor cells and target both neoplastic and healthy proliferating cells, resulting in undesirable side effects such as nausea, vomiting, myelosuppression and even thrombocytopenia [1-4]. The frequent development of multi-drug resistance by cancer cells is another factor that hinders conventional chemotherapy. There is a need to develop new approaches to cancer therapy that specifically targets tumor cells, sparing effects on all other cells, thus eliminating side effects, and avoiding the problem of drug resistance. A potential therapy has been found in certain cationic antimicrobial peptides (AMPs), which have been shown to specifically destroy cancer cells [5-7].

AMPs are innate immunity peptides known as peptide antibiotics or natural antibiotics, and are produced by many organisms as a first line of defense to fight microbial invaders [6]. AMPs constitute a ubiquitous and broadly effective component of innate immunity in the host organism. AMPs have been found in many diverse species including insects, fish, amphibians and mammals. More than 1200 AMPs of different origins have been identified or described [8-12] since the first discoveries of plant thionins in 1972 [13] and insect Cecropin in 1981 [14]. Each AMP is encoded by a distinct gene. These peptides show great structural diversity but have some common features including: 1) relatively small size (usually less than 50 amino acid residues), 2) cationic nature (net charge from +2 to +9 at neutral pH), 3) amphipathic (which enables the peptides to interact with and disrupt lipid membranes), and 4) a substantial portion of hydrophobic amino acids (approximately 50%) [6,15]. Cationic AMPs disrupt cell membranes and induce cell death through electrostatically interaction with anionic membrane components of cells [1,16]. Cancer cell membranes typically carry a net negative charge due to a higher than normal expression of anionic molecules, while normal cell membranes are neutral [1,7]. Electrostatic interactions between cationic AMPs and negatively charged membranes allow cationic AMPs to highly selectively kill cancer cells while sparing normal cells [17]. This ability to directly and selectively attack the membranes of microbes and tumour cells is potentially advantageous in overcoming tumour cell drug resistance.

Cecropins are one of the cationic antimicrobial peptides, first isolated from the Cecropin moth, and have been shown to have anti-tumor capabilities against a variety of cancer cell types, including bladder cancer [18], hepatocellular carcinoma [19], leukemia [20], colon cancer [21], small cell lung cancer [22], and gastric carcinoma [23]. In this study, we report the anti cancer effect of Cecropin B purified from silk worms infected with *staphylococcus aureus* on breast cancer cells.

Methods and Materials

Animal

Female BALB/c (H-2Kd) mice 6 weeks of age were purchased from The Jackson Laboratories, (Jackson Laboratories, Bar Harbor, ME), and kept in filter-top cages at the Animal Care and Veterinary Services Facility at the University of Western Ontario (UWO) according to Canadian Council for Animal Care Guidelines. Mice were fed by food and water ad libitum and allowed to acclimatize for a week after delivery before initiation of experiments. All animal procedures were ethically approved by The UWO Animal Use Subcommittee.

Cell culture and treatment with cecropin

Mouse breast cancer cell line 4T1 cells and human breast cancer cell line MDA-MB231 were obtained from the ATCC and plated on 12-well plates, with 2×10^5 cells in 2 ml of culture medium-RPMI 1640 supplemented with 2 mmol/l l-glutamine, 100 U/ml penicillin, 100 µg streptomycin and 10% fetal bovine serum (FBS). Following overnight incubation, varying concentrations of Cecropin B (2 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml) in complete medium were added. Cells were incubated with the described concentration of Cecropin-B for four

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hours, eighteen hours, or twenty-four hours, after which they were subjected to the following analyses.

Mouse kidney epithelial cells and human umbilical vein endothelial cells (HUVEC) were obtained from the ATCC and cultured in DMEM medium supplemented with 2 mmol/l l-glutamine, 100 U/ml penicillin, 100 µg streptomycin and 10% FBS.

Cecropin B preparation and synthesis

Cecropin B sequence (RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK) was selected from our previous study [24,25] in which the peptide was identified from silk worms infected with *staphylococcus aureus*. The selected peptide was proved to have high killing ability against *E. coli*. The peptide and control peptide (sequence: DSHAKRRHHGYKRKFHEKHHSHRGY) were synthesized and purified by high performance liquid chromatography by Biomatik Corporation (Cambridge, Ontario, Canada).

CCK-8 assay

Tumor Cells or normal cells (10,000/well) were plated in a 96-well plate and treated with Cecropin-B for 18 h 10 µl Cell Counting Kit-8 (CCK-8) reagent was added to the cells. Two hours after addition of CCK-8, the absorbance at 450 nm was measured. Cell viability was normalized with the control peptide.

Cell morphology

Cells were cultured in a 12-well plate overnight and treated with Cecropin-B. Cell morphology was studied under a light microscope.

Reverse transcript quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Burlington, Ontario, Canada). To synthesize the cDNA with oligo-dT and reverse transcriptase (Invitrogen), 3µg total RNA was used in a reaction volume of 20 µl. DNA primers used to amplify murine caspase 3, Fas, HMGB 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were: caspase3 5'- GGGCTTTGCTCTACCACATC-CACT-3' (forward) and 5' - ACATCGTCATCCCCTCGGTTCC-3' (reverse); Fas 5'- GGGCTTTGCTCTACCACATCCACT-3', 5'-GGGCTTTGCTCTACCACATCCACT-3'; HMGB 1 5'- GGGCTTTGCTCTACCACATCCACT-3' and 5'- GGGCTTTGCTCTACCA-CATCCACT-3'; and GAPDH 5'-TGATGACATCAAGAAGGTGGT-GAA-3' (forward) and 5'-TGGGATGGAAATTGTGAGGGAGAT-3' (reverse). Quantitative polymerase chain reaction (qPCR) was performed using an Applied Biosystems MX 4000 PCR instrument (Applied Biosystems, Carlsbad, CA) and SYBR Green reagent (Bio-Rad Life Science, Mississauga, ON) according to the manufacturer's protocol. The data were used to measure the amount of caspase3 or Fas or HMGB mRNA present in samples, relative to GAPDH mRNA levels.

Apoptosis assay by flow cytometry

Cultured 4T1 cells were harvested using Trypsin and washed with PBS for two times. Cells were suspended in 100 µl binding buffer. 1 µl FITC-labeled Annexin-V (CalBiochem, San Diego, CA) was added to cells and incubated at room temperature for 15 min. 15 µl Propidium iodide (PI) were directly added to the above stained cells. The fluorescence from the cells was immediately detected by flow cytometry using a Becton Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA).

Cell proliferation

To test cell proliferation, a ³H thymidine incorporation assay was used. 4T1 or MDA-MB231 cells (10⁴/well) were plated in a flat-bottom 96 well plate (Corning). Cecropin B at the designed concentrations was added to the cells one hour after culture. 48 h after treatment, 1 µCi ³H thymidine (Amersham, Quebec, Canada) was added to the samples and was incorporated by the cells into their DNA during each cell division. The levels of incorporation were obtained by assessing radioactivity. The cultures were harvested onto glass fiber filters (Wallac Filtermat B, Wallac 1205-404). Radioactivity was counted using a Wallac Betaplate liquid scintillation counter.

Administration of cecropin-B to mice harboring 4T1 breast tumors

Cultured 4T1 cells were collected, washed with PBS and suspended in PBS (10⁶ cells/ml). Isolated 4T1 cells (10⁵cells) were injected into the mammary fat pads of six week old female BABL/c mice (n=5, per group). When tumors had grown to 5 mm in diameter, mice were treated with intratumoral injection of Cecropin-B. Cecropin-B injection was repeated every other day for 2 weeks. Tumor size was measured by caliper and the volumes were estimated using the following formula: tumor volume = 0.5 × (tumor width)² × (tumor length).

Statistical Analyses

Data are shown as means ± SEM. Statistical analyses were carried out with GraphPad Prism software (Graph-Pad Software, Inc., San Diego, CA, USA). Comparisons between two groups were performed using an unpaired two sided t test and all others were subjected to one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison tests (p < 0.05 was considered significant).

Results

Cecropin-B induced 4T1 cell death

Previously, our group had isolated a Cecropin (named as Cecropin B) from silk worms infected with *staphylococcus aureus*, demonstrated its killing ability against microorganisms, and identified its structure and sequence [25]. In this study, we hypothesized that Cecropin B would have killing activity against breast cancer cells but not normal nonmalignant cells. To test our hypothesis, we used a chemically synthesized Cecropin B based on its sequence. We cultured murine breast cancer line 4T1 and human breast cancer cell line MDA-MB231 cells in vitro. 24 h after culture, we added various concentration of Cecropin-B onto the cells. After exposing cells to varying concentrations of Cecropin-B, an increase in cell death was noticed as early as 4 h after an addition of Cecropin-B. Cell death was relative to the exposure time, and the concentration of the peptide. Healthy 4T1 cells have a rounded triangular shape and grow in a single layer on the plate. Cell death is indicated by the shrinking of cells into spheres, as well as the absence of healthy cells. Cells treated with Cecropin-B shrank into spheres, lost their normal morphology indicating that they were dying, and the number of cells decreased (Figure 1A). Cells were still healthy at the same time points in the culture medium alone and with a control peptide. The tumoricidal effect was also observed in MDA-MB 231 (Figure 1B). The killing ability of Cecropin-B was confirmed by CCK-8 assay (Figure 1C). In order to exclude Cecropin-B toxicity to normal nonmalignant cells, we treated mouse normal kidney epithelial cell and human umbilical vein endothelia cells (HUVEC) with the same amount of Cecropin-B. Figure 1C shows that the cell viability of non-malignant cells including mouse kidney epithelial cells and HUVEC

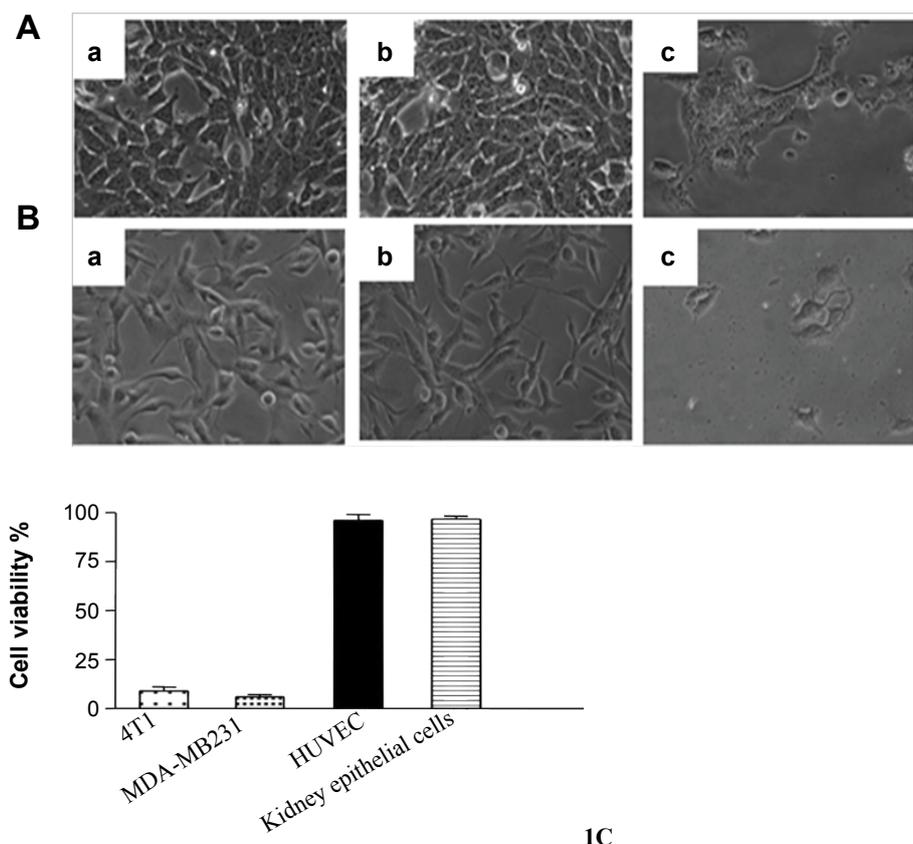


Figure 1: Cecropin-B caused breast cancer cell death, not normal cells. Tumor cells or normal cells (100,000 cells/well) were plated in a 24 well-plate and cultured in RPMI1640 medium with supplement of 10% FBS for overnight. The culture medium were removed and replaced with fresh medium. 20 μ l of 0.1mg/ml Cecropin-B was added to the culture. 18 h after addition of the peptide, cell morphology was examined under microscope (200 X). Cell viability was detected. **(A)** Mouse breast cancer cells 4T1; (a) complete medium; (b) control peptide; (c) Cecropin B. **(B)** human breast cancer cell line MDA-MB231; **(C)** Cell viability determined by CCK-8 assay. Tumor Cells or normal cells (10,000/well) were plated in a 96-well plate and treated with Cecropin-B for 18 h. 10 μ l Cell Counting Kit-8 (CCK-8) reagent was added to the cell. Two hours after addition of CCK-8, the absorbance at 450nm was measured. Cell viability was normalized with the control peptide.

was not affected by Cecropin-B, indicating that Cecropin-B did not induce nonmalignant cell death.

Cecropin-B caused cancer cell apoptosis

Studies have shown that cecropin induces cell apoptosis leading to cell death [19,26]. Accordingly, we determined whether Cecropin B affects tumor cell apoptosis. We treated 4T1 cells with Cecropin or control peptide for certain time periods and then double stained these cells with Annexin-V and PI, followed by flow cytometry analysis for measuring cell apoptosis and death. About 9% of 4T1 cells treated with Cecropin-B was apoptotic 4 h after treatment with 2 μ g/ml Cecropin-B, however only about 1.5% of the cells displayed Annexin V positive in the control group treated with either control peptide or complete medium (Figure 2). The treatment with Cecropin B induced more than two times cell apoptosis as compared with control groups. 38% of the cells treated with AMP died 24 hours after treatment, while only 7% of the cells in the control group were dead. The higher the concentration of Cecropin that was used, the more apoptotic and necrotic cells were observed. The effect of Cecropin-B on cell apoptosis and death was in a dose and time-dependent manner.

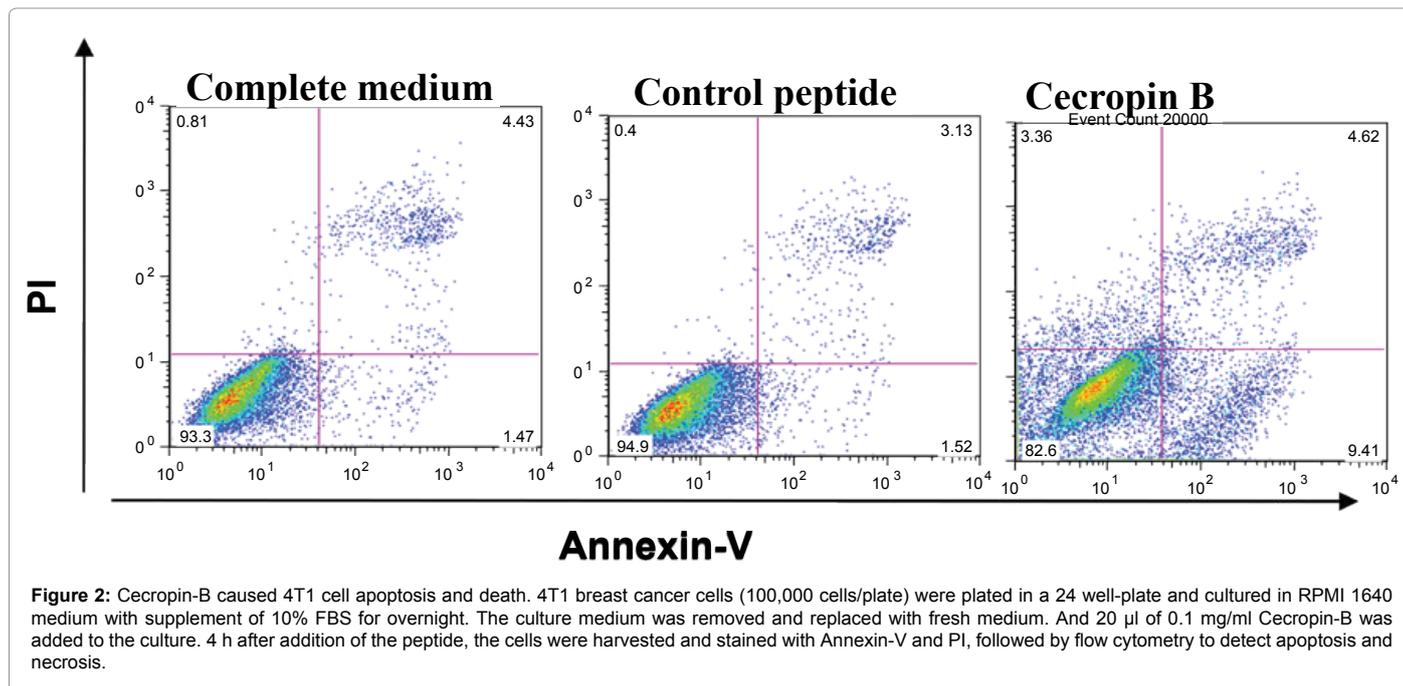
Cecropin-B inhibited cell proliferation

Uncontrolled cell proliferation is one of the hallmarks of cancer cells. We detected the effect of Cecropin-B on the cell proliferation of

4T1 and MDA-MB231 cells in vitro using ^3H -thymidine incorporation assay. 4T1 or MDA-MB231 cells were cultured and treated with various concentrations of Cecropin-B for 72 h. In the last 18 h, 1 μ Ci ^3H -thymidine was added onto the culture medium. The incorporation of ^3H thymidine into cells was measured for cell proliferation. As shown in Figure 3, the cell proliferation was inhibited by Cecropin B and the inhibition of cell proliferation for Cecropin-B was dose-dependent.

Cecropin-B up-regulated cell apoptosis and death associated gene expression

Given that Cecropin-B induces tumor cell apoptosis, we next examined the effect of Cecropin B on expression of genes associated with cell apoptosis and death such as caspase 3, Fas and HMGB. We treated 4T1 cells with Cecropin-B and examined its effect on the gene expression of caspase-3, Fas and HMGB by qRT-PCR. We found that the gene expression of caspase-3, Fas, and HMGB in the 4T1 cells treated with Cecropin-B was significantly increased 4 h after Cecropin-B treatment as compared with the untreated cells or those treated with control peptide (Figure 4). The data were consistent with the Annexin-V staining results, suggesting the caspase pathway may be activated by cecropin-B. Because most cells were dead 24 h after treatment, we did not observe any increase in these genes expression (data not shown).



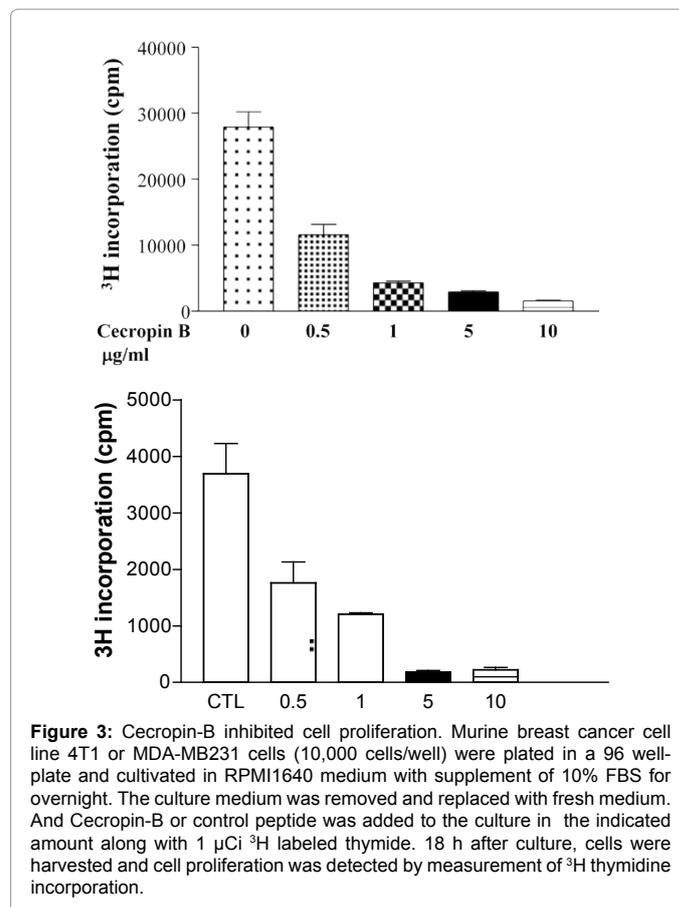
Treatment with Cecropin-B arrested tumor growth in a murine breast cancer model

Cecropin-B induced breast cancer cell apoptosis/death and inhibited cell proliferation *in vitro*. We further explored the therapeutic anticancer effect of cecropin-B in a xenograft model. Considering that Cecropin B administrated intravenously will be enzymatic degraded and inactivated by negatively charged serum proteins [4], we chose intratumor injection to deliver the peptide into tumor bearing mice in this study. We injected mouse breast cancer 4T1 cells into the mammary fat pad of female Babl/C mice. When developing tumors reached 0.5 cm in diameter, the mice were given intratumoral injections of Cecropin-B or control peptide. Tumor size was measured every other day using a caliper. Tumors treated with Cecropin-B treatment grew slower than control groups (Figure 5A). At the end point of the experiments, we isolated and weighed primary tumors. The tumors from mice treated with Cecropin-B showed smaller and lower weight than those treated with the control peptide (Figure 5B).

Discussion

Development of treatment resistance and lack of specificity are common problems in current cancer chemotherapies, resulting in diverse and negative side effects. This holds true for current cancer treatments. Emerging studies have shown that certain AMPs have no or less effect on normal eukaryotic cells but a destructive effect on cancer cells [4,27-29]. In this study, we demonstrated the anti-tumor effect of Cecropin B both *in vitro* and *in vivo*. Treatment of murine breast cancer cell line 4T1 cells with the peptide increased cell death and apoptosis through up-regulating the expression of apoptotic genes caspase-3 and Fas. A correlation between increasing concentration of peptide and increasing cell death was noted. Similarly, increased time of exposure to the peptide caused an increase in cancer cell death. Increasing inhibition of cell proliferation with increasing concentrations of Cecropin-B carries further implications for antitumor therapy. Intratumoral administration of Cecropin-B reduced tumor growth. This study demonstrates, for the first time, the potential of development of Cecropin B as an anti-cancer drug.

Cecropins are one of the AMPs that are present in mammals and many insects [4,14]. These cationic peptides consist of 34 – 39 amino acids and can be divided into cecropin A and B peptide families [14]. Irrespective of their fundamental role as antimicrobial agents, both



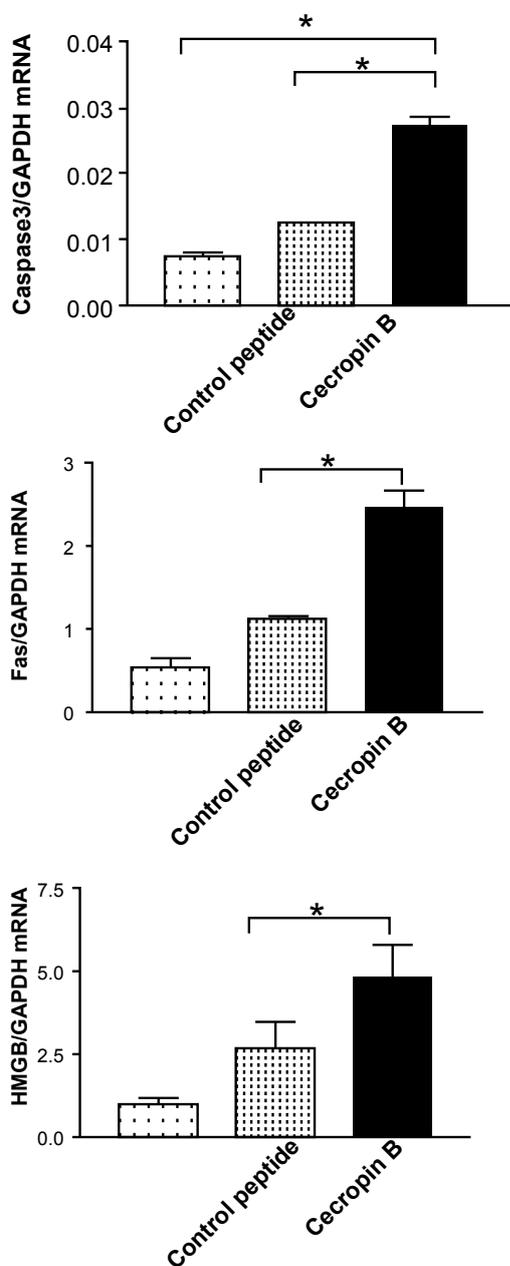


Figure 4: Cecropin-B up-regulated caspase-3, Fas and HMGB gene expression 4T1 breast cancer cells (100,000 cells/plate) were plated in a 24 well-plate and cultured in RPMI 1640 medium with supplement of 10% FBS for overnight. The culture medium was removed and replaced with fresh medium. And 0.1mg/ml cecropin-B was added to the culture. 4 h after addition of the peptide, the cells were harvested and total RNA was extracted from the above cells using a Trizol kit. 3µg RNA was used to synthesize cDNA with oligdT and reverse transcriptase. The gene expression of caspase-3 (A), Fas (B) and HMGB1(C) and GAPDH was detected by quantitative PCR using SYBRGreen. The gene expression was normalized by GAPDH. *, P value < 0.05.

cecropin A and B family members show cytolytic activity against several different human cancer cell lines, including leukaemic and lymphoma cells. Cecropins show selective cytotoxicity and antiproliferative activities in bladder cancer cells [18], leukemic cells [20], colonic adenocarcinoma cells [21] and gastric cancer cells [21,23]. It is also

found that breast and ovarian carcinoma cell lines with a multi-drug-resistant phenotype are sensitive to cecropin B [4].

The exact mechanisms by which cecropins kill cells are not clearly understood. One possible mechanism reported is that cecropins attack membranes of cells [7,30]. Through their net positive surface charge (which interacts electrostatically with negatively-charged phospholipid headgroups and/or amphipathic structures on bacterial/tumor cell membranes), cecropins generate membrane pores by a process of attachment, insertion and permeabilization, and consequent membrane disruption [10,31,32]. In our study, we observed the green fluorescent dye FITC labeled peptide was bound to the cell membrane. Recent studies have shown, apart from disrupting cell membranes, AMPs are endowed with cytotoxic mechanisms in cancer cells involving apoptosis [4,33]. The anticancer activity of these peptides was accompanied by changes in cell growth and proliferation signaling pathways [33]. Our data also showed that cecropin-B caused cancer cell apoptosis, indicating the peptide is at least partially acting via this mechanism. The up-regulation of Caspase 3, Fas and HMGB further pointed towards involvement of the apoptotic pathway. For a potential cancer therapy, apoptosis would be the preferred method of cell death as it causes minimal leakage of cell content, thereby avoiding a large inflammatory response. Certainly, additional research on possible

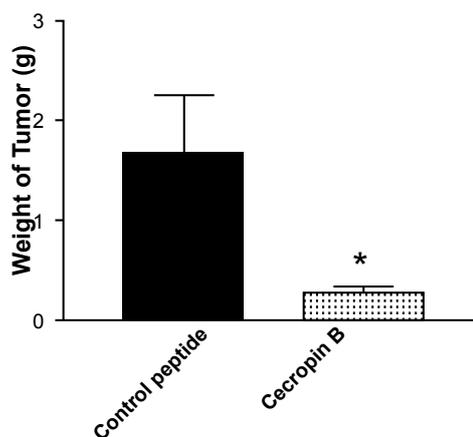
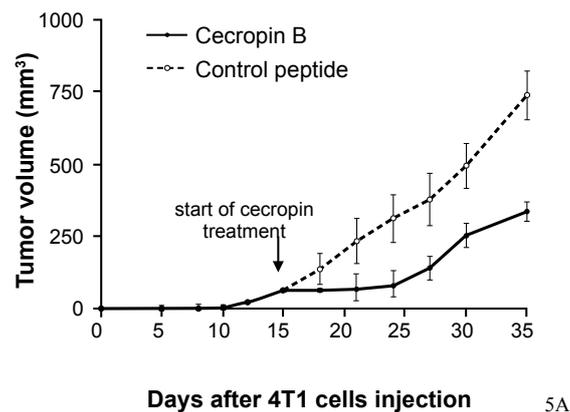


Figure 5: Cecropin-B arrested tumor growth. I. 4T1 cells (10^5 cells/injection) were injected into the fat pad of Balb/C mice (n=5 per group). When tumor size reached 5mm in diameter, the mice were daily treated with intratumoral injection with cecropin-B or control peptide. Tumor growth was monitored every other day with a caliper as described in the Material& Methods. At the endpoint of experiments, tumors were excised and weighed. *, p value < 0.05. (A) Tumor growth; (B) Tumor weight.

mechanisms leading to anti tumor effects of cecropin B needs to be conducted in future.

Cancer cell membranes typically carry a net negative charge due to a higher than normal expression of anionic molecules, while normal cell membranes are neutral [1,7]. Cationic cecropins toxic to mammalian cells interact electrostatically with anionic membrane components to disrupt those membranes and induce cell death [1,16]. Electrostatic interactions between cationic cecropins and negatively charged membranes make cationic cecropins highly selectively kill cancer cells while sparing normal cells. The ability to directly and selectively attack the membranes of tumor cells is potentially advantageous in overcoming tumor cell drug resistance: cationic cecropins that spare normal cells can be administered in higher concentrations capable of killing drug resistant tumor cells. Our data showed that Cecropin B attacked cancer cells including breast cancer cells and melanoma cells (B16F10, data not shown) but not nonmalignant cells. Intratumoral injection of Cecropin reduced tumor growth in a murine breast cancer model while notable toxicity to adjacent normal tissue was not observed. Optimization of the treatment, other administration routes (e.g. intravenous injection or intraperitoneal injection) and cytotoxicity of Cecropin B will be investigated in future. Nonetheless, the results from the current study indicate its potential for the development of anti-cancer therapies.

Despite the promise that cationic cecropins are demonstrated as anticancer agents, a number of issues and obstacles must be addressed and overcome before it can be effectively employed in a clinical setting. The enzymatic degradation and inactivation of cationic cytotoxic peptides through interaction with negatively charged serum proteins is the most serious obstacle to the clinical use of anticancer cationic peptides. Chemical modification such as using D-amino acid analogues or encapsulation of peptides into liposomes might protect peptides from degradation and inactivation by serum components. The cost of cytotoxic peptides synthesis is one of concerns too. More economic peptide preparation approaches are needed.

In conclusion, Cecropin B possesses specific killing ability against tumor cells. There is the potential of development of cecropins as anti-cancer drugs.

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