PEPTIDES 30 (2009) 283–290

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Epinecidin-1, an antimicrobial peptide from fish (*Epinephelus coioides*) which has an antitumor effect like lytic peptides in human fibrosarcoma cells

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ARTICLE INFO

Article history:
Received 14 August 2008
Received in revised form
1 October 2008
Accepted 1 October 2008
Published on line 21 October 2008

Keywords:
Epinecidin-1
Tumor cell inhibition
Lytic peptide

ABSTRACT

Epinecidin-1, a synthetic 21-mer antimicrobial peptide originally identified from grouper (*Epinephelus coioides*), specifically exhibited high antimicrobial activities against both Gram-negative and Gram-positive bacteria. In the current study we report on the *in vitro* cytotoxicity of the peptide, an important factor before it can be considered for further applications in cancer therapy. The cytotoxicity of epinecidin-1 was investigated against several cancer cells (A549, HA59T/VGH, HeLa, HepG2, HT1080, RAW264.7, and U937) and normal cells (AML-12, NIH3T3, and WS-1) with the MTT assay, and the inhibition of cancer cell growth was confirmed by a soft agar assay and scanning electron microscopy. However, cell variations were detected with AO/EtBr staining, while apoptosis and necrosis gene expressions in HT1080 cells after treatment with the epinecidin-1 peptide and Nec-1 showed that epinecidin-1 had an anti-necrosis function in HT1080 cells. The data presented here indicate that epinecidin-1 has *in vitro* antitumor activity against the HT1080 cell line, and functions like lytic peptides. In addition, our results suggest that epinecidin-1 may prove to be an effective chemotherapeutic agent for human fibrosarcoma cells in the future.

1. Introduction

Recent reports have demonstrated that antimicrobial peptides (AMPs) which exhibit anticancer activities can be divided into two categories: AMPs that specifically act only against bacteria and cancer cells and do not attack normal mammalian cells; and AMPs which are cytotoxic to bacteria, cancer cells, and normal mammalian cells [5]. However, these AMPs were initially discovered due to their role in innate immunity, and they are being applied to lyse bacterial membranes for further development of therapies against drug-resistant bacterial infections [11]. AMPs are natural-source antibiotics that mostly possess cationic and amphipathic molecules which have the potential to be bactericidal and antitumor agents [14]. AMPs may be used in early treatment strategies against tumors, and when a cream mixed with CAP18 AMP was brushed over tumors

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2008.10.007

of the external surface, it showed successful treatment of intradermal and epithelial skin cancers [16]. However, some AMPs from non-mammalian organisms, such as cecropins, melittin, magainin 2, and tachyplesin, exhibited cytotoxic activity against cancer cells in *in vitro* experiments which may affect their application to cancer research [8].

AMPs from marine organisms have not been exploited to their full potential in human cancer research due to a lack of information. We previously isolated an AMP gene of epinecidin-1 from a cDNA library and genomic DNA library of the grouper (*Epinephelus coioides*). Epinecidin-1 contains a prepropeptide of 67 amino acids (aa), and the epinecidin-1 gene is composed of three epinecidin-1 peptides from the fish genome. A 21-aa sequence from the C-terminal domain of epinecidin-1 synthesized from epinecidin-1 amino acids number 22–42 exhibited high antimicrobial activities against Gram-negative and -positive bacteria [11]. The grouper epinecidin-1 gene is predicted to possess an α-helical structure that is similar to many other AMPs, whose function may be to form holes in bacterial membranes [12]. On the other hand, the results of a few in vivo studies showed that AMPs are capable of disrupting membranes by forming holes and subsequently inducing cancer cell death. Thus, it would be advantageous to be able to use epinecidin-1 to target cancer cells.

In the current study we compared the cytotoxic activities of a synthesized peptide of epinecidin-1 against normal human cells, mouse cell lines, and several different human cancer cell lines. We demonstrated that epinecidin-1 selectively kills some cancer cells at lower dosages, and the results obtained were also supported by a soft agar assay. Using electron microscopy, we documented morphologic changes in human fibrosarcoma cell membranes treated with epinecidin-1. Furthermore, the results of the necrosis inhibition test and real-time PCR suggest that epinecidin-1 not only possesses cytolytic activity but also may have an anti-necrosis function in cancer cells.

2. Materials and methods

2.1. Peptide

Epinecidin-1 was synthesized with an amidated C-terminus (GFIFHIIKGLFHAGKMIHGLV-NH2) by Genesis Biotech (Taipei, Taiwan) at >95% purity. Synthetic peptides were reconstituted in phosphate-buffered saline (PBS; pH 7.4) for the experiments.

2.2. Cell culture

The A549 (human lung carcinoma cell), HeLa (human cervix adenocarcinoma cell), HepG2 (human hepatocellular carcinoma cell), HT1080 (human fibrosarcoma cell), U937 (human histiocytic lymphoma), NIH3T3 (mouse fibroblast cell), RAW264.7 (mouse macrophage from a tumor induced by the Abelson murine leukemia virus), and WS-1 (human kidney cell) cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). The AML-12 (murine hepatocyte cell) and HA59T/VGH (human hepatic tumor-derived cell) cell lines were a gift from Dr. Chun-Yao Chen (Department of Life Science, Tzu Chi University, Hualien, Taiwan). Cells were cultured using ATCC-suggested medium and conditions, while the two other cell lines from Tzu Chi University were cultured in medium and conditions following the suggestions of the provider.

2.3. Cell viability assays

Cell viability was determined using MTT assays. Cells were plated at a density of 5000 cells/well in 96-well plates for 24 h, and treated with different concentrations of epinecidin-1 or PBS alone for 24 h. This was followed by adding 30 μl of the tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 270 μl of fresh culture medium were for 6 h at 37 °C. The optical density was measured spectrophotometrically at 570 nm on a microtiter plate reader. Experiments were done in triplicate. The results are expressed as a percentage of the inhibition rate for viable cells, and values were deducted from the PBS-treated group.

2.4. Colony formation in the soft agar assay

A549, HeLa, and HT1080 cells were resuspended at 4×10^3 cells in 1 ml of 0.35% agar solution containing cell culture medium at a final concentration of 2 μg/ml epinecidin-1, and layered on top of a 0.7% agar layer in six-well plates. Epinecidin-1 was not added to the PBS groups (control group), and only PBS buffer was used as the control. Plates were incubated for 10 days at 37 °C in a humidified atmosphere containing 5% CO_2. Cell colonies were visualized following treatment with 0.5 ml *p*-iodonitrotetrazolium violet (Sigma, Steinheim, Germany) for 16 h and were observed by microscopy. Colony growth in peptide- or PBS-treated wells was expressed as a count number. Colonies of >50 μm were counted 10 days after plating. These experiments were repeated three times and at least five wells were replicated each time for each condition.

2.5. Scanning electron microscopy

HT1080, RAW264.7, and WS-1 cells were seeded in six-well round-bottom trays with 3×10^5 cells and treated with 2 μg/ml epinecidin-1 or left untreated for 1, 2, 3, and 4 h. Samples were analyzed and prepared according to a published report [2] and then analyzed by scanning electron microscopy (SEM).

2.6. Detection of cell variations by AO/EtBr staining

HT1080 cells were grown to confluence at 3×10^5 cells/dish. For AO/EtBr staining, cultures were treated with 0.5 μM Nec-1 (an inhibitor of necroptosis) for 2 h, followed by incubation with 2 or 4 μg/ml of the synthesized epinecidin-1 peptide which was treated for 0, 4, 24, and 48 h and processed for staining using cold PBS containing 1 μg/ml ethidium bromide (EtBr) and 1 μg/ml acridine orange (AO). Cells were washed with cold PBS to remove excess dye and subsequently observed, and pictures were taken under a fluorescence microscope. Experiments were performed in triplicate.

2.7. Real-time reverse-transcription polymerase chain reaction (RT-PCR)

A real-time RT-PCR analysis was used to analyze the gene expressions of calpain 5 (F: CAGGTCCTCTCAGAGGCAGATAC;

R: ACCTCTCCAGGGACCTTAACG), cathepsin G (F: TCAAGTTTCCTGCCCTGGAT; R: CCTGTGTCCCCGAGAAGAAG), caspase 3 (F: ATACCAGTGGAGGCCGACTTC; R: CAAAGCGACTGGATGAACCA), c-Jun (F: TGGACTTGGAGGAGAGAACCA; R: CGACGATGATGATGGATGCT), and β-actin (F: ATTGGCAATGAGCGGTTC; R: GGATGCCACAGGACTCCAT) by HT1080 cells following either co-treatment with epinecidin-1 and Nec-1 (an inhibitor of necroptosis; from Sigma–Aldrich Chemical, St Louis, MO), treatment with only epinecidin-1, or treatment with no chemicals, according to the manufacturer's

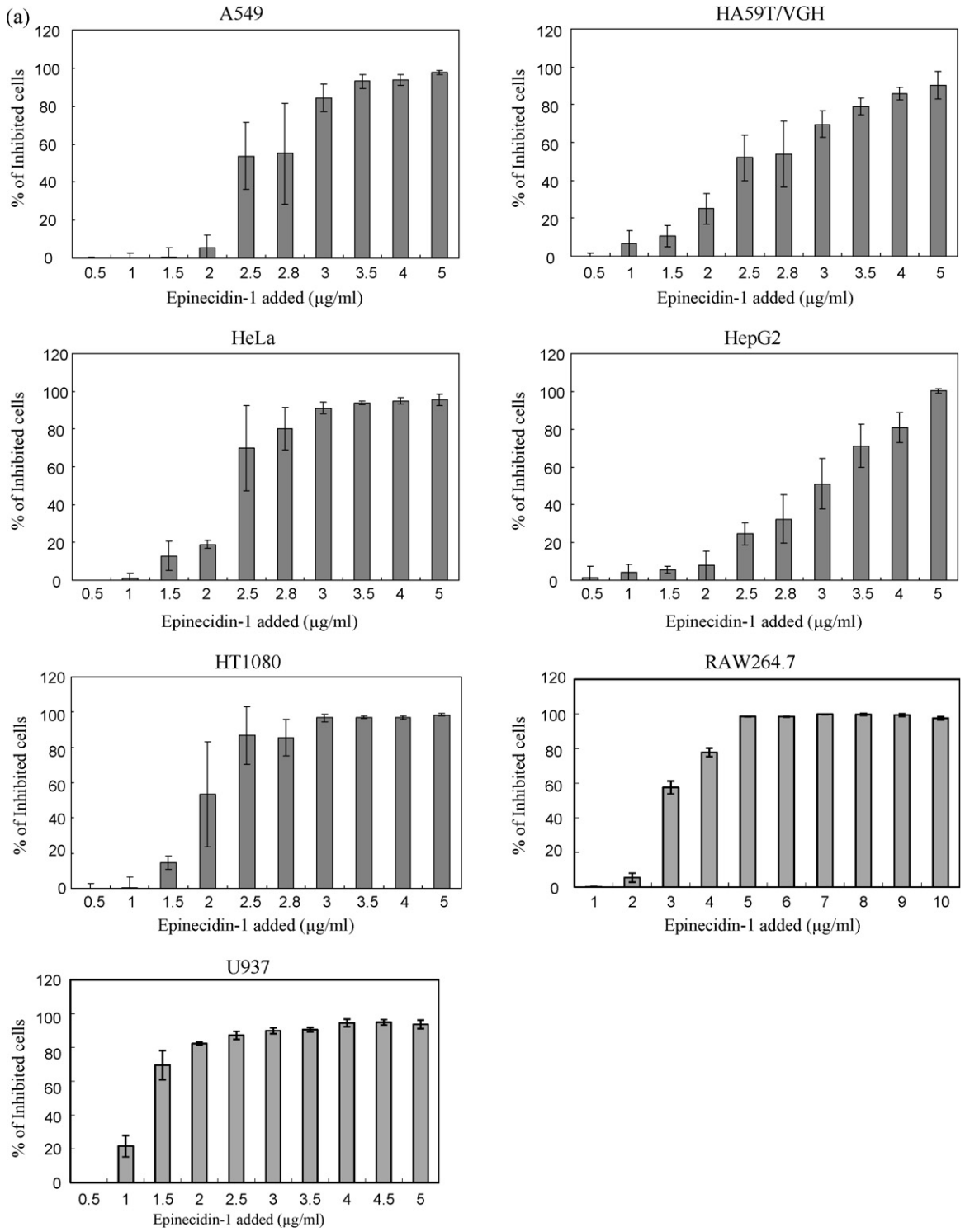

Fig. 1 – Effects of epinecidin-1 on cell proliferation. Tumor cells (a) and normal cells (b) were treated with different doses of epinecidin-1 for 24 h, followed by the MTT assay. Each concentration was repeated in eight wells for three independent experiments. Epinecidin-1 treatment affected cell viability in a dose-dependent fashion. There were significant differences between treatment groups (Student's t-test, $p < 0.05$). Clonal assay (c) for HT1080, A549, and HeLa cell lines treated with 2 μg/ml epinecidin-1 indicated reduced colony formation. Data are from five separate experiments.

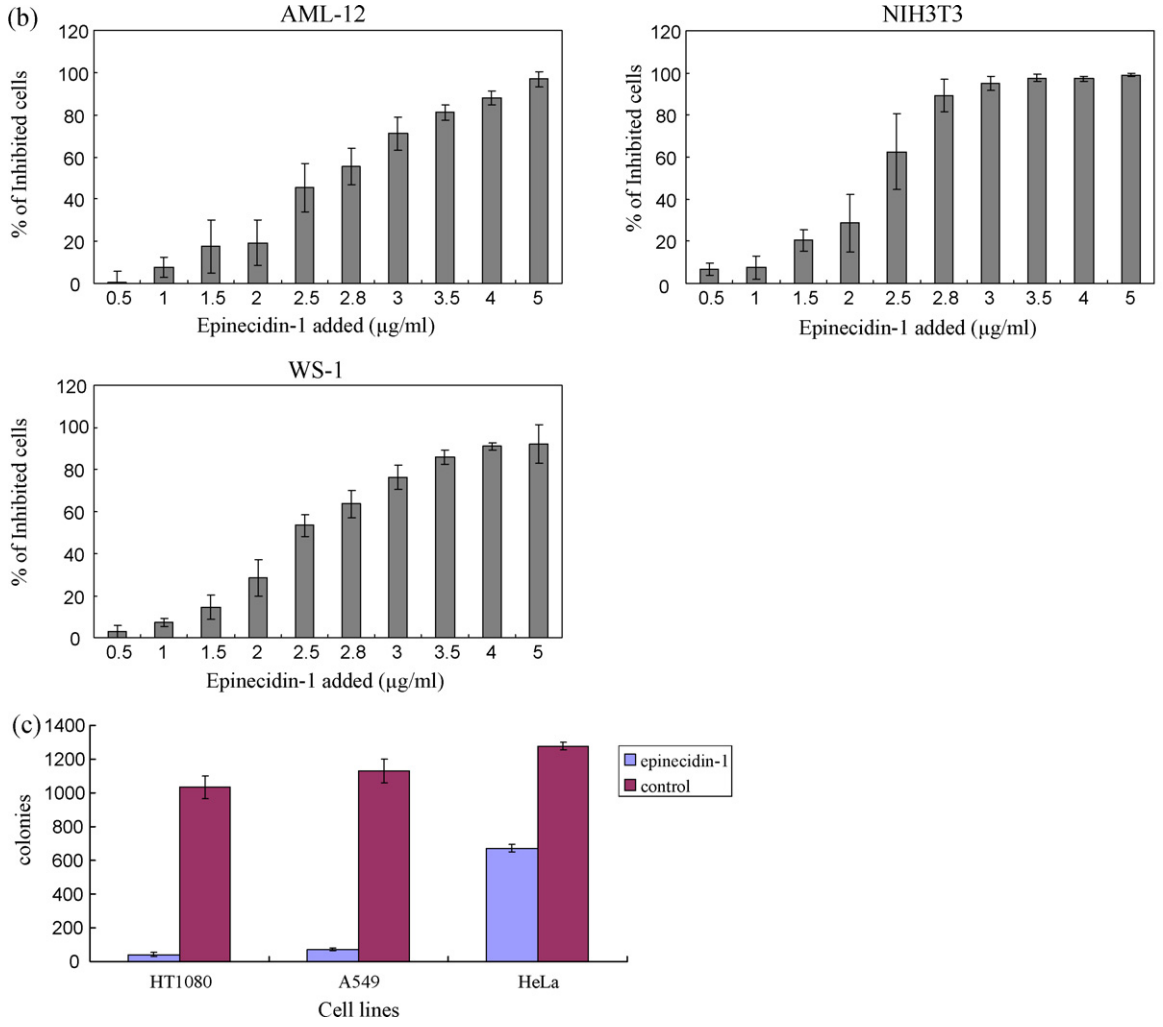

Fig. 1. (Continued).

instructions. The SYBR® Green PCR Master Mix (ABI, USA) and specific primer pairs were used for selected genes, and the primer pair for β-actin was used as the reference gene. Quantitative PCR was performed according to the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C using 0.5 μl of complementary cDNA, 2X SYBR Green PCR Master Mix, and 500 nM of the forward and reverse primers. The threshold cycle number (Ct) was calculated with ABI software. Relative transcript quantities were calculated using the ΔCt method with β-actin as the reference gene amplified from the same samples. ΔCt is the difference in the threshold cycles of messenger mRNA for selected genes relative to those of β-actin mRNA. The real-time RT-PCR was performed in triplicate for each experimental group.

3. Results

3.1. *Effects of epinecidin-1 on cell viability and clonal growth*

To examine the effects of the synthesized epinecidin-1 peptide, several tumor cell lines (Fig. 1a) and normal cell lines (Fig. 1b) were exposed to different concentrations (0.5–10 μg/ml) of epinecidin-1 for 24 h, and cell viability was determined by the MTT assay (Fig. 1). The results consistently showed higher growth inhibition ratios after 2.5 μg/ml treatment with epinecidin-1, which indicates that epinecidin-1 was very effective at inhibiting the growth of both tumor cell lines and normal cell lines (Fig. 1). In contrast to the selective cytotoxic activity of epinecidin-1 at 2 μg/ml treatment in each cell line which was like a threshold concentration, it was observed that 2.5 μg/ml epinecidin-1 produced an inhibition ratio of <60% in normal cells, while it produced an inhibition ratio of >60% in cancer cells except for HepG2 cells. Epinecidin-1 treatment was used to determine the *in vitro* antineoplastic effect. Fig. 1c shows the results of the clonal assay for the A549, HT1080, and HeLa cell lines. From these results, it is evident that the 21 aa of the synthesized epinecidin-1 peptide caused >90% inhibition of colony formation in these two cell lines (A549 and HT1080), indicating that epinecidin-1 exhibits a potential antineoplastic effect.

3.2. *Epinecidin-1-induced cell lysis*

A concentration of epinecidin-1 of 2 μg/ml was sufficient to induce apparent lysis of HT1080, RAW264.7, and WS-1 cells

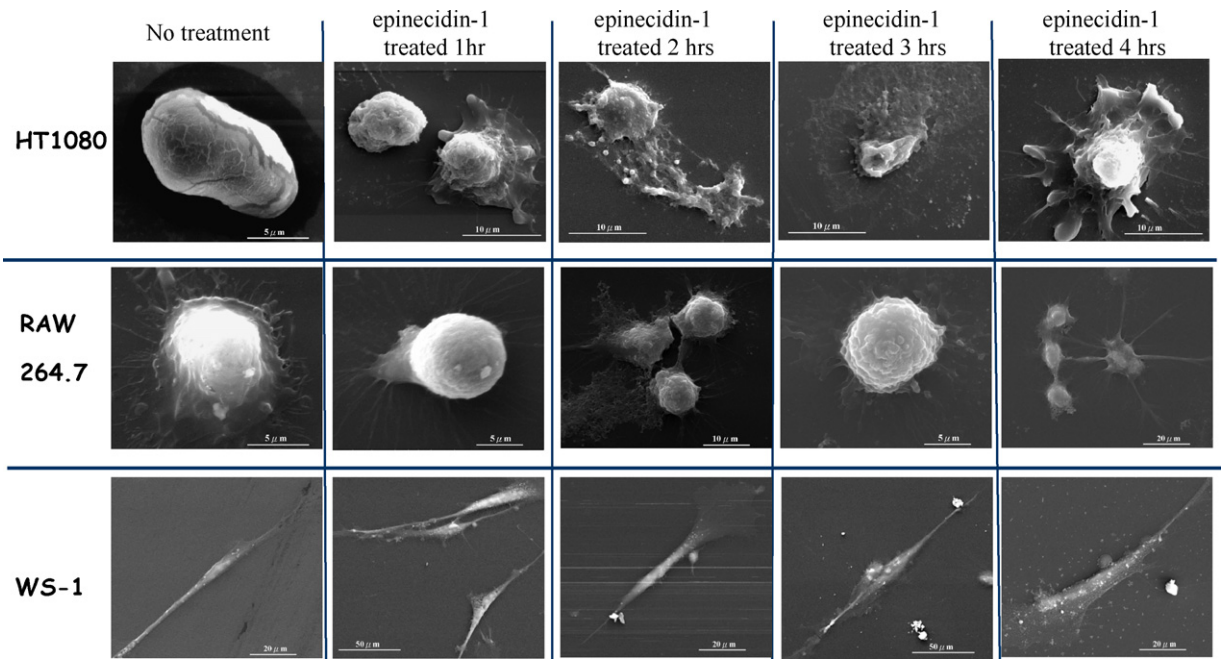

Fig. 2 – Effects of epinecidin-1 on cell membranes of tumor cells (HT1080 and RAW264.7) and normal cells (WS-1) using scanning electron microscopy. Untreated cells show a normal smooth surface, while cells treated with epinecidin-1 (2 μg/ml) for different times reveal a disrupted cell membrane.

with different treatment durations (Fig. 2). The pictures show that untreated cells had an intact cell membrane. However, after 60 min of incubation, HT1080 cells showed significant morphological changes: the cell was almost disrupted and the intracellular material was discharged after 120 min of treatment. But at the same concentration of epinecidin-1 (2 μg/ml), disrupted membranes of RAW264.7 cells were observed until 240 min, and after 120 min, WS-1 cells began to appear to bulge out on one side.

3.3. *Epinecidin-1-evoked anti-necrosis function in HT1080 cells*

Our primary study revealed that incubation of HT1080 cells with different concentrations of epinecidin-1 (2 or 4 μg/ml) for 4 h caused the induction of drastic changes in cellular morphology and caused a large percentage of cells to detach from the culture dishes in comparison to the untreated group. By using AO/EtBr staining, floating cells appeared like late apoptotic or necrotic cells (supplemental Fig. 1). To identify if epinecidin-1 induces a reduction in viability of HT1080 cells through apoptosis, HT1080 cells were treated with epinecidin-1 (2 or 4 μg/ml) for different durations, and another group of cells was treated with epinecidin-1 and Nec-1 together for different durations. The integrity of DNA and the cell morphology were examined by DNA electrophoresis and microscopic observations, respectively. Microscopic observations of the nuclei of live cells revealed that they were green, while nuclei of apoptotic cells containing condensed or fragmented chromatin appeared orange (Fig. 3). With this staining method, we failed to find any apoptotic cells at time zero in cells treated with or without Nec-1 (Fig. 3). From 4 to 24 h of incubation, most HT1080 cells showed an orange color following an increase in the epinecidin-1 dosage (Fig. 3a and b). In contrast to Nec-1 co-treated with 2 or 4 μg/ml epinecidin-1 after 48 h, the ratio of cells exhibiting orange color was reduced compared to cells with no Nec-1 treatment. Nec-1 also exhibited inhibitory effects on both cell death and vacuolization. These results suggest that the degree of apoptosis observed was related to necrosis inhibition during the experimental period. Epinecidin-1 exhibited significant lytic activity in HT1080 cells, and the lytic activity may have destroyed the cellular or nuclear membrane, thus presenting an orange color in the morphological observations. AO/EtBr staining was used to evaluate the effects of epinecidin-1 and seemed to indicate an apoptotic phenomenon (supplemental Fig. 1). The results of DNA electrophoresis showed that epinecidin-1 did not stimulate DNA fragmentation in a dose- or time-dependent manner when either treated alone or in combination with Nec-1 (supplemental Fig. 2). We observed orange color in HT1080 cells with both AO/EtBr staining and the DNA fragmentation analyses, indicating that epinecidin-1 induced lysis of cell membranes, and this effect appeared to be mediated after 48 h of treatment by necrosis inhibitory activity.

3.4. *Modulation of gene expression in HT1080 cells by treatment with the epinecidin-1 peptide and Nec-1*

To investigate the effects of epinecidin-1 on cell function, we performed a real-time RT-PCR analysis of calpain 5, cathepsin G, caspase 3, and c-Jun. As shown in Fig. 4, we observed decreases in the necrosis-related expressions of calpain 5 and cathepsin G after treatment with epinecidin-1 (but not Nec-1) from 4 to 24 h, although there was no statistical difference. However, comparison of the control (treated only with Nec-1) showed that calpain 5 gene expression with co-treatment of 2 μg/ml epinecidin-1

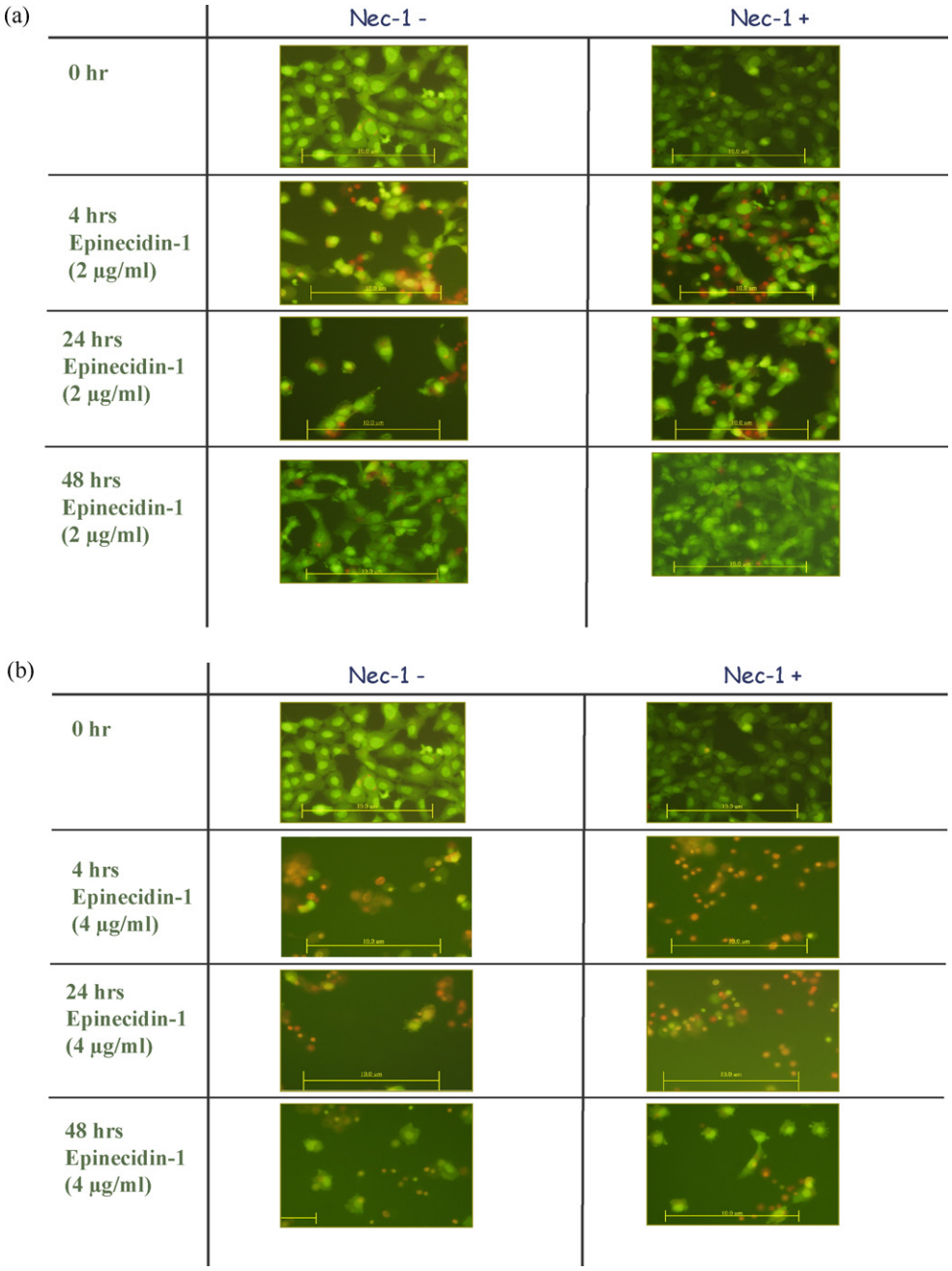

Fig. 3 – Membrane disruption in HT1080 cells after treatment with Nec-1 for 120 min followed by incubation without or with 2 (a) or 4 μg/ml (b) epinecidin-1. Cells show a necrotic pattern. Cells were harvested at the indicated times and were stained with AO/EtBr.

and Nec-1 significantly differed from 4 to 48 h. We also observed a significant increase in the expression of caspase 3 after co-treatment with 2 or 4 μg/ml epinecidin-1 and Nec-1 from 4 to 24 h. These results suggest that the expressions of necrosis and apoptosis genes appeared to respond differently when exposed to epinecidin-1 alone and when co-administered with Nec-1.

4. Discussion

Epinecidin-1 plays a crucial role in the early defense against bacterial infection in marine fish. Treatment with the synthesized epinecidin-1 peptide was effective in promoting a significant increase in fish survival after injection of *Vibrio vulnificus* in tilapia (*Oreochromis mossambicus*) and grouper [11]. But, up to now, there has been no report about epinecidin-1's antitumor activity. Interestingly, our results support the lytic and antiproliferative actions of epinecidin-1, like other AMPs which possess an antitumor effect such as the function of cecropin on bladder cancer cells [17]. Epinecidin-1 increased the cytotoxicity of HT1080 cells and other tumor cell lines in dose- and time-dependent manners, indicating that cell death occurred by membrane disruption. Little information about the cytotoxicity of AMPs or their interactions with cancer cells

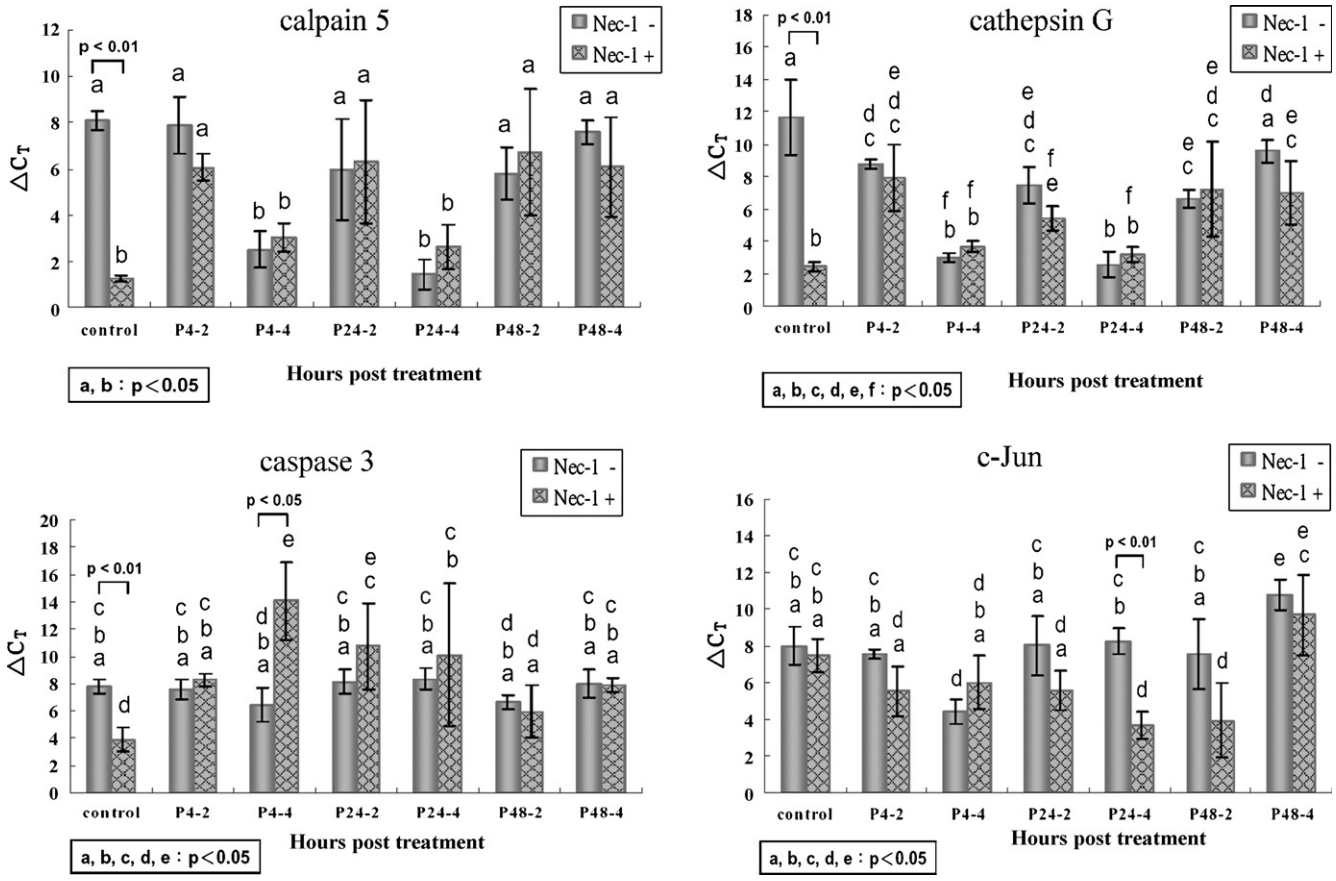

Fig. 4 – Quantification of transcript levels by comparative real-time RT-PCR. RNA from HT1080 cells was co-treated with 2 or 4 μg/ml epinecidin-1 and Nec-1, or only treated with 2 or 4 μg/ml epinecidin-1. Samples were collected after treatment for 4, 24, and 48 h. Transcript abundance, normalized to β-actin expression, is expressed as the relative expression and graphed on a rational scale. Each bar represents the mean value from three determinations with the standard error. Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among treatments. An example *x*-coordinate is P4-2, where 4 is the number of hours, and 2 is the dosage treatment (μg/ml) for epinecidin-1; i.e., P4-2 was treated for 4 h with 2 μg/ml epinecidin-1.

is available [9]. AMPs' destruction of cancer cells is divided into two categories: AMPs that act against cancer cells and bacteria but do not harm normal mammalian cells, and AMPs that are cytotoxic to all kind of cells [5]. The results of the present study demonstrated that epinecidin-1 significantly inhibited HT1080 tumor cell proliferation following treatment with 2 μg/ml compared with other cells suggesting that 2 μg/ml may be a threshold value (Fig. 1). Additionally, epinecidin-1 caused direct tumor cell lysis and probably functions by targeting cell membrane disruption. Thus, the results of the present study indicated that most AMPs, including LL-37/hCAP18 and defensin, induce pore formation in cellular membranes [4,19]. The reasons may be due to cationic AMPs (such as LL-37/hCAP18) causing leakage of cytoplasmic molecules and precipitating cell death by binding to the external surfaces of negatively charged cytoplasmic membranes of bacteria [6].

SEM was utilized to directly observe the morphologic effects of epinecidin-1 on tumor cells (HT1080 and RAW264.7) and normal cells (WS-1). Tumor cells treated with epinecidin-1 also showed potent disruption of cell membranes likely to represent pore formation with short-term treatment (Fig. 2). However, Magainin 2 causing vesiculation and pore formation belongs to a carpet effect identified by SEM technology [7], suggesting that similar results cannot be ruled out for epinecidin-1. In addition to disrupting the surface membrane of tumor cells which induces cytolysis, the cecropin family also showed potent antitumor activity against bladder cancer cells as observed by SEM [17].

The anti-neoplastic activity of AMPs has been studied in many cell lines [1]. A necrotic mechanism was suggested to be involved in epinecidin-1's killing of tumor cells, but the details of how epinecidin-1 kills HT1080 cells are still not understood very well. Although many AMPs have been reported to inhibit function in tumor cells, the mode of action on cancer cells of only a few peptides have been discussed [18]. To understand the mechanism, we used AO/EtBr staining to observe the morphology. In the late stage of apoptosis, we observed granules in the cytoplasmic space of the cell (supplemental Fig. 1), which differs from other chemotherapeutics such as C5b-9 which induces apoptosis through a caspase-dependent pathway and forms apoptotic bodies [10]. Programmed cell death is primarily mediated by Fas receptor signaling and

shows DNA fragmentation by BMAP-27. The BMAP-28-treated U937 cell line revealed that some AMPs are endowed with cytotoxic mechanisms also involving triggering of cancer cell suicide by apoptosis [15]. Although our results could not demonstrate apoptosis by DNA fragmentation after treatment of HT1080 cells with epinecidin-1 (Supplemental Fig. 2), they indicate the potential of epinecidin-1 as a drug that triggers anti-necrosis through a cell membrane lytic effect which acts in a similar way to many other native and de novo-designed AMPs [13].

Our results suggest that epinecidin-1 downregulates necrosis-related genes. Therefore, epinecidin-1's involvement in an anti-necrotic function was indicated by staining with AO/EtBr in HT1080 cells and the necrosis genes, calpain 5 and cathepsin G, being activated after Nec-1 treatment and inactivated in the absence of Nec-1 treatment (Figs. 3 and 4). It is noteworthy that necrotic cell death is the result of several signaling cascades induced by specific receptors, and RIP1 appears to be a central initiator of necrosis [3]. Finally, our observation that epinecidin-1 is endowed with cytotoxic mechanisms, involving perturbation of cancer cell membranes, and inhibition of necrosis gene expression suggests that epinecidin-1 may have the potential to overcome multi-drug resistance activity by increasing the permeability of cancer cells, thus providing opportunities to develop new drugs for chemotherapeutic use.

Acknowledgments

This study was supported by a research grant from the Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2008.10.007.

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