Anticancer activity of a synthetic peptide derived from harmoniasin, an antibacterial peptide from the ladybug *Harmonia axyridis*

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Received March 28, 2013; Accepted May 13, 2013

DOI: 10.3892/ijo.2013.1973

Abstract. Harmoniasin is a defensin-like antimicrobial peptide identified from the ladybug *Harmonia axyridis*. Among the synthetic homodimer peptide analogues derived from harmoniasin, HaA4 has been found to have antibacterial activity without hemolytic activity. In this study, we investigated whether HaA4 has anticancer activity against human leukemia cell lines such as U937 and Jurkat cells. HaA4 manifested cytotoxicity and decreased the cell viability of U937 and Jurkat cells in MTS assay and LDH release assay. We found that HaA4 induced apoptotic and necrotic cell death of the leukemia cells using flow cytometric analysis, acridine orange/ethidium bromide staining and nucleosomal fragmentation of genomic DNA. Activation of caspase-7 and -9 and fragmentation of poly (ADP-ribose) polymerase was detected in the HaA4-treated leukemia cells, suggesting induction of a caspase-dependent apoptosis pathway by HaA4. Caspase-dependent apoptosis was further confirmed by reversal of the HaA4-induced viability reduction by treatment of Z-VAD-FMK, a pan-caspase inhibitor. In conclusion, HaA4 caused necrosis and caspase-dependent apoptosis in both U937 and Jurkat leukemia cells, which suggests potential utility of HaA4 as a cancer therapeutic agent.

Introduction

Living organisms are exposed daily to microbial infections and pathogens. In order to defend themselves against such infectious agents, they have developed potent defensive mechanism, i.e., innate and adaptive immunity. In innate immunity, antimicrobial peptides (AMPs) that possess potent antibiotic activity against bacteria, fungi and even certain viruses play important roles in the host defense mechanisms of most living organisms including plants, insects, amphibians and mammals (1-3).

Insect AMPs are cationic and amphipathic. Although insect AMPs display variable length, sequences and structures, most AMPs have relatively small (<5 kDa) molecular masses (4,5). In case of insect defensins, that was first isolated from the culture medium of an embryonic cell line of the flesh fly, *Sarcophaga peregrine* (6), are members of a widely distributed family of AMPs. Insect defensin contains six conserved cysteine residues engaged in three intradisulfide bonds (4,5) and have antimicrobial activity against Gram-positive bacteria and fungi (5,7).

Interestingly, several insect AMPs show cytotoxic effects against a broad range of cancer cell lines such as mouse myeloma, melanoma, lymphomas, leukemia, breast cancer and lung cancer (8-12). Coprisin belongs to the defensin family of insect AMPs, and has been identified from dung beetle, *Copris tripartitus* (13) and its analogue CopA3 showing cytotoxicity against cancer cell lines as well as strong antibacterial activity against microbes (14-16).

Previously, we characterized the antibacterial activity of the synthetic analogue of harmoniasin, HaA4 that was identified from the ladybug, *Harmonia axyridis*. Active region of harmoniasin was defined and selected to be modified as a homodimeric peptide. HaA4 displayed more potent antibacterial activity than that of the native peptide (17). HaA4 might also retain cytotoxic effect on cancer cells similarly to some other AMPs. Therefore, we investigated the anticancer activity of the HaA4 peptide against two human leukemia cell types in the present study and report that the anticancer effect of HaA4 is caused by necrosis and apoptosis.

Materials and methods

Peptide synthesis. Harmoniasin is a defensin-like peptide consisting of 50 amino acid residues with three intra-disulfide

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Key words: Harmonia axyridis, anticancer activity, necrosis, apoptosis, caspase

bonds. Because of the large molecular weight and disulfide bonds, we designed a variety of analogues based on the harmoniasin sequence in a previous study (17). The resulting homodimer peptide, named HaA4, was synthesized and provided by Anygen Co., Ltd. (Gwangju, Korea).

Cell culture. Raw 264.7, Jurkat and U937 cells were maintained in DMEM and RPMI-1640 medium containing 10% FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA), respectively. Cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

Cell viability assay. Cells were plated into 96-well tissue culture plates ($2x10^4$ cells/well) and treated with various concentrations (50, 100, 150 and 200 μg/ml) of HaA4 or without HaA4. After incubation for 24 h, viability of the cancer cells was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay according to the manufacturer's protocol (Promega, Madison, WI, USA). Optical density was measured at 490 nm with a microplate reader (Beckman DTX 8800 multi detector). Reversal of viability reduction by HaA4 was attempted by treatment with Z-VAD-FMK (Promega), a broad-spectrum caspase inhibitor at indicated concentration.

LDH release assay. Cell membrane integrity was analyzed by measuring LDH activity. LDH activity was measured using a Cytotoxicity Detection kit (Roche Applied Science). In brief, the cells were seeded at $1x10^4$ cells/well into a 96-well tissue culture plate in assay medium (RPMI-1640 containing 1% FBS). The cells were treated with different doses of HaA4. After 24 h of incubation, 5 μl of lysis solution was added to high control samples as a positive control and the plate incubated for an additional 15 min. Then, 100 μl reaction mixture was added to each well on the 96-well plate and incubated for 15 min. Finally, 50 μl stop solution was added to each well on the plate and the absorbance at 490 nm was measured using a microplate reader. The percent cytotoxicity was calculated by the following equation: Cytotoxicity (%) = (exp. value - low control)/(high control - low control) x 100.

Annexin V/propidium iodide (PI) staining. Jurkat and U937 cells were plated into 6-well tissue culture plates ($1x10^6$ cells/well) and treated with various concentrations (50, 100, 150 and 200 μg/ml) of HaA4 or without HaA4. After incubation for 4 h, cells were harvested and washed twice with cold PBS and once with 1X binding buffer (0.01 M HEPES/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM $CaCl_2$). Cells were prepared in 100 μl of the binding buffer ($1x10^5$ cells) and then, added with 5 μl of FITC Annexin V and PI. The cells were gently mixed by vortex and incubated for 15 min at room temperature in the dark. After the incubation, 400 μl of 1X binding buffer was added to each tube. Stained cells were measured by flow cytometry with a BD FACSCalibur cytometer (BD Biosciences) and CellQuest software (BD Biosciences) was used for analysis of the results.

Acridine orange/ethidium bromide staining. Cells were seeded in 6-well tissue culture plates ($1x10^6$ cells/well), treated without or with HaA4 (50, 100 and 150 μg/ml) for 24 h and the cells were washed with PBS. Then, the cells were stained with mixture of acridine orange (3 μg/ml) and ethidium bromide (10 μg/ml) and observed immediately using AxioImager Z1 fluorescence microscope (Carl Zeiss, Germany).

Table I. Sequence of harmoniasin analogues.

Peptide	Amino acid sequence	Mass (Da)	
		Measured	Theoretical
HaNP	IGGYCSELDL-NH_2 | IGGYCSELDL-NH_2	2134.8	2134.4
HaA4	IGGYCS<u>W</u>L<u>R</u>L-NH_2 | IGGYCS<u>W</u>L<u>R</u>L-NH_2	2330.2	2330.8

Vertical bar represents a disulfide linkage between cysteine residues in the sequence. Substituted residues are underlined.

DNA fragmentation assay. For the DNA fragmentation assay, $2x10^6$ cells were seeded into 6-well plates and treated with 200 μg/ml HaA4 or without HaA4 for 24 h. Cells were collected, washed once with PBS, lysed in a solution containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0) and 0.5% Triton X-100 on ice for 30 min and then centrifuged at 15,000 rpm for 5 min. The supernatant was digested with 0.1 mg of RNase A/ml and 1 mg of proteinase K/ml for 1 h at 55˚C in the presence of 1% sodium dodecyl sulfate (SDS). DNA was extracted from the digested supernatant with phenol and chloroform, precipitated in cold ethanol and subjected to electrophoresis on 2% agarose gels containing ethidium bromide. DNA fragments were visualized by UV light.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL assay). Jurkat and U937 cells were plated into 6-well plates ($2x10^6$/ml) and treated with or without HaA4 (200 μg/ml) for 24 h. TUNEL assay was performed with DeadEnd™ Fluorometric TUNEL system according to the manufacturer's instructions (Promega) to determine apoptotic cells.

Immunoblot analysis. Cells were washed with cold PBS and lysed in buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA and 1% Nonidet P-40]. Equal amount of protein was separated by SDS-polyacrylamide gel electrophoresis (12% SDS-PAGE) and transferred onto a nitrocellulose membrane. The antigen-antibody complexes were detected using FluorChem (Alpha Innotech, USA). Polyclonal antibodies against caspase-7, -9, PARP and AIF were obtained from Cell Signaling Technology (MA, USA). The β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the broad-spectrum caspase inhibitor, Z-VAD-FMK, was obtained from Promega.

Results and Discussion

The peptide. The primary amino acid sequence of the synthetic harmoniasin analogues are shown in Table I. HaA4 peptide was used in this study. Dimerized peptides by disulfide bond

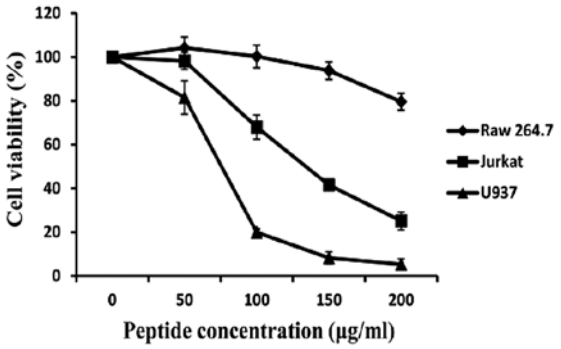

Figure 1. Cell viability of the human leukemic cell lines after HaA4 treatment. Cells were treated with different concentrations (0, 50, 100, 150 and 200 μg/ml) of HaA4 for 24 h and cell viability was measured by the MTS assay. Error bars represent the standard deviation derived from three independent experiments.

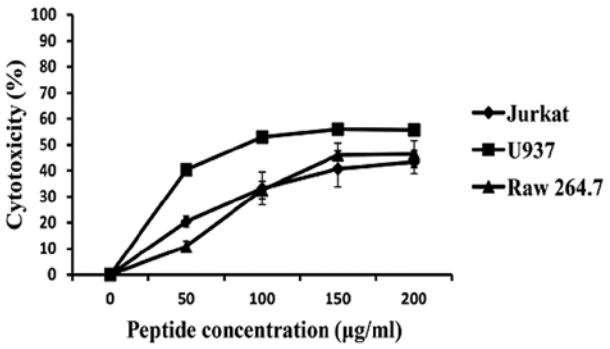

Figure 2. HaA4 causes cell membrane disruption. Cells were incubated with HaA4 for 24 h and treated samples assessed by LDH release assay. The results are expressed as the means ± SD from three individual measurements.

such as magainin 2 and melittin analogues showed stronger antimicrobial activity than the monomeric forms (18,19). Moreover, halocidin dimer congeners derived from halocidin, a dimeric α-helical structure peptide that was purified from the tunicate *Halocynthia aurantium* showed more potent antibacterial activity than the its monomer forms (20). Therefore, dimerization of the AMPs is suggested to potentiate their biological activity in an undefined way.

HaA4 markedly decreases cell viability of leukemia cell lines. Recently, we showed that synthetic HaA4 exerts antibacterial effect without hemolytic activities (17). We attempted to determine the effect of the synthetic peptide HaA4 on cell growth and survival of human leukemia cells (Jurkat and U937) in this study. Cancer cells were treated with various concentrations (50, 100, 150 and 200 μg/ml) of HaA4 for 24 h and the cell viability was measured by MTS assay. As shown in Fig. 1, HaA4 decreased the viability of the leukemia cells in a dose-dependent manner. In particular, viability of the cells precipitated by >70% at 200 μg/ml of HaA4, while >70% of Raw 264.7 cells remained viable. Therefore, our results suggest that HaA4 should exert a potent anticancer activity against human leukemia cells.

Effect of HaA4 on the integrity of cancer cell membrane. We attempted to characterize the effects of HaA4 on the integrity of cancer cell membranes by detecting the LDH activity. As shown in Fig. 2, the amount of LDH release increased in a dose-dependent manner in both cancer cell types and the percentage of cytotoxicity appeared to reach plateau with the elevation of the peptide concentration. Although level of LDH release from both was similar, LDH release from U937 was a little higher than that from Jurkat. Maximal cytotoxicity at 200 μg/ml HaA4 was 43.3 and 55.7% for Jurkat and U937 cells, respectively. However, HaA4 showed cytotoxic activity against Raw 264.7 cells and the LDH release was similar to Jurkat cells. Base on the results, we surmised that lytic activity of HaA4 is influenced by the presence of serum. Although HaA4 had no hemolytic activity in our previous report (17), cell selectivity of HaA4 including serum stability needs to be examined further. Finally, when compared to the results from the MTS assay, the reduction in cell viability was 30-40% higher than expected from cytotoxicity. The observed discrepancy suggested that additional factors including apoptosis and growth inhibition as well as necrosis could play a critical role in the viability reduction by HaA4.

HaA4 induces apoptosis and necrosis in leukemia cells. In order to further characterize mechanism of the viability reduction, we assessed the involvement of apoptosis. Apoptosis (programmed cell death) is a pivotal physiological process that is required for the normal development and maintenance of tissue homeostasis in multicellular organisms (21). During apoptosis, certain morphological characteristics are involved, such as membrane blebbing, phosphatidyl inositol exposure, nuclear and cytoplasmic shrinkages, chromatic condensation and DNA fragmentation (22). Apoptosis was examined by Annexin V/PI staining of the HaA4-treated leukemic cells. Annexin V binding to the HaA4-treated leukemia cells was gradually increased as the peptide concentration elevated. Annexin V-positive cell population reached maximum at 150 μg/ml HaA4, while Annexin V/PI-positive at 200 μg/ml HaA4 (Fig. 3). These results indicated that HaA4 should induce both apoptosis and necrosis depending on the concentration of HaA4. Necrosis appeared prevailing over apoptosis at higher concentration of HaA4.

Previously it has been reported that piscidin-1, a cationic peptide isolated from the mast cells of hybrid striped bass (23), also causes apoptosis and necrosis at a low concentration and necrotic effect at a high concentration for a short period in HT1080 cells (24). Piscidin-1 has a net charge of +3 and HaA4 has a net charge of +2 at pH 7.0, which might function in the anticancer activity. Net charge of a peptide is an important parameter for antitumor activity (25). Thus, it is supposed that the positively charged cationic peptide could interact with anionic cancer cell membrane electrostatically and damage the membrane integrity.

Acridine orange/ethidium bromide staining. To verify Annexin V/PI assay results, HaA4-treated Jurkat cells were stained with acridine orange/ethidium bromide. After HaA4 treatment for 24 h, majority of cells exhibited green fluorescence in control, while diffused or orange-colored nuclei were increased in HaA4-treated cells with increase of HaA4 concen-

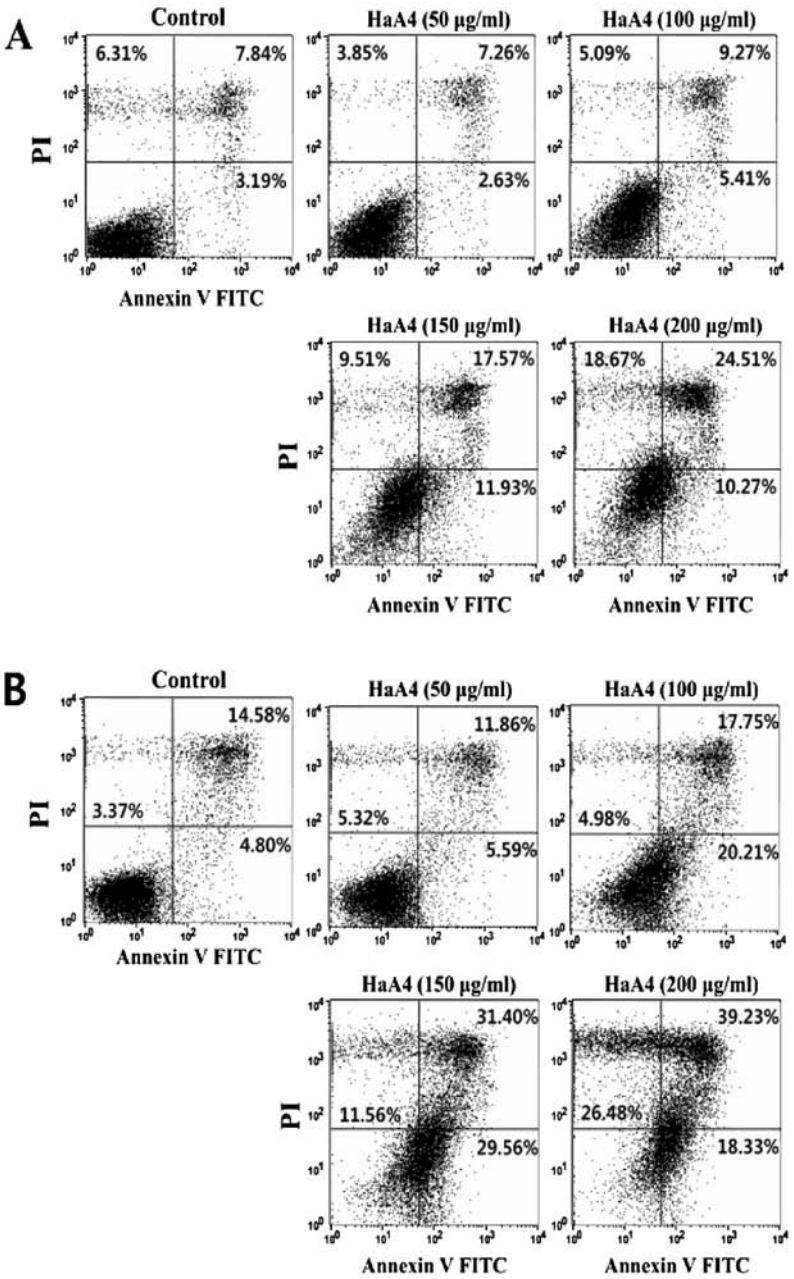

Figure 3. HaA4 induces apoptotic and necrotic cell death of the human leukemic cells. Cancer cells were treated with various concentration of HaA4, stained with FITC-conjugated Annexin V/propidium iodide and subjected to flow cytometry. The lower left part represents viable cells and the lower right part represents early apoptotic cells and the upper left part represents necrotic cells and the upper right part represents secondary necrotic and late apoptotic cells. (A) Jurkat cells. (B) U937 cells.

tration. The cells treated with 150 μg/ml HaA4 developed orange and orange-red fluorescence, indicating membrane disruption (Fig. 4). U937 cells presented similar results (data not shown). These results support that HaA4 could induce both apoptosis and necrosis at high concentrations.

HaA4-induced DNA fragmentation. In order to further determine whether apoptosis is involved in the viability reduction of the leukemia cells, we performed TUNEL assay and agarose gel electrophoresis for chromosomal DNA after treating these cells with 200 μg/ml of HaA4 for 24 h. As shown in Fig. 5A, the number of TUNEL-positive apoptotic cells was significantly increased in Jurkat and U937 cells treated with HaA4 when compared with the untreated cells. In agreement with the results, the chromosomal DNA of Jurkat and U937 cells was fragmented in nucleosomal ladder by HaA4 (Fig. 5B). Based on these results, we assured that such pro-apoptotic effects of HaA4 should contribute to the viability reduction of the leukemia cells.

HaA4 induces apoptosis in the leukemia cells via a caspase-dependent pathway. Since apoptosis can proceed via either caspase-dependent or -independent signaling pathways (26,27), the involvement of caspases in HaA4-induced leukemia cell apoptosis was assessed. As shown in Fig. 6, a marked increase in the cleavage of caspase-7, -9 and PARP was observed. Subsequently, the potential role of apoptosis inducing factor (AIF), a caspase-independent apoptosis regu-

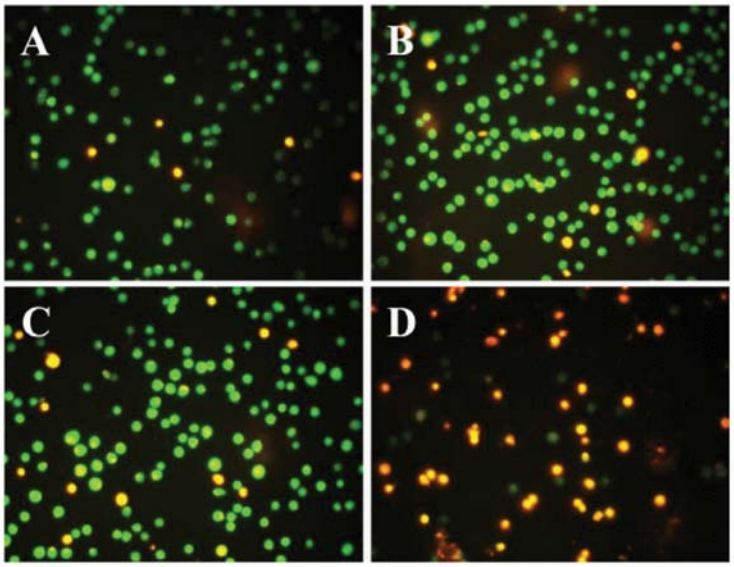

Figure 4. Jurkat cells were stained with acridine orange/ethidium bromide after incubation with HaA4. Cells were observed under fluorescence microscope (x400). Viable cells show green fluorescence. Necrotic and apoptotic cells show orange and yellow fluorescence. (A) No treatment control. (B) Cells were treated with 50 μg/ml HaA4. (C) Cells were treated with 100 μg/ml HaA4. (D) Cells were treated with 150 μg/ml HaA4.

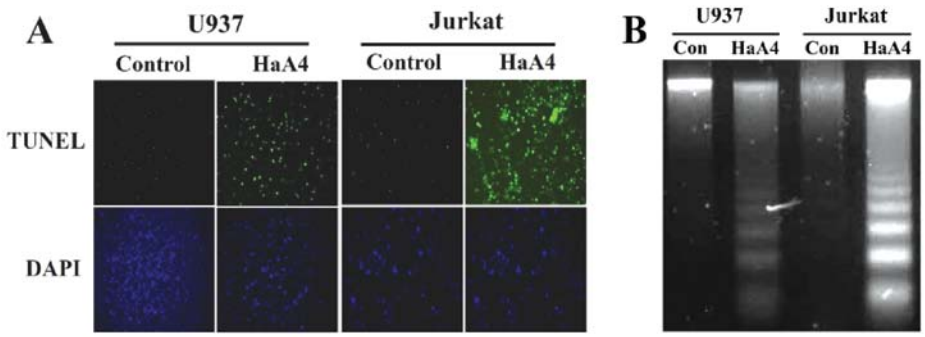

Figure 5. TUNEL assay (A) and DNA fragmentation analysis (B) were carried out to demonstrate apoptotic cancer cells following treatment with 200 μg/ml of HaA4 for 24 h. The fluorescence microscopic images were taken at x400 magnification.

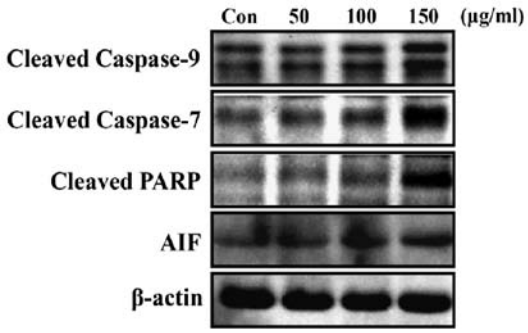

Figure 6. HaA4-induced apoptosis is associated with a caspase-dependent pathway. Jurkat cells were exposed to medium (con) or HaA4 (50, 100 and 150 μg/ml) for 24 h. Caspase-7 and -9 were activated and PARP was cleaved by HaA4 in Jurkat cells.

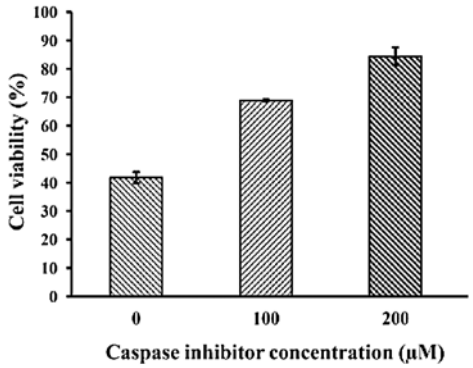

Figure 7. Jurkat cells were treated with Z-VAD-FMK (0, 100 and 200 μM) in HaA4 (150 μg/ml). Cell viability recovered depending on the concentration of Z-VAD-FMK. The bars represent the mean ± SEM of 3 independent experiments performed in triplicate.

lator on HaA4-induced apoptosis was investigated. However, we could not observe converted mature form of AIF (Fig. 6). These results suggest that HaA4-mediated leukemia cell apoptosis might be associated with the activation of caspase. Moreover, the decreased cell viability by HaA4 treatment in the MTS assay recovered in the presence of Z-VAD-FMK, a pan-caspase inhibitor (Fig. 7), demonstrating that HaA4-

induced leukemia cell apoptosis is dependent on the activation of the caspase family proteins.

Most antibacterial and anticancer peptides employ cell membrane disruption by lytic activity, or some peptides employ apoptosis in cancer cells through mitochondrial damage. It is believed that the mode of action originates from electrostatic interaction between cationic peptides and anionic cell wall components of bacterial and cancer cells. To date, there are four possible different models (toroidal, carpet, barrel-stave and aggregate channel) of AMP action mechanisms for membrane permeabilization (28). In previous studies, α-helical peptides were shown to need more than 20 amino acid residues to span the entire thickness of the eukaryotic cell membranes for the barrel-stave mechanism (29,30). Thus, the relatively small size of HaA4 along with aurein 1.2 (31) and citropin 1.1 (32), isolated from frogs suggest that these AMPs mediate their membranolytic effect through the carpet mechanism (33). In addition, it has been reported that bovine lactoferricin binds to the cell membrane and causes cell membrane disruption followed by entry of the peptide to the cytoplasm of Jurkat T-leukemia cells and damage to mitochondrial membrane (34). Based on the results of our previous study (17), it was postulated that HaA4 acts on anticancer activity similar to bovine lactoferricin, although the exact mechanism of HaA4 has to be elucidated.

In this report, we have shown that HaA4 is a good candidate for a new anticancer therapeutic agent as described above. As a consequence, we could identify necrotic effects of HaA4 via LDH activity detection (Fig. 2) and Annexin V and PI staining (Fig. 3) and we also observed that HaA4 indicates apoptotic effects. Additionally, apoptosis of the leukemic cells by HaA4 was dependent on the activation of caspase (Figs. 6 and 7), a regulator of a caspase-dependent pathway. Overall, our present study revealed that HaA4 should retain anticancer activity against human leukemia cells (Jurkat and U937) and the activity might ascribe to necrosis and apoptosis of the leukemia cells.

Acknowledgements

This study was supported by a grant from the Next-Generation BioGreen 21 Program (no. PJ008158) and partially supported by a grant (no. PJ008706) from the Agenda Program, Rural Development Administration, Republic of Korea.

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