Original Article

Antitumor effect of the antimicrobial peptide GLI13-8 derived from domain of the avian β-defensin-4

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We previously reported that GLI13-8, one of cationic antimicrobial peptides from linear avian β-defensin-4 (RL38) analogs, exhibited high antimicrobial activities against both Gram-negative and Gram-positive bacteria. In the present study, we reported the in vitro cytotoxicity of GLI13-8 using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Results showed that the cytotoxicity of GLI13-8 in three human carcinoma cells (HepG2, SGC7901, and A375) was in a dose-dependent manner. When the concentration of GLI13-8 is <128 μM, it had no toxicity towards the normal human fibroblasts (MRC-5). The Annexin-V-FITC/PI staining assay, the Hoechst 33258/PI staining assay, the permeability of fluorescent macromolecules and scanning electron microscope assays, mitochondrial membrane potential assay, caspases-3 and poly ADP-ribose polymerase (PARP) assays have been carried out. Results indicated that apoptosis was induced by GLI13-8 in HepG2 cells, and demonstrated that GLI13-8 induced loss of mitochondrial membrane potential, disruption of HepG2 cell membranes, and activation of caspase-3 and PARP. These findings suggested that GLI13-8 may be an effective agent for HepG2 cells.

Keywords antimicrobial peptide; AvBD-4 derivative; carcinoma; apoptosis

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Introduction

Traditional anticancer drugs can kill both cancer cells and normal cells due to their inability to distinguish between them. Moreover, cancer cells develop resistance to these drugs mediated by the overexpression of multidrug-resistance proteins that pump the drugs out of the cells and thus render the drugs ineffective [1]. Due to these limitations, more attention has been paid to finding alternatives to traditional cancer drugs in recent years. Antimicrobial peptides (AMPs), also known as genetically encoded antibiotic peptides, represent essential components of the innate immune system [1,2]. AMPs showed various activities against bacteria, fungi, enveloped viruses, protozoa, and some human tumor cells [2–5]. Some AMPs have already entered pre-clinical and clinical trials to treat wound healing, cystic fibrosis, acnel, catheter-site infections, and patients undergoing stem cell transplantation [6–8]. For example, magainin peptides have been shown to improve the survival of animals with ascites-producing tumors in vivo [9]. With increasing resistance to conventional chemotherapy in tumors, cationic AMPs, as novel anticancer agents, have potential desirable features.

A few mechanisms of AMP-mediated cell death have been proposed. One is that AMPs affect cell membrane’s net charge, owing to the peptides’ net positive charge and amphipathic α-helical structure [3,10–12]. The other is that AMPs may kill cancer cells via membrane permeation by breaking the cell membrane into peptide-coated vesicles or forming transient transmembrane pores. The disruption may lead to the permeation and swelling of mitochondria, the release of cytochrome c, and the induction of apoptosis [13–15]. In addition, apart from direct membrane damage, AMPs may activate intracellular signaling pathways and molecules such as the Fas/Fas-L and tumor necrosis factor (TNF)/TNF receptor (TNFR) system, p53, ERK1/2, caspase-7, Bax, and Bcl-2, which mediate apoptosis in cancer cells [16–18]. Therefore, the precise details of the antitumor mechanism of AMPs’ action still remain unknown.

Avian defensins are cationic AMPs that provide the first line of defense against potential pathogens in the chicken innate immune system [19]. Avian β-defensin (gallinacin) belongs to a family of cationic AMPs, and it was originally isolated from chicken neutrophilic granulocytes [20]. We previously found that avian β-defensin-4 (AvBD-4) induced hemolysis of human red blood cells [21]. However, GLI13-8, the 13-mer peptide (RLIRRKRRIIRWL-NH₂) derived from AvBD-4, was found to preferentially permeabilize bacterial membranes rich in anionic phospholipids and exhibit high cell selectivity between bacteria and mammalian cells [21].
In this study, we compared the cytotoxic activities of a synthesized peptide of GLI13-8 against three human carcinoma cell lines (human hepatocellular carcinoma HepG2, human gastric carcinoma SGC7901, and human melanoma A375) and human embryonic lung fibroblasts (MRC-5). In addition, Annexin-V-fluorescein isothiocyanate (Annexin-V-FITC)/propidium iodide (PI) staining, the permeability determination of fluorescein macromolecules, and scanning electron microscopy assays were performed to elucidate the peptide–membrane interactions. Hoechst 33258/PI staining, mitochondrial membrane potential, caspases-3, and poly ADP-ribose polymerase (PARP) assays were further carried out to explore the antitumor mechanism of the peptide GLI13-8. These results indicated that apoptosis was induced by GLI13-8 in HepG2 cells.

Materials and Methods

Peptide synthesis

The peptide was synthesized at GL Biochem Corporation (Shanghai, China). The peptide was amidated at the C-terminus and the purity was over 95% as determined by reverse-phase high-performance liquid chromatography. Electrospray mass spectrometry was used to characterize the peptide.

Cell culture and treatment

Three human cancer cell lines (human hepatocellular carcinoma HepG2, human gastric carcinoma SGC7901, and human melanoma A375) and normal human embryonic lung fibroblasts (MRC-5) were obtained from Cell Bank of Shanghai, Shanghai Institutes for Biological Sciences (Shanghai, China). Cells were cultured in RPMI-1640 or Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco, Gaithersburg, USA) in a humidified incubator with 5% CO2 at 37°C. Cells were washed three times with binding buffer (10 mM HEPES, pH 7.4 NaOH, 140 mM NaCl, and 2.5 mM CaCl2) by centrifugation at 2000 rpm for 5 min at 4°C. Cells were then suspended in 200 μl of binding buffer, and 10 μl of Annexin-V-FITC was added into the solutions. After incubation at 4°C for 30 min in the dark, an additional 300 μl of binding buffer was added. Finally, 5 μl of PI was added 5 min prior to analysis by the flow cytometer (Becton–Dickinson, San Jose, USA). The experiment was performed in triplicate.

Analysis of cell viability

Cell viability was measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (5 × 10^3 per well) were cultured in a 96-well plate for 24 h before peptide treatment. Cells were treated with various concentrations of peptide for 3, 24, and 48 h. The control cells were treated in the same way without the peptide. Then 30 μl of 5 mg/ml MTT solution was added to each well, and the plate was incubated at 37°C for 4 h. Then, 150 μl of dimethyl sulfoxide was added into each well and incubated for 10 min to dissolve the formazan precipitate. The absorbance was measured at 492 nm using a microplate reader (Tecan GENios F129004; Tecan, Männedorf, Swiss). The absorbance of the control group was considered 100% cell survival, and the experiment was performed in triplicate.

Annexin-V-FITC/PI double staining

Apoptosis of HepG2 cells was determined using the Annexin V-FITC/PI apoptosis detection kit (Biosea Biochemicals, Beijing, China). Briefly, after peptide treatment, HepG2 cells were washed three times with binding buffer (10 mM HEPES, pH 7.4 NaOH, 140 mM NaCl, and 2.5 mM CaCl2) by centrifugation at 2000 rpm for 5 min at 4°C. Cells were then suspended in 200 μl of binding buffer, and 10 μl of Annexin-V-FITC was added into the solutions. After incubation at 4°C for 30 min in the dark, an additional 300 μl of binding buffer was added. Finally, 5 μl of PI was added 5 min prior to analysis by the flow cytometer (Becton–Dickinson, San Jose, USA). The experiment was performed in triplicate.

Hoechst 33258/PI double staining

Analysis of nuclear morphology was performed using a Hoechst 33258/PI double staining assay. The peptide GLI13-8-treated cells were fixed with a 50% solution of fixative (3 : 1 methanol: acetic acid) for 10 min. Fixed cells were then stained with Hoechst 33258 at 1 μg/ml in the dark for 10 min at room temperature, and then stained with PI at 1 μg/ml in the dark for 20 min at 4°C. Then, the cells were washed three times with phosphate-buffered saline (PBS) and the nuclear morphology was analyzed using a laser scanning confocal microscope (TCS, SPI; Leica, Wetzlar, Germany).

Analysis of cell membrane permeability

HepG2 cells were treated with GLI13-8 for 3 and 24 h. The controls were treated without GLI13-8. Then, cells were incubated with 50 μM of Dextran-FITC in the dark for 30 min at 37°C. Cells were washed three times with PBS and photographed using a laser scanning confocal microscope.

Scanning electron microscopy

HepG2 cell membranes were detected using a scanning electron microscope (SEM) (5-3400N; Hitachi, Tokyo, Japan) as previously reported [22]. At least 3 × 10^4 cells were cultured on 1/4 sterilized coverslip and incubated overnight. The final concentrations of GLI13-8 were added the next day, and the cultures were incubated for 24 h. The medium was then removed, and 1 ml of 4% glutaral solution was added to each well and incubated for 1.5 h. Cells were then washed in PBS (PH 7.2) three times, dehydrated in ethanol, and dried in a critical point dryer (ES-2030; Hitachi). Cells on coverslips were coated with gold and analyzed using SEM.
Mitochondrial membrane potential assay
The loss of mitochondrial membrane potential was detected with the cationic dye JC-1 (5 μg/ml; Beyotime, Haimen, China) [23]. Briefly, HepG2 cells (1 × 10⁶ cells per well) were incubated with 5 μg/ml of JC-1 staining solution at 37°C for 20 min and washed twice with PBS. Mitochondrial depolarization was indicated by an increase in the green/red fluorescence intensity ratio using a laser scanning confocal microscope and the flow cytometer. The experiment was performed in triplicate.

Western blot analysis
HepG2 cells were harvested and lysed in 1 ml of lysis buffer. Total protein was loaded onto 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), separated by electrophoresis, and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, USA). The membrane was blocked with 5% non-fat milk and 1% polyvinylpyrrolidone in PBS for 30 min and then incubated with antibodies to caspase-8 and PARP (BioLegend, San Diego, USA). After being washed with PBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG) for 2 h, and observed in enhanced chemiluminescence method (Bio-Rad, Hercules, USA). Gray values were determined by a gel image analysis system (Bio-Rad) normalized with the gray values of β-actin. The experiment was performed in triplicate.

Statistical analysis
All data were presented as the mean ± standard deviation. Groups were compared using an unpaired t-test. All statistical analyses were conducted using the SPSS 16.0 (SPSS Inc. Chicago, USA) software. P < 0.05 was considered as statistically significant.

Results

Effect of GLI13-8 on viability of tumor cells and MRC-5 cells in vitro
Cell viability was quantified by the MTT assay. At the concentration of 0 μM, the cell survival rate of GLI13-8 was nearly 100% (data not shown). The results showed a consistent inhibition on the growth of tested tumor cell lines after treatment with 64 μM of GLI13-8.

In contrast to the selective cytotoxic activity with the treatment of 128 μM GLI13-8 in each cell line that was considered as the threshold concentration, it was observed that 128 μM GLI13-8 produced an inhibition ratio of <20% in normal cells (MRC-5), while it produced an inhibition ratio of >80% in test cancer cells except for SGC7901 cells [Fig. 1(A)]. As shown in Fig. 1(B), HepG2 cells were more sensitive to GLI13-8 than other tested cell lines.

GLI13-8 treatment for 3 h showed a potent antitumor activity, which was comparable with the activity achieved with 24 and 48 h of incubation, as indicated by the similar curve shapes [Fig. 1(A)]. These results indicated that GLI13-8 exerted its antitumor activity in a rapid and non-time-dependent manner. We proposed that GLI13-8 had no effect on the tested cells at the concentration of 8 μM, and it achieved about 50% cell inhibition at the concentration of 64 μM (except the MRC-5 cells). The peptide GLI13-8 had a greater toxicity to the tested cells (except MRC-5 cells) at the concentration of 128 μM, and the cell survival rate of GLI13-8 was nearly 0% at the highest concentration of 256 μM. Therefore, the concentrations of 8, 64, and 128 μM were used in the following experiments on HepG2 cells.

Apoptosis determination by an Annexin V-FITC/PI assay
Phosphatidylserine (PS) shifts from the inner layer of the plasma membrane to the outer layer in the early stage of apoptosis. Annexin V, a calcium dependent, phospholipid-binding protein, binds to PS with high affinity, and the binding is a marker of cell apoptosis. The Q₁, Q₂, Q₃, and Q₄ gates, respectively, represented dead cells, the late stage of cell apoptosis, normal cells, and the early stage of cell apoptosis. Figure 2 showed that no apoptotic cells were detected in the control and 8 μM treated groups, and HepG2 cells treated with 64 or 128 μM of GLI13-8 had an increase in the percentage of apoptotic cells. The number of apoptotic cells increased significantly with 64 μM at the early stage of cell apoptosis (Fig. 2).

Morphological characterization of HepG2 cell apoptosis determined by Hoechst 33258/PI double staining
Apoptosis was further analyzed by examining the nuclear morphology of GLI13-8-treated HepG2 cells. Hoechst 33258, a fluorescence dye related to membrane permeability, was used to examine apoptotic cells. PI was used to examine the late stages of cell apoptosis and dead cells. Figure 3 showed representative Hoechst 33258/PI fluorescence photomicrographs of cultured HepG2 cells treated with or without GLI13-8, respectively. In the control and the 8 μM treated groups, the majority of nuclei were only stained blue with Hoechst 33258, and appeared with regular particles in the nuclei. In contrast, most nuclei of 64 μM GLI13-8-treated HepG2 cells were smaller, and condensed chromatin and light blue were observed. Most nuclei were stained by both dyes at the 128 μM concentration, suggesting that HepG2 cells were in the late apoptosis or death.
The permeability of GLI13-8 to fluorescein macromolecules

After HepG2 cells were treated with GLI13-8 for 3 h, the fluorescent macromolecular probe Dextran-FITC was individually distributed on the membranes under a laser scanning confocal microscope (Fig. 4). However, Dextran-FITC entered the cells and formed bright blocks in HepG2 cells treated with GLI13-8 for 24 h. This result indicated that the HepG2 membranes were not permeable to the macromolecules after a short incubation with GLI13-8. However, after a longer incubation, the membranes were obviously damaged and allowed the 40 kDa macromolecules through the cell membranes.

Effect of GLI13-8 on the cell membranes of HepG2 cells

The pores in the HepG2 cell membranes after treatment with GLI13-8 were further examined by SEM. In the untreated HepG2 cells and the 8 μM treated groups, the microvillus on HepG2 cell surfaces was long and thin. In contrast, HepG2 cells treated with 64 and 128 μM GLI13-8 revealed a disrupted cell membrane likely to represent pore formation on the cell membranes. Additionally, the microvillus on HepG2 cell surfaces disappeared at the concentrations of 64 and 128 μM (Fig. 5).

Effect of GLI13-8 on mitochondrial membrane potential

The loss of mitochondrial membrane potential is a critical step of cell apoptosis. JC-1 accumulates as J-aggregates in the mitochondria (red) in non-apoptotic cells and exists as a monomer (green) in the cytosol in apoptotic cells. Figure 6 was representative JC-1 stains of apoptotic and non-apoptotic HepG2 cells. In the negative control (0 μM) and 8 μM of the GLI13-8 groups, most HepG2 cells showed red fluorescence, indicating that they were live cells. However, we observed a significant change in JC-1 staining at 64 and 128 μM for 24 h, suggesting that GLI13-8-induced HepG2 cell apoptosis. Carbonylcyanidem-chlorophenylhydrazone-
induced mitochondrial membrane depolarization was shown as the positive control.

Caspase-3 and PARP protein expression
We measured caspase-3 and PARP activity in HepG2 cells that had been treated with GLI13-8 [Fig. 7(A)]. The expression levels of cleaved caspase-3 reflected the activity of caspase-3 and cell apoptosis situation. PARP, as a substrate of caspase-3, was a key mediator of apoptosis. Western blot analysis showed that cleavage of caspase-3 and PARP appeared and caspase-3 (p17) and PARP (p89) protein expression levels were increased after cells were treated with GLI13-8 for 24 h [Fig. 7(B)]. These results suggested that GLI13-8 caused cell apoptosis by the mitochondrial pathway, through the activation of procaspase-3 and PARP.

Discussion
We previously demonstrated that the GLI13-8 peptide derived from the linear AvBD-4 (RL38) exerted antimicrobial activities against Gram-positive and Gram-negative bacteria and showed a low hemolytic activity towards human erythrocytes [21]. In this study, it was shown that the peptide GLI13-8 rapidly initiated cytotoxicity and significantly inhibited the viability of three tumor cell lines, especially HepG2 cells in a dose-dependent manner. However, the viability of MRC-5 cells was unaffected by GLI13-8 treatment at concentrations < 128 μM. These results showed the cell selectivity of GLI13-8 to a certain extent, which may be related to the different interactions of GLI13-8 with tumor and normal cell membranes [15,22,24]. Net negative charges are located in the outer leaflet of the cancer cell membranes [25]. Additionally, O-glycosylated mucin, a type of glycoprotein, exists in the cancer cell membranes and can increase negative charges on the cancer cell surfaces [26]. However, normal mammalian cell membranes are mainly composed of
neutral zwitterionic phospholipids and sterols [15]. Cationic AMPs typically contain net positive charges, and anionic cancer cell membranes are more susceptible to the lytic action of cationic AMPs. The net positive charge and hydrophobicity, which have the ability to adopt an amphipathic conformation, are critical structural parameters for antitumor activity according to the previous reports [27,28]. Our data showed that GLI13-8 had selective activity between the tested human carcinoma cells and the normal human fibroblasts, which may also be correlated to its high net positive charges (+8).

The mechanism by which host defense peptides kill cancer cells is poorly understood in most cases. It is currently known that apoptosis is mediated by two major pathways [29–31]. One pathway is the disruption of the surface membrane of tumor cells, which induces cell apoptosis/death. The other one is that subgroups of AMPs are able to cause disruption of mitochondrial membranes, which subsequently leads to the activation of apoptosis pathways. In this study, we demonstrated (i) biochemical marker (exposure of PS on the cell membrane) by Annexin-V-FITC/PI double staining; (ii) cell membrane permeability by Dextran-FITC labeling, and (iii) cell membrane disruption by SEM. SEM was used to directly observe the morphological effects of GLI13-8 on HepG2 cells. Results showed that the pore formation of HepG2 cell membranes represented the membrane disruption (Fig. 5). Previous studies reported that most AMPs, including magainin II and defensins, induced pore formation in cellular membranes [22,25,32]. We previously reported that GLI13-8 contained high net positive charges [21]. The positive charges interact electrostatically with the negative charges on the cancer cell surface and the amphipathicity favors the peptide to insert into the membrane. Therefore, the peptide GLI13-8–HepG2 cells interaction was initiated by electrostatic interactions, which may be essential for the peptide to closely interact and integrate into HepG2 cell membrane interface, thereby leading to disrupt HepG2 cell membranes.

Moreover, in the present study, we investigated the effect of GLI13-8 on the mitochondrial membrane potential. Green fluorescence is indicative of the mitochondrial membrane potential decline by JC-1 staining [33]. The loss of mitochondrial membrane potential is an important step in the induction of cell apoptosis. In this study, the percentage of HepG2 cells with green fluorescence increased in GLI13-8-treated groups. Results showed that mitochondrial membrane potential was disrupted by GLI13-8-induced apoptosis in HepG2 cells.
Caspase activation is a common pathway to induce cell apoptosis in many systems. Caspase-dependent apoptosis involves the mitochondrial pathway, death receptor pathway, and endoplasmic reticulum pathway [34]. Caspase-3 plays a very important role in the cancer cell apoptosis process [35]. In this study, it was shown that the activation of caspase-3 led to increased cleavage of PARP, indicating that the caspase-3-dependent pathway plays a major role in
GLI13-8-induced apoptosis in HepG2 cells. Mitochondria-dependent apoptosis in the presence of GLI13-8 was further confirmed by western blot analysis (Fig. 7).

In summary, we found that GLI13-8 showed selective cytotoxicity between the tested tumor cells and normal cells when its concentration is <128 μM. The mechanism of GLI13-8 activities may be dependent on the cell apoptosis according to the assays shown in Figs. 3, 6, and 7.

We also demonstrated that mitochondria and caspase-3 play critical roles in GLI13-8-induced apoptosis in HepG2 cells. The peptide GLI13-8 may kill HepG2 cells through the disruption of the cell membrane by forming transmembrane pores and the induction of apoptosis in HepG2 cells probably through triggering mitochondria-dependent pathway.

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References