Novel cationic antimicrobial peptide GW-H1 induced caspase-dependent apoptosis of hepatocellular carcinoma cell lines

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ABSTRACT

Due to its malignancy, the development of effective therapeutic strategies for hepatocellular carcinoma (HCC) is of urgent needs. Natural antimicrobial peptides (AMPs), also known as host defense peptides (HDPs), not only act as direct antimicrobial agents, but also represent important regulators of the innate immune system. It has been reported that cationic AMPs may exhibit cancer-selective toxicity. We have designed a series of novel AMPs with potent antimicrobial activity against a broad spectrum of bacterial pathogens. In the current study, we evaluate the antitumor potency of these AMPs toward HCC cell lines J5, Huh7, and Hep3B. Selected AMPs inhibit the viability of HCC cells in a dose-dependent fashion, while the normal 3T3 cells were significantly less susceptible to these AMPs. GW-H1 treatment (20 μM) of J5 cells for 24–72 h resulted in the induction of apoptosis, as revealed by flow cytometry (increased sub-G1 populations), and western blot analysis for the appearance of activated caspase-3, -7, and -9 cleavages. Two-dimensional gel electrophoresis was applied to further analyze the AMP-responsive protein profiles of HCC, down-regulation of Hsp27, phosphoglycerate kinase 1 and triosephosphate isomerase indicated that GW-H1 may induce apoptosis, and further inhibit progression and metastasis of J5 HCC cells. FITC-labeled GW-H1 was found to attach to cell membrane initially, then translocated into the cytoplasm, and eventually membranous organelles or nucleus. GW-H1 induced a marked growth suppression of J5 xenografts in nude mice in a dose dependent manner. These findings provided support for future application of GW-H1 as potential therapeutic agent for HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is a prevalent cancer throughout the world. In general, the main curative therapies for cancers are surgery and radiation, which generally are only successful if the cancer is diagnosed at an early stage. The conventional chemotherapies used to treat advanced tumors, although quite effective, also have adverse effects that limit a patient’s tolerance to the chemotherapeutic drug. Therefore, new therapeutic options are necessary.

Many types of cancer cells and microorganisms have relatively more anionic phospholipids in the outer layer of their external membrane compared with normal eukaryotic cells. Several studies have demonstrated that certain antimicrobial peptides (AMPs) are cytotoxic against transformed cells but are less cytotoxic against non-transformed cells [3,5,11,14,21,33,37–39]. Certain AMPs, when administered locally to solid tumors, exhibit anticancer activity [3,4]. Recently, several AMPs from fish were shown to possess antitumor activities against certain cancer cells [4,6,18–20,26–28]. Additionally, a cytotoxic peptide that was resistant to enzymatic degradation inhibited metastasis of a mouse primary tumor to lung without producing detectable side effects [34]. Therefore some AMPs may be anticancer drug candidates alone or in combination with conventional therapies.

The mechanisms of action of AMPs against prokaryotic cells have been widely investigated [2,8,16,31,36], whereas much less is known regarding their interactions with and their effects on eukaryotic cells. Certain AMPs conjugated to a functional domain that allowed receptor-mediated or receptor-independent internalization into eukaryotic cells caused mitochondrial membrane disruption, the release of mitochondrial cytochrome c, and the induction of apoptosis [15,29]. Some AMPs translocate spontaneously across eukaryotic membranes into the cytoplasm where they depolarize inner mitochondrial membranes, but whether...
these peptides disrupt mitochondria directly or indirectly remains known [15,29].

In the current study, we initially assessed the cytotoxicities of five cationic α-helical AMPs (which had been designed for use as antibacterial agents in another study, based on four structural parameters: charge, polar angle, hydrophobicity, and polarophilic moment) [7] and two naturally occurring peptides plus their C-terminally amided forms against three HCC cell lines. The effects of the AMP GW-H1, which was most cytotoxic against the cell lines, were then examined further using the HCC cell line J5. Our findings suggested that AMP GW-H1 induced caspase-dependent apoptosis of J5 cell line, and may have chemotherapeutic potential for the treatment of HCC.

2. Materials and methods

2.1. Synthesis and purification of AMPs

Peptides used in the current study were synthesized on a solid-phase peptide synthesizer (Model 433A; Applied Biosystems, Foster City, CA) as previously described [7] with slight modifications. For peptides with carboxylic acid terminus, hydroxymethyl resin (Wang resin) was used, and for those with amidated C-terminus, amide resin was used instead. Single coupling was carried out for each Fmoc-amino acid residue using the FastMoc chemistry (HBTU/HOBt activation strategy). The crude peptides were cleaved from the resin by 95% trifluoroacetic acid (TFA), separated by filtration, precipitated from diethyl ether and lyophilized to yield white solid powders. Peptides were analyzed and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) with a linear gradient of water (0.1% TFA)–80% acetonitrile (0.08% TFA). The peptides were further characterized by MALDI-TOF mass spectrometry, as previously described [7].

2.2. Cell lines and cell culture

The human hepatocellular carcinoma cell line Hep3B (BCRC 60434) and the mouse fibroblast cell line 3T3 (BCRC 60071) were obtained from Bioresources Collection and Research Center (Hsin Chu, Taiwan). The human hepatocellular carcinoma cell line Huh7 (ATCC CCL-185) was obtained from American Type Culture Collection. The J5 line of human hepatocellular carcinoma cells was kindly provided by Dr. M.J. Chou (Graduate Institute of Basic Medical Science, Chang Gung University, Tao-Yuan, Taiwan). The Hep2 cells and the 3T3 cells were maintained with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, and the J5 cells were maintained with RPMI 1640 medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO₂, as previously described [40].

2.3. Cell viability assay

The viability of the cells after treated with various AMPs was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays performed in triplicate. Briefly, cells were plated at a density of 3000 cells/well in 96-well plates, and were permitted to adhere for 12–18 h then washed with phosphate buffered saline (PBS). Solutions were always prepared freshly by dissolving 1 × PBS or drugs in culture medium and added to Huh7, Hep3B, J5 cell lines and mouse fibroblast cell line 3T3. After 24 h of exposure, the drug containing medium was removed, washed with PBS and replaced by fresh medium. The cells in each well were then incubated in culture medium with 500 µg/mL MTT for 2 h. After the media were removed, 200 µL of DMSO was added to each well. Absorbance at 570 nm of the maximum was detected by a multimode microplate reader SpectraMax M2 (Molecular Devices, USA). The absorbance for DMSO-treated cells was considered as 100%. The results were determined by three independent experiments.

2.4. Flow cytometric analysis of cell cycle

After treatment with GW-H1, J5 cells were harvested and washed twice with PBS and fixed in 70% cold ethanol at 4 °C overnight. Before analysis, cells were washed twice with PBS containing 1% bovine serum albumin (BSA), then resuspended with 400 µl PBS and treated with 100 µg/ml RNase A (Roche Diagnostics) and 20 µg/ml propidium iodide (PI) (Sigma). After incubation for 30 min at 37 °C, the cells were subjected to DNA content analysis. PI fluorescence was analyzed with FACS caliber flowcytometer (Becton Dickinson). Data from at least 10,000 cells were analyzed with Flowjo software (Becton Dickinson). Cell cycle distributions were calculated with Flowjo software.

2.5. Analysis of mitochondrial transmembrane potential

Flow cytometric analysis of cells stained with DiOC₆ was used to measure changes in mitochondrial transmembrane potential. DiOC₆ was stored at −20 °C as a 1 mM stock in DMSO. Cells (2 × 10⁵ per treatment) were exposed to media or GW-H1 (20 and 40 µM) for 4 h in a 6-well round-bottomed tissue culture plate (37 °C, 5% CO₂). DiOC₆ was then added for 30 min to untreated or GW-H1 treated cells at a final concentration of 40 nM. Cells were analyzed by flow cytometry using the FL1 channel. DiOC₆ enters healthy, intact mitochondria and is released into the cytosol on mitochondrial transmembrane potential loss. This is observed as a left-shift in fluorescence intensity.

2.6. Western blot analysis

Approximately 1 × 10⁶ cells were cultured in 60-mm² dishes and then incubated in 20 µM GW-H1 for the indicated time. The cells were lysed on ice with 200 µl of protein extraction buffer (50 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, and 50 µg/ml leupeptin) and centrifuged at 12,000 × g at 4 °C for 10 min. The protein concentration of the cell lysates was measured with a Bio-Rad protein assay (Bio-Rad Laboratories, USA) following the manufacturer’s instructions. Aliquots (20 µg) of the cell lysates were separated by 12.5–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA, USA). Resolved proteins were then transferred to polyvinylidenefluoride (PVDF) membranes. Filters were blocked with 5% non-fat milk overnight and 1:500 dilutions of primary antibodies for 1 h at room temperature. Membranes were washed with three times with 0.05% Tween-20 and incubated with 1:5000 dilution of HRP-conjugated secondary antibody for 1 h at room temperature, then visualized with an enhanced chemiluminescence (ECL) plus chemiluminescence system (Millipore, USA).

2.7. Two-dimensional gel electrophoresis (2-DE), gel staining and image analysis

Approximately 1 × 10⁶ cells were cultured in 60-mm² dishes and then incubated in 20 µM GW-H1 for the indicated time. The cells were lysed on ice with 200 µl of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 2% pH 3–10 ampholyte), 2-DE was performed according to the method of O’Farrell [32] with slight modifications. Aliquots (200 µg) of the cell lysates were resolved in 125 µl of rehydration buffer [8 M urea, 4% CHAPS, 0.5% IPG 4–7 buffer, and 18.2 mM N-dithiothreitol (DTT)] and were loaded onto 7-cm Immobiline DryStrips (pH 4–7, linear; GE Healthcare, Chalfont

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St Gilea, UK) for the first-dimension isoelectric focusing (IEF). Sample application and gel rehydration were managed in one step (14 h, 30 V) on an IPGphor (GE Healthcare). IEF was conducted with a total of 13,400 Vh at an end voltage of 5000 as follows: 300 and 500 V constant each for 0.5 h; gradient 500–1000 V over 0.5 h; gradient 1000–5000 V over 2 h, 5000 V constant to the desired Vh. Focused proteins were reduced and alkylated during the equilibration step, immediately prior to the second dimension. Equilibration, 15 min in 50 mM Tris (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 75 mM DTT followed by 15 min in an identical buffer with DTT replaced by 135 mM iodoacetamide. The second dimension was performed on 12.5% SDS-polyacrylamide gels using the Protean III System (Bio-Rad), as described by Laemmli [23]. 2-D gels were stained with Coomassie Brilliant Blue G-250 (CBB G-250), scanned with the ImageScanner ver. 5.0, and analyzed with the Prodigy SameSpots software ver. 6.0 (Nonlinear). All 2-D analyses were performed on triplicate repeats. Spots were compared based on their volume percentages in the total spot volume over the whole gel image. Proteins were considered significantly altered if difference in spot intensity was more than 1.5 fold.

2.8. In-gel trypsin digestion

In-gel trypsin digestion was performed according to our previous report with slight modifications [22]. Protein spots of interest were excised, cut into about 1 mm x 1 mm pieces and placed in a microcentrifuge tube. 100 μL of 50 mM DTT in 25 mM ammonium bicarbonate (pH 8.5) was added into the tube and shaken at 37 °C for 1 h. After removal of excess DTT by centrifuge, 100 μL of 100 mM iodoacetamide (IAA) in 25 mM ammonium bicarbonate (pH 8.5) was added and the tube was shaken for 30 min at RT in the dark environment. Then excess IAA was removed by centrifuge. 100 μL of 50% acetonitrile in 25 mM ammonium bicarbonate buffer (pH to 8.5) was added and the gel pieces were soaked for 15 min, then the buffer was removed completely. The destaining step was repeated twice or more depending on the intensity of the dye. The gel pieces were soaked in 100 μL of 100% acetonitrile for 5 min and dried by SpeedVac to remove the remaining acetonitrile. Trypsin (0.1 μg) in 10 μL 25 mM ammonium bicarbonate (pH 8.5) was added to the gel pieces, and digestion was performed for 16 h at 37 °C. 50 μL of 50% acetonitrile in 5% TFA was added to quench the trypsin digestion and the solution was sonicated for 10 s to release the tryptic peptides from the gel. The peptide solution was concentrated and collected for further LC-MS/MS analysis.

2.9. LC-nano ESI-Q-TOF MS/MS analysis

The resulting peptide mixture was subjected to the CapLC system (Waters, Milford, MA) utilizing a capillary column (75 μm i.d., 10 cm in length, C5, SUG, Taiwan) with a linear gradient from 5% to 50% acetonitrile containing 0.1% formic acid over 46 min. The separated peptides were on-line analyzed under positive survey scan mode on a nano-ESI-Q-TOF (Micromass, UK) instrument as previously described [24]. The scan range was from m/z 400 to 1600 for MS and m/z 50 to 2000 for MS/MS. The raw data was processed into a text file format of PKL with MassLynx 4.0 (subtract 30%, smooth 3/2 Savitzky Golay and center three channels 80% centroid).

2.10. Protein identification

For protein identification, the PKL files generated from MS/MS spectra were uploaded to MASCOT search engine v2.2 (Matrix Science, UK) [http://www.matrixscience.com]. The parameters for database search were as follows: Protein database was set to be NCBI nr. Taxonomy was set as eubacteria. One trypsin missed cleavage was allowed. The mass tolerance was set to be 0.4 Da for both precursor and product ions. Carbamidomethyl (C) was chosen as a fixed modification. Deamidated (NQ) and oxidation (M) were chosen for variable modifications. Data format was chosen as Micromass(.pkf) and instrument was chosen as ESI-QUAD-TOF. The proteins with the scores above the significant threshold (p < 0.05) are shown as identified proteins.

2.11. HCC xenografts and GW-H1 treatment

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of National Ilan University. To evaluate the antitumor activity of GW-H1 in vivo, human HCC xenografts were established by subcutaneous injection of ∼1 × 10^7 J5 cells into the backs of nude mice. After the tumors were ∼100–250 mm^3, the mice (n = 6/group) were randomly subdivided into a control group (no treatment) and two treatment groups that were given a daily subcutaneous injection of GW-H1 (60 or 120 mg/kg) for five successive days. Tumor volumes were measured using caliper measurements once every 3 days and calculated with the formula V = πr^2h.

3. Results

3.1. Peptide design

We previously designed and synthesized a series of cationic AMPs with cytotoxicity and selectivity against a broad spectrum of G(+)- and G(-) bacteria [7]. In the current study, we assessed the chemotherapeutic potential of five synthetic AMPs (GW-Q3, -Q4, -Q6, -H1, -A5) against HCC cell lines. Two natural potent AMPs and their C-terminally amidated derivatives: magainin 2, magainin-2-amide, pleurocidin, and pleurocidinamide were also synthesized and tested (Table 1). These AMPs were chosen for the present study since they exhibited highest selectivity against bacterial cells and showed the least hemolytic activity in our previous report [7]. All AMPs were prepared by solid-phase synthesis and were purified by reverse-phase HPLC to at least 95% homogeneity (data not shown).

3.2. Cationic AMPs inhibit the viability of several HCC cell lines

The three HCC cell lines, J5, Huh7, and Hep3B, were treated with different concentrations of the individual cationic AMPs for 24 h, and then cell viability (reported as IC_{50} values; Table 2) was determined using the MTT assay (Fig. 1). The cells treated with the AMPs exhibited reduced viability in a dose-dependent manner. The IC_{50} values for the AMPs ranged from 20.3 μM to 257.1 μM. The J5 cell line was most sensitive to GW-H1 (IC_{50} value of 20.3 μM). The non-transformed murine fibroblast 3T3 line was less susceptible to the peptides, having significantly higher IC_{50} values as compared with the HCC cell lines. In summary, the peptides had a selective inhibitory effect on the viabilities of the HCC cell lines as compared with the 3T3 cell line. Among them, AMP GW-H1 and J5 cell line were selected for further analysis of the anticancer mechanism.

3.3. GW-H1 alters J5 cell morphology and translocates into its cytoplasm and nuclei

J5 cells were treated with 0, 20, or 40 μM of GW-H1, and GW-H1 localization and cell morphology were assessed at 30 min and 24 h (Fig. 2A). After incubation for 30 min, GW-H1 was found at the cell membrane, and it induced the appearance of membrane-bound particles producing irregular shapes that are smaller in size than the untreated cells in a dose- and time-dependent manner. After the J5 cells had been incubated with GW-H1 for 24 h, they had significantly shrunk in size and lost their capacity to adhere to
Table 1
Characteristics of AMPs used in the current study.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Charge</th>
<th>Polar angle</th>
<th>Hydrophobicity</th>
<th>Hydrophobic moment</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic AMPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW-Q3</td>
<td>GANLAKKEYTINKEFINYW</td>
<td>+3</td>
<td>140</td>
<td>−0.043</td>
<td>0.343</td>
<td>2425.81</td>
</tr>
<tr>
<td>GW-Q4</td>
<td>GANAAKFIATUARKFINYW</td>
<td>+4</td>
<td>140</td>
<td>−0.043</td>
<td>0.344</td>
<td>2255.69</td>
</tr>
<tr>
<td>GW-Q6</td>
<td>GIKAIKATIUARKFANYW</td>
<td>+6</td>
<td>140</td>
<td>−0.043</td>
<td>0.343</td>
<td>2257.88</td>
</tr>
<tr>
<td>GW-H1</td>
<td>GYNYAKKFIATUARKFANALW</td>
<td>+4</td>
<td>140</td>
<td>−0.115</td>
<td>0.344</td>
<td>2284.69</td>
</tr>
<tr>
<td>GW-A5</td>
<td>GATYAIKTIATUARKTAW</td>
<td>+4</td>
<td>180</td>
<td>−0.042</td>
<td>0.344</td>
<td>2179.63</td>
</tr>
</tbody>
</table>

| Natural AMPs |                     |        |             |                |                    |                  |
| M2           | GIKFLHSAAKFKAFVGEIMNS | +4     | 140         | −0.046         | 0.325              | 2506.95          |
| M2α          | GIKFLHSAAKFKAFVGEIMNS-NH2 | +5 | 140       | −0.026         | 0.287              | 2711.17          |
| Ple          | GWGSFFKAHAVGKHAALTYL | +4     | 140         | −0.026         | 0.287              | 2710.18          |
| Ple-α        | GWGSFFKAHAVGKHAALTYL-NH2 | +5 | 140       | −0.026         | 0.287              | 2710.21          |

The bold-face type letters indicate positively charged lysine residues.

M2, magainin 2 from frog; M2α, C-terminally amidated magainin 2; Ple, pleurocidin from winter flounder; Ple-α, C-terminally amidated pleurocidin.

Fig. 1. Effects of synthetic and natural AMPs on the cell viability of three HCC cell lines J5 (A), Huh7 (B), Hep3B (C) and a non-transformed murine fibroblast 3T3 (D). Cells were treated with different concentrations (0–100 μM) of AMPs each for 24 h, followed by an MTT assay. Each concentration was repeated in eight wells for three independent experiments.
the substratum. Cells were also incubated with fluorescein isothiocyanate (FITC)-labeled GW-H1 for 30 min and 2 h to confirm that GW-H1 initially bound to the cell membrane (Fig. 2B), then could permeate the cells. Confocal microscopy images revealed that FITC-labeled GW-H1 was present in both the cytoplasm and nuclei (Fig. 2C).

**Table 2**

Effect of AMPs on cell viability in HCC cell lines J5, Huh7, Hep3B and mouse fibroblast cell line 3T3.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cell viability IC_{50} [μmol/L=μM]a</th>
<th>J5</th>
<th>Huh7</th>
<th>Hep3B</th>
<th>3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic AMPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW-Q3</td>
<td>30.8</td>
<td>n.d.b</td>
<td>257.1</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>GW-Q4</td>
<td>29.9</td>
<td>70.0</td>
<td>34.4</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>GW-Q6</td>
<td>24.3</td>
<td>107.4</td>
<td>53.4</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>GW-H1</td>
<td>20.3</td>
<td>87.2</td>
<td>69.2</td>
<td>234.3</td>
<td></td>
</tr>
<tr>
<td>GW-A5</td>
<td>28.9</td>
<td>n.d.</td>
<td>88.2</td>
<td>336.9</td>
<td></td>
</tr>
<tr>
<td>Natural AMPs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>128.9</td>
<td>145.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>M2a</td>
<td>96.0</td>
<td>n.d.</td>
<td>169.2</td>
<td>433.8</td>
<td></td>
</tr>
<tr>
<td>Ple</td>
<td>54.9</td>
<td>n.d.</td>
<td>340.9</td>
<td>350.2</td>
<td></td>
</tr>
<tr>
<td>Ple-a</td>
<td>11.0</td>
<td>60.0</td>
<td>77.5</td>
<td>39.6</td>
<td></td>
</tr>
</tbody>
</table>

*a*: IC_{50} values were assessed by logarithmic extrapolation.

*b*: n.d.: not determined or IC_{50} values > 500 μM.

**3.4. GW-H1 increases the length of the sub-G1 phase and decreases the mitochondrial membrane potential of J5 cell**

To investigate whether GW-H1 induced cytotoxicity of J5 cell lines through the apoptotic pathway, flow cytometry was applied to monitor J5 cells in the absence or presence of GW-H1 (0, 20, or 40 μM) for 0–72 h, with visualization by propidium iodide (PI) staining. An increased percentage of cells in the sub-G1 phase was observed in a dose- and time-dependent manner (Fig. 3A and B), which is indicative of apoptotic cells. Furthermore, the loss of mitochondrial membrane potential (MMP) is considered to be a critical mediator of apoptosis. To determine whether GW-H1 triggers mitochondrial injury, DiOC_{6}, which is a fluorescent dye that is incorporated into mitochondria in a Δψ_{m}-dependent manner, was used to evaluate changes in MMP during GW-H1 treatment. As shown in Fig. 3C and D, treatment with GW-H1 caused significant MMP reduction in a dose- and time-dependent manner, suggesting the partial role of the loss of MMP in GW-H1-induced apoptosis of J5 cells.

**3.5. GW-H1 induces caspase-dependent apoptosis in J5 cells**

To further investigate whether GW-H1 induced apoptosis of J5 cells through the caspase-dependent pathway, we determined if caspase-3, -7, -9, and poly(ADP-ribose) polymerase (PARP) were
cleaved in cells treated with GW-H1. According to the results of western blot analysis, these enzymes were all cleaved in a time-dependent manner (Fig. 4), indicating that GW-H1 induced caspase-dependent apoptosis of J5 HCC cell lines.

3.6. Two-dimensional gel electrophoresis and mass spectrometry

To further delineate the mechanism of GW-H1-induced apoptosis of J5 cells, we performed two-dimensional gel electrophoresis on J5 cells treated with GW-H1 for 0 to 72 h. Gel images analyzed by Prodigy SameSpots (Nonlinear) software (Fig. 5), two proteins upregulated and three proteins downregulated in a time-dependent manner were selected for LC-ESI-Q-TOF MS/MS analysis. These proteins were identified by Mascot program as Cleavage and polyadenylation specificity factor subunit 5, elongation factor 1-delta, heat shock protein β-1 (also known as heat shock protein 27, Hsp27), phosphoglycerate kinase 1 and triosephosphate isomerase (Table 3).

3.7. GW-H1 inhibits the growth of J5 xenografts in nude mice

To evaluate the antitumor activity of GW-H1 in vivo, human HCC xenografts were established by subcutaneous injection of ~1 × 10^7 J5 cells into the backs of nude mice. After the tumors were ~100–250 mm^3, the mice (six animals per group) were randomly subdivided into a control group (no treatment) and two treatment groups that were given a daily subcutaneous injection of GW-H1 (60 or 120 mg/kg) for five successive days. Tumor growth was significantly suppressed in mice treated with GW-H1 as compared with those of the control group (Fig. 6A). The total body weight
Fig. 5. Two-dimensional gel electrophoresis on J5 cells treated with GW-H1 (20 μM) for 0–72 h. 2-DE were performed and gels stained with CBB G-250. Gel images were analyzed by SameSpots (Nonlinear) software, and the protein spots significantly altered were selected and then subjected to LC-ESI-Q-TOF MS/MS analysis.

Fig. 6. GW-H1 inhibited tumor growth of J5 xenografts in nude mice. (A) Human HCC xenografts were established by subcutaneous injection of ~1 × 10⁷ J5 cells into the backs of nude mice. After the tumors were ~100–250 mm³, the mice (six animals per group) were randomly subdivided into a control group (no treatment, PBS only) and two treatment groups that were given a daily subcutaneous injection of GW-H1 (60 or 120 mg/kg) for five successive days. (B) The body weight was recorded from the first day of GW-H1 administration. There was no notable side effect on the body weight of mice during GW-H1 treatment procedure. (C) Immunohistochemical staining using anti-cleaved caspase-3 antibody was also performed for tumor tissues collected from control mice and mice treated with GW-H1, respectively at day 36 after initiation of treatment [8].
of groups treated with 60 or 120 mg/kg were similar to those in the control group, indicating that there was no notable side effect on the body weight of mice during GW-H1 treatment procedure (Fig. 6B). In addition, compared with tumors of control mice, more cleaved caspase-3 was present in the tumors of mice treated with GW-H1 at day 36 after initiation of treatment, as confirmed by immunohistochemical staining (Fig. 6C).

4. Discussion

In the current study, AMPs had a greater effect on the antiproliferation of J5 cells than on HuH7 and Hep3B, while no significant growth inhibitory effect was detected on 3T3 cells (Fig. 1, Table 2). Generally, AMPs exhibit selectivity for cancer and microbial cells primarily due to their elevated levels of negative membrane surface charge as compared to non-cancerous eukaryotic cells [13]. These cationic and amphipathic AMPs are able to discriminate between neutral or non-neutral cells interacting specifically with negatively charged membrane components such as phosphatidylserine (PS), sialic acid or heparan sulfate, which differ between cancer and non-cancer cells [35]. The anticaner activity of AMPs normally occurs at micromolar levels but is not accompanied by significant levels of hemolysis or toxicity to other mammalian cells. In most cases the mechanisms underlying such killing involves disruption of mitochondrial membrane integrity and/or that of the plasma membrane of the target tumor cells [13]. We thereby reasoned that the differences in plasma membrane contents and mitochondrial membrane potential between J5, HuH7 and Hep3B HCC cell lines may account for their discrepancies in sensitivity against AMPs. J5 is a more aggressive cell line than HuH7 and Hep3B [40], resulting in its significantly higher susceptibility to AMP GW-H1.

In our previous report, AMP GW-H1 has been tested to exert its cytolytic activity by folding into an amphipathic helix upon selectively binding and insertion into the target membrane, leading to breakdown of the membrane structure, thus causing leakage of cell contents, resulting finally in cell death [8]. In the current study, after J5 HCC cells were treated with FITC-labeled GW-H1 for 30 min, peptides were found to attach to the cell membrane, and they induced the appearance of membrane-bound particles producing irregular shapes, as revealed by confocal microscopy (Fig. 2A and B). However, when incubated for 2 h, FITC-labeled GW-H1 was found to penetrate J5 cells, as confirmed by fluorescence all over the cytoplasm, and also in several nuclei (Fig. 2C). Whether GW-H1 directly permeates the cell membrane of J5 cells using its hydrophobic contents, or through certain receptors is still unknown. Further experiments are now under investigation to clarify this issue.

Proteomic analysis based on 2-DE revealed five proteins of J5 cell with significantly altered expression upon treatment of AMP GW-H1. Among them, cleavage and polyadenylation specificity factor subunit 5 (spot no. 1) and elongation factor 1-delta (spot no. 2) were up-regulated (Fig. 5), indicating that GW-H1 treatment may have certain effects on mRNA processing and protein biosynthesis of J5 HCC cells. Spot no. 3, heat shock protein B-1 (also known as heat shock protein 27, Hsp27) was down-regulated. Hsp27, one of the low molecular weight molecular chaperone that is highly induced during the stress response, functions in regulating apoptosis through an ability to interact with key components of the apoptotic signaling pathway, in particular, those involved in caspase activation and apoptosis [9,10]. In previous reports, Hsp27 has been found to inhibit apoptosis by inhibition of cytochrome c-mediated activation of caspase-3, thus preventing the correct formation and function of the apoptosome complex [10]. Furthermore, Hsp27 protein expression and its phosphorylation levels have been found to increase in parallel with enhanced metastatic potentials of HCC cells through activation of NF-kB signaling pathway [17,30]. In the current study, downregulation of Hsp27 in GW-H1-treated J5 cells may induce its apoptosis, and may further suppress metastasis ability of HCC cells. Furthermore, protein expression of phosphoglycerate kinase 1 (PGK1, spot no. 4) and triosephosphate isomerase (spot no. 5) were also inhibited after GW-H1 treatment. Recently, glycolysis has emerged as a potent driving force of tumor growth and therapy failure [12]. It has been reported that PGK1 protein and gene expression was scant in normal liver, elevated in cirrhotic livers and most intense in HCC [12]. Ai et al. also showed that PGK1 was highly expressed in HCC cell lines with high metastatic potential [1]. Similarly, the protein expression of triosephosphate isomerase was found to be significantly higher in HCC than in chronic hepatitis and normal individuals [25]. In the current study, GW-H1 treatment results in down-regulation of PGK1 and triosephosphate isomerase in J5 HCC cells, suggesting that GW-H1 may have the ability to inhibit tumor progression and metastasis of HCC cells.

In the present study, our findings suggested that the novel cationic amphipathic AMP GW-H1 exerts highly selective cytotoxicity against J5 HCC cell lines via caspase-dependent apoptosis. Optimization of these cationic and amphipathic AMPs using various strategies to enhance further selectivity is expected to yield novel anticancer drugs with chemotherapeutic potential for the treatment of hepatocellular carcinoma.

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References


