Caspase-8 acts as a key upstream executor of mitochondria during justicidin A-induced apoptosis in human hepatoma cells

Chun-Li Su\textsuperscript{a,1}, Lynn L.H. Huang\textsuperscript{b,1}, Li-Min Huang\textsuperscript{b}, Jenq-Chang Lee\textsuperscript{c}, Chun-Nan Lin\textsuperscript{d}, Shen-Jeu Won\textsuperscript{e,*}

\textsuperscript{a} Department of Nursing, Chang Jung Christian University, Tainan 711, Taiwan
\textsuperscript{b} Institute of Biotechnology, College of Science, National Cheng Kung University, Tainan 701, Taiwan
\textsuperscript{c} Department of Surgery, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
\textsuperscript{d} School of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan
\textsuperscript{e} Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan

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Abstract \textit{Justicia procumbens} is a traditional Taiwanese herbal remedy used to treat fever, pain, and cancer. Justicidin A, isolated from \textit{Justicia procumbens}, has been reported to suppress in vitro growth of several tumor cell lines as well as hepatoma cells. In this study, justicidin A activated caspase-8 to increase tBid, disrupted mitochondrial membrane potential (Δ\textit{Ψ}\textsubscript{m}), and caused the release of cytochrome \textit{c} and Smac/DIABLO in Hep 3B and Hep G2 cells. Justicidin A also reduced Bax and Bak in mitochondria. Caspase-8 inhibitor (Z-IETD) attenuated the justicidin A-induced disruption of Δ\textit{Ψ}\textsubscript{m}. Growth of Hep 3B implanted in NOD-SCID mice was suppressed significantly by oral justicidin A (20 mg/kg/day). These results indicate that justicidin A-induced apoptosis in these cells proceeds via caspase-8 and is followed by mitochondrial disruption. Supplementary materials are available at http://myweb.ncku.edu.tw/~a725/.
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1. Introduction

Human hepatocellular carcinoma (HCC) is the fifth most frequent cancer and is the third most common cause of cancer-related death worldwide [1,2]. In Taiwan, HCC is also a leading malignant neoplasm [3]. Unfortunately, it does not respond well to chemotherapy and has a poor prognosis [4,5]. To develop a more effective chemotherapeutic agent for this disease, we concentrated our efforts on natural compounds traditionally used to treat the disease. \textit{Justicia procumbens} (\textit{J. procumbens}) is a traditional herbal remedy in Taiwan for fever, pain, and cancer [6,7]. Justicidin A, purified from a methanolic extract of \textit{J. procumbens}, has various biological activities including suppression of tumor cell growth [8,9] and release of TNF-\alpha [10]. The purpose of present study was to determine whether apoptosis is stimulated by justicidin A-treatment of human HCC. Two major apoptotic pathways (intrinsic and extrinsic) have been concluded. The intrinsic apoptotic pathway operates via mitochondria [11]. The extrinsic apoptotic pathway activates caspase-8 and its downstream regulators [12]. The results of this study reveal that justicidin A induces both intrinsic and extrinsic apoptotic pathways in HCC cells since both caspase-8 and mitochondria are affected. Induction of apoptosis is characterized by phosphatidylserine externalization, accumulation of sub-G\textsubscript{1} cells, and DNA fragmentation. Activation of caspase-8 increases the amount of tBid to change mitochondrial membrane potential (Δ\textit{Ψ}\textsubscript{m}), which in turn causes the release of cytochrome \textit{c} and second mitochondria-derived activator of caspase/direct IAP binding protein with low pl (Smac/DIABLO) from mitochondria to activate caspase-9 and caspase-3. The increase of Bax and Bak and decrease of Bcl-x\textsubscript{L} in mitochondria further promote the process of apoptosis. The cytotoxicity of justicidin A is also effective in vivo.

2. Materials and methods

2.1. Materials

Justicidin A was isolated from \textit{J. procumbens} plants \textsuperscript{9}. DiOC\textsubscript{6}(3) was obtained from Molecular Probes (Eugene, OR). Anti-cytochrome \textit{c} mouse monoclonal antibody (mAB) and Annexin V-FITC were purchased from BD Pharmingen (San Diego, CA). Anti-caspase-3 mouse mAB and anti-Smac/DIABLO rabbit polyclonal antibody (pAB) were purchased from IMGENEX (San Diego, CA). Anti-caspase-8 mouse mAB and anti-poly(ADP-ribose) polymerase (PARP) rabbit pAB were purchased from Cell Signaling Technology (Beverly, MA). Anti-caspase-9 mouse mAB was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-receptors for activated C-kinase (RACK1) mouse mAB was purchased from BD Transduction Laboratories (Lexington, KY). Z-IETD, Z-LEHD, anti-Bax and anti-Bcl-x\textsubscript{L} mouse mAB, anti-Bak, anti-DNA fragmentation factor (DFF) 45 and anti-DFF40 rabbit pAB, and goat anti-mouse conjugated HRP secondary antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA).
Goat anti-rabbit conjugated HRP secondary antibody was purchased from Amersham Pharmacia Biotech (Quebec, Canada). Other reagents were purchased from Sigma (St. Louis, MO).

2.2. Cell cultures

Human HCC (Hep 3B and Hep G2 cells), and Chang liver cells from American Type Culture Collection (ATCC, Rockville, MD), were maintained in complete Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY). Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors’ whole blood (Tainan Blood Bank Center, Tainan, Taiwan) by centrifugation over a Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) density gradient at 400 × g for 30 min in a Sorvall RT6000B (Du Pont, Wilmington, DE) [13]. The cells collected at the interface were washed thrice with serum-free RPMI-1640 (Gibco BRL, Grand Island, NY) and subsequently resuspended in complete DMEM.

2.3. Cell viability assay

Cytotoxicity was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [13]. Cells on 96-well plates (Nunc, Roskilde, Denmark) were treated with different concentrations of the test agent for 6 days. After addition of 10 μl MTT to a final concentration of 0.5 mg/ml, cells were incubated at 37 °C for 4 h. After adding 100 μl of 10% SDS/0.01 N HCl, cells were left overnight at 37 °C. The absorbance of each well was measured at 950 nm in a Multiscan photometer (MRX II, Dynatech, McLean, VA).

2.4. Flow cytometric detection of phosphatidylserine exposure and cell cycle distribution

Cells were trypsinized, and resuspended in HEPES buffer solution (HBS) containing 1.25% (v/v) of Annexin V–FITC to stain phosphatidylserine on the cell surface [14]. Stained cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) [13]. For cell cycle distribution analysis, cells were washed with HBS and resuspended in 70% ethanol at 4 °C. After centrifugation at 800 × g for 10 min, cells were resuspended in HBS containing 40 μg/ml propidium iodide (PI) and 0.1% NP-40 for flow cytometric analysis [16,17].

2.5. Analysis of DNA fragmentation

Extraction and electrophoresis of DNA were performed as described [13]. Cells were incubated with lysis buffer (10 mM Tris–HCl [pH 7.6], 200 mM EDTA, and 1% NP-40) for 20 min at 37 °C. After centrifugation, the supernatants were incubated with 50 μl of RNase A (20 mg/ml) and 20 μl of SDS (10%) at 56 °C for 2 h. Proteinase K (35 μl of 20 mg/ml) was mixed with the cell lysates and incubated for another 2 h at 37 °C. Precipitated DNA fragments were resuspended in 15 μl of Tris-EDTA buffer, and separated by electrophoresis on a 1% (w/v) agarose gel in TBE buffer. The patterns of DNA ladders were examined, the supernatants were centrifuged at 12000 × g at 4 °C for 30 min. The supernatants were centrifuged again at 100000 × g for another 1 h. The resulting supernatants were used as cytosolic fractions, and the resulting pellets were lysed with lysis buffer and used as mitochondrial fractions. For nuclear extract preparation, cells (1 × 10⁴) were lysed in 400 μl of buffer A (10 mM HEPES [pH 7.9], 5 mM MgCl₂, 10 mM KCl, 3 mM Na₂VO₄, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of leupeptin, antipain, aprotinin, and pepstatin A) on ice for 20 min. After centrifugation at 11000 × g for 20 s at 4 °C, the pellets were resuspended in 60 μl of buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM Na₂VO₄, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, antipain, aprotinin, and pepstatin A) for 15 min on ice with occasional mixing. Nuclear debris was removed by centrifugation again at 12000 × g for 15 min at 4 °C. All isolated proteins were stored at −70 °C before immunoblotting analysis [22].

2.6. Measurement of Δψm

Change in Δψm was determined by flow cytometry using the mitochondrial-sensitive dye rhodamine 123 in the dark [16,18]. After treatment, cells were stained with 5 μM rhodamine 123 for 30 min. After mixing with 2.5 μg/ml of PI, the stained cells were subjected to flow cytometry, and the data were analyzed using CellQuest software. Change of Δψm was also determined by laser scanning confocal microscopy (Leica TCS-SP2, Germany) [16,19]. Cells were seeded in 6-well plates containing methanol-sterilized glass cover slips. After treatment, the cells were stained with 5 μM rhodamine 123 at 37 °C, washed twice with PBS at room temperature, fixed with 4% paraformaldehyde for 15 min, and then examined using a Leica TCS NT laser scanning confocal imaging system coupled to a Leica DMRBE microscope with a Leica 630 fluorot objective.

2.7. Subcellular fractionation and Western blot analysis

Whole cells (1 × 10⁶) were mixed with 200 μl of lysis buffer and then centrifuged at 15000 × g for 10 min [16]. The supernatant was used as total protein for immunoblotting. The cytosolic, mitochondrial, and nuclear proteins were prepared as previously reported [16,20,21]. Briefly, harvested cells (1 × 10⁶) were resuspended in TSE buffer (10 mM Tris, 0.25 M sucrose, and 0.1 mM EDTA [pH 7.4]), and homogenized with 10 strokes in a Dounce homogenizer (Glas-Col, Terre Haute, IN) using a Teflon pestle. After removing the cell debris by centrifuging the homogenates at 750 × g at 4 °C for 30 min, the supernatants were centrifuged at 12000 × g at 4 °C for 30 min. The supernatants were centrifuged again at 100000 × g for another 1 h. The resulting supernatants were used as cytosolic fractions, and the resulting pellets were lysed with lysis buffer and used as mitochondrial fractions. For nuclear extract preparation, cells (1 × 10⁴) were lysed in 400 μl of buffer A (10 mM HEPES [pH 7.9], 5 mM MgCl₂, 10 mM KCl, 3 mM Na₂VO₄, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml of leupeptin, antipain, aprotinin, and pepstatin A) on ice for 20 min. After centrifugation at 11000 × g for 20 s at 4 °C, the pellets were resuspended in 60 μl of buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM Na₂VO₄, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, antipain, aprotinin, and pepstatin A) for 15 min on ice with occasional mixing. Nuclear debris was removed by centrifugation again at 12000 × g for 15 min at 4 °C. All isolated proteins were stored at −70 °C before immunoblotting analysis [22].

2.8. Animal study

NOD.CB17-PRKDC(Scid)/J (NOD-SCID) mice were bred and maintained at the Animal Center of National Cheng Kung University (NCKU, Tainan, Taiwan) in a specific pathogen-free environment. Mice at 6–7 weeks of age were used in the experiments as described previously [16]. Food and water were provided ad libitum. Tumor volume was measured using calipers (2–3 times/week) [23].

2.9. Statistical analysis

All of the experimental data are expressed as means ± S.E.M. Differences in tumor volumes were determined by Student’s t test using the Mininab (version 10.2) software package. We assigned statistical significance if P < 0.05.

3. Results

3.1. Growth inhibition and apoptosis of justicidin A-treated cells

Low dosages of justicidin A suppressed the viability of HCC Hep 3B and Hep G2 cells, and the IC₅₀ at day 6 was 0.048 ± 0.020 and 0.052 ± 0.050 μM, respectively. The IC₅₀ of justicidin A for non-malignant Chang liver cells was 0.95 ± 0.12 μM, which is at least 10-fold higher than the IC₅₀ of justicidin A for HCC cells. Human PBMC were much more resistant to justicidin A treatment with an IC₅₀ of 23 ± 1 μM. To examine the possible mechanism of justicidin A on cell viability, three parameters of apoptosis (exposure of phosphatidylserine, cell cycle redistribution and DNA fragmentation) were analyzed. As shown in Fig. 1A, the percentage of Annexin V–FITC positive cells were increased significantly in a dosage- and time-related manner. Dose-related elevations in the sub-G₁ fraction of both tumor cells were also observed (Supplementary Fig. 1). Justicidin A also caused time- and dose-related enhancement of apoptotic DNA fragmentation in these cells (data not shown).

3.2. Activation of caspases and involvement of mitochondria in justicidin A-induced apoptosis

The process of apoptosis involves a cascade of proteolytic activity, much of it carried out by caspases [21]. In this study, both procaspase-8 (Fig. 1B) and procaspase-9 (Supplementary Fig. 2) were cleaved into their active forms in a time-related manner. Cleavage of their downstream molecule procaspase-3 was also revealed (Supplementary Fig. 2). Since PARP and DFF are substrates of activated caspase-3 [24–26], increase
Fig. 1. Induction of apoptosis and expression of caspase-8, cytochrome c and Smac/DIABLO in hepatoma cells in response to justicidin A. Externalization of phosphatidylserine in Hep 3B and Hep G2 cells (A). After treatment with the indicated concentrations of justicidin A for 48 h or with 1 μM of justicidin A for the indicated time periods, cells (2 × 10^5) stained with Annexin V–FITC were analyzed by flow cytometry. The percentages in the figure indicate the proportion of apoptotic cells with externalization of phosphatidylserine. Activation of caspase-8, and release of cytochrome c and Smac/DIABLO in Hep 3B and Hep G2 cells (B). Total proteins from justicidin A-treated cells were subjected to Western blot analysis to determine the activation of procaspase-8 by using anti-caspase-8 mouse mAb. RACK1 was served as a loading control. Blots of the cytosol- and mitochondria-enriched fractions were used to demonstrate the translocation of cytochrome c and Smac/DIABLO using anti-cytochrome c mouse mAb or anti-Smac/DIABLO rabbit pAb. Relative protein expression is shown at the bottom of each panel, with control levels arbitrarily set to 1. JA, justicidin A.
in the cleavage PARP and nuclear DFF40 (Supplementary Fig. 2) displayed the enzymatic activity of caspase-3. The involvement of caspase-8 and caspase-9 in the process of apoptosis was confirmed by showing that the presence of caspase-8 inhibitor (Z-IETD) or caspase-9 inhibitor (Z-LEHD) reduced the number of justicidin A treated cells in the sub-G1 phase (Supplementary Fig. 3).

Activation of caspase-9 (Supplementary Fig. 2) implied the involvement of mitochondria in justicidin A-induced apoptosis. Maintenance of Δψm is important for normal function and survival of cells [27]. Changes in Δψm can cause the release of apoptogenic proteins. In this study, the time-related release of cytochrome c and Smac/DIABLO from mitochondria to cytosol (Fig. 1B) further demonstrated the pivotal role of mitochondria in justicidin A-induced apoptosis. Conversely, mitochondrial cytochrome c decreased gradually in both cells (Fig. 1B). Similar patterns of increased cytosolic and decreased mitochondrial Smac/DIABLO were observed (Fig. 1B). Anti-X-linked apoptosis-inhibiting protein (XIAP), an antagonist of caspase-3 and caspase-9 [28], was reported to be antagonized by cytosolic Smac/DIABLO [29]. Decrease in XIAP expression (data not shown) may therefore favor the increase in caspase-9 and -3 activities (Supplementary Fig. 2). Involvement of mitochondria was also confirmed by changes in Δψm upon justicidin A stimulation. A dose- and time-related decrease in the intensity of rhodamine 123 fluorescence was detected in the mitochondria of justicidin A-treated Hep 3B cells (Supplementary Fig. 4). A similar time-related decrease in fluorescence intensity was observed in Hep G2 cells (data not shown). Confocal microscopy also showed that justicidin A induced Δψm. Decrease in fluorescence emission was found in both tumor cells after justicidin A treatment (Fig. 2A).

Fig. 2A reveals the relationship between mitochondria and caspase-8. In both cells, Z-IETD (an inhibitor of caspase-8) alleviated the justicidin A-induced decrease in mitochondrial fluorescence signals (Fig. 2A) and justicidin A-induced increase in the number of apoptotic cells in the sub-G1 fraction (Supplementary Fig. 3). Similar results were observed when cells were treated with justicidin A plus cyclosporin A, a permeability transition pore inhibitor that blocks the release of cytochrome c from mitochondria (Fig. 2A).

3.3. Involvement of Bcl-2 family in justicidin A-induced apoptosis

Members of Bcl-2 family regulate apoptosis by interacting with mitochondria [28]. Bcl-2 and Bcl-xL protect against mitochondrial dysfunction and therefore inhibit apoptosis. In contrast, Bid, Bax, and Bak induce Δψm and thus promote apoptosis. To test the involvement of the proteins of the Bcl-2 family in justicidin A-induced apoptosis, total cell lysates, and cytosolic and mitochondrial fractions of justicidin A-treated tumor cells were prepared. In Fig. 2B, justicidin A significantly decreased total and mitochondrial Bcl-xL. Translocation of Bax was also observed in these two cells after treatment of justicidin A (Fig. 2B). The expression of cytosolic Bax decreased and the mitochondrial Bax increased (Fig. 2B). In Hep 3B cells, mitochondrial Bak expression increased a small amount, whereas, in Hep G2 cells it increased markedly (Fig. 2B). Cytosolic Bid was decreased after treatment with justicidin A (Fig. 2B). The translocation of the tBid fragment to mitochondria began at 6 h of justicidin A treatment, kept increasing between 12 and 24 h, and peaked at 72 h in Hep 3B cells or at 48 h in Hep G2 cells (Fig. 2B).

3.4. Tumor growth in mice

To examine the antitumor effect of justicidin A in vivo, Hep 3B cells were implanted in mice before they were fed justicidin A (20 mg/kg/day) for 60 consecutive days. As shown in Fig. 3, the tumors were sensitive to justicidin A, and their growth was halted throughout the period of justicidin A administration.

4. Discussion

Our experimental findings suggest the following signaling cascades in justicidin A-treated HCC. At 6 h of justicidin A treatment, activation of caspase-8 (Fig. 1B) triggers the cleavage of Bid into tBid and causes the translocation of tBid to mitochondria (Fig. 2B). Mitochondrial tBid may oligomerize with the mitochondrial Bax (Fig. 2B) and Bak (Fig. 2B) (both proteins first increase in the mitochondria at 6 h of justicidin A treatment) to damage Δψm (Fig. 2A and Supplementary Fig. 4) and result in releasing cytochrome c and Smac/DIABLO into the cytosol at a later time point (at 24 h in Hep 3B and 48 h in Hep G2 cells) (Fig. 1B). Since Bcl-xL, can bind to Bax and prevent Bax insertion into the outer membrane of mitochondria [30], the decrease in total and mitochondrial Bcl-xL, (Fig. 2B) promotes the changes of Δψm. The released cytochrome c may contribute to the formation of apoptosomes in the cytosol to activate caspase-9 (Supplementary Fig. 2). Decrease in XIAP (data not shown), because of interaction with the cytosolic Smac/DIABLO, may further increase apoptosisome formation and therefore facilitate caspase-9 activation [28]. The activation of caspase-8 or caspase-9 subsequently activates caspase-3 (Supplementary Fig. 2). The activated caspase-3 then cleaves PARP (Supplementary Fig. 2). Since PARP participates in DNA repair mechanism [31,32], the increase in cleaved PARP disables the function of DNA repair in both cells. The activated caspase-3 also cleaves DFF45 (Supplementary Fig. 2). Since DFF45 can bind DFF40 to prevent DFF40-mediated DNA fragmentation [33], the decrease in DFF45 allows release of DFF40 into the nucleus. The increase in nuclear DFF40 (Supplementary Fig. 2) may result in the formation of DNA ladders in justicidin A-treated HCC (data not shown). Greater increase in the level of cleaved PARP than nuclear DFF40 (Supplementary Fig. 2) in Hep 3B cells suggests that PARP is the more important determinant of DNA fragmentation in these cells. The small increase in pro-apoptotic Bak (Fig. 2B) in Hep 3B cells suggests that caspase activation and Bax-dependent release of mitochondrial apoptogenic proteins are sufficient to increase the level of cleaved PARP and nuclear DFF40 (Supplementary Fig. 2) and thereby to provoke Hep 3B cell death. Our results indicate that Hep G2 but not Hep 3B cells express Bcl-2 (data not shown). Since Bcl-2 protein has been reported to inhibit caspase-8 activity [34,35], the greater increase in cleaved caspase-8 in Hep 3B cells (Fig. 1B) may be explained. Greater cleavage of procaspase-8 (Fig. 1B) may result in more predominant increase in mitochondrial tBid and tBid-induced translocation of Bax (Fig. 2B), and thereby lead to the greater increase in cleaved caspase-3 (Supplementary Fig. 2) in Hep 3B cells. Recently, Bak has been reported to be a mitochondrial membrane pro-
Fig. 2. Changes of Δwm and expressions of proteins involved in mitochondria-dependent apoptosis induced by justicidin A treatment. Inhibitory effects of Z-IETD or cyclosporin A on Δwm and on the percentage of cells in the sub-G1 fraction were evaluated respectively by confocal microscopy and flow cytometry (A). In the experiment, cells were pretreated with 20 μM of Z-IETD or 10 μM of cyclosporin A for 4 h prior to the addition of justicidin A (1 μM) for 30 h. Cells were either stained with rhodamine 123 for confocal microscopy or stained with PI for flow cytometry. Expression of death related proteins in justicidin A-treated hepatoma cells (B). Blots were developed with anti-Bcl-xL or anti-Bax mouse mAB, or with anti-Bak or anti-Bid rabbit pAB.
In this study, mitochondrial Bak expression increases upon justicidin A treatment in HCC cells. Similar result was also reported in Hep G2 cells [36]. The predominant increase in mitochondrial Bak in Hep G2 than in Hep 3B cells may be due to the greater increase in total Bak protein expression in Hep G2 cells upon justicidin A stimulation (data not shown). However, the mitochondrial Bak in Hep G2 cells might not all be in oligomerized form for cytochrome c release (Fig. 1B). Of note, the control of caspase-8 inhibitor (Z-IETD) on Δψm (Fig. 2A) indicates that caspase-8 is an upstream regulator of mitochondria in justicidin A-induced apoptosis. The total blockage of justicidin A-induced apoptosis by either cyclosporine A (Fig. 2A) or caspase-9 inhibitor (Z-LEHD) (Supplementary Fig. 3) demonstrates that this apoptotic process is mitochondria- and caspase-9-dependent, and the direct activation of caspase-3 by caspase-8 only plays a minor role in justicidin A-induced apoptosis.

Both the intrinsic and extrinsic pathways were induced in the HCC cells since both caspase-8 (Fig. 1B) and mitochondria (Fig. 2A and Supplementary Fig. 4) were affected by justicidin A. Our previous experiments indicated that only the intrinsic pathway is stimulated by justicidin A in colorectal carcinoma cells [16], in which, caspase-8 was not activated in either HT-29 or HCT 116 cells. The justicidin A-induced apoptotic pathway in colorectal carcinoma cells begins with the suppression of Ku70, which causes the translocation of Bax to mitochondria. The change of Δψm causes the release of apoptogens (cytochrome c and Smac/DIABLO) to further activate their downstream regulators. In contrast, in these HCC cells, alteration of Ku70 expression was not detected (data not shown).

In conclusion, justicidin A inhibits the growth of HCC cells in vitro and in vivo. Induction of apoptosis is the result of justicidin A cytotoxicity. The lower sensitivity of PBMC to justicidin A illustrates that this natural compound, justicidin A, is selective against malignant cells.

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Appendix A. Supplementary data


References

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