行政院國家科學委員會補助專題研究計畫成果報告
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※爵床之粗萃取物及其純化物 justicidin-A 引起 ※
※類肝癌細胞 Hep 3B 及 Hep G2 凋死之作用機轉 ※
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計畫類別：口個別型計畫
口整合型計畫
計畫編號：NSC 90-2320-B-006-064
計畫編號：NSC 91-2320-B-006-075
執行期間：90年08月01日至92年07月31日

計畫主持人：翁紳誌

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口國際合作研究計畫國外研究報告書一份

執行單位：國立成功大學微免所

中華民國 92 年 12 月 9 日
The antitumor mechanism of *Justicia procumbens* L crude extracts and its pure compound justicia-A mediated apoptosis in human hepatocellular carcinoma Hep 3B and Hep G2

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Abstract

The natural product justicidin A (JA), isolated from the Chinese herb *Justicia Procumbens*, inhibits the growth of human Hep3B and HepG2 hepatocellular carcinoma cells via apoptosis. Fifty percents of effective concentrations (IC₅₀) of JA in Hep3B and HepG2 cells are 0.038 μM and 0.078 μM, respectively, compared to 0.949 μM for Chang liver cells and 23 μM for PBMC at day 6 post-treatment. Cells that succumb to JA undergo apoptosis, which is demonstrated by phosphatidylserine exposure, an increase in sub-G1 content, and DNA fragmentation. The molecular evidence of JA-triggered apoptosis includes caspase-8 activation, increased mitochondrial tBid, Bax and Bak expression, and decreased expression of cellular anti-apoptotic Bcl-2 and Bcl-XL. Concomitantly, we detected loss of ΔΨₘ, mitochondrial release of Smac/DIABLO and cytochrome c into the cytosol, a decrease in XIAP protein levels, and activation of caspase-9 and -3, which in turn increase cleaved PARP levels and decrease DFF45 expression, ultimately causing DNA fragmentation. In addition, JA-induced loss of ΔΨₘ and apoptosis in both cell lines can
be reversed by the caspase-8 inhibitor Z-IETD-fmk and cyclosporin A, indicating that JA activates caspase-9 via caspase-8 activation and mitochondrial dysfunction. Moreover, animal studies showed that oral administration of JA into NOD-SCID mice remarkably inhibited pre-established Hep3B s.c. xenografts without any side effects. Our findings indicate that JA is a promising chemotherapeutic/chemopreventive agent that can induce apoptosis in Hepatocellular carcinoma.

Keywords: Justicidin-A, hepatocellular carcinoma, apoptosis, Bcl-2, cytochrom c, caspases, Smac/DIABLO, SCID mice

二、緣由與目的

Herbaceous plants, traditional Chinese herbal medicines, have been used as food and as medicine for thousands of years. These traditional Chinese medicines have significant roles in treating and maintaining Chinese health, and improving the qualities of Chinese life for many centuries. Many herbal medicines have been verified by the National Center Institute (NCI) in USA as of anticancer and cancer-preventive properties. The whole plant water extracts of *Justicia procumbens* L. is used for treatments of pain, fever, and cancer in Taiwan. In addition, the water extracts of *Justicia procumbens* are very common folklore used in the treatment of viral hepatitis and primary hepatocellular carcinoma (HCC) in National Kengdin park of Pingtung. Previous report have shown that the methanolic extract of the whole plant of *Justicia procumbens* L. (*J. procumbens*) suppressed P-388 lymphocytic leukemia growth in BDF1 mice and caused the cytotoxicity in the 9-KB cell in vitro. Recently, five 2,3-naphtalide lignans including justicidin A (JA), justicidin E, neojusticin A, B and diphyllin have been isolated from methanolic extract of the whole plant of *J. procumbens*. An earlier report indicated that JA exhibited significant inhibition of KB cell growth in vitro.

Apoptosis (programmed cell death) represents a general and a delicately autonomous cellular suicide pathway that helps to govern cell numbers and can occur throughout normal development. Cell death by apoptosis is characterized by reduced cell volume, blebbing, condensed chromatin in the nucleus, DNA fragmentation, and phosphatidylserine externalization, as well as generation of apoptotic bodies. The apoptotic bodies are engulfed by phagocytes and lead to little or no inflammation. Apoptosis has been observed in vitro systems, and can be induced by stimuli including radiation, hyperthermia, and chemotherapeutic agents. These results suggest that different type of cellular damage may lead to the production of common signal pathways that initiate the death of cells.

The current aim of this research is to ascertain therapeutic agents with fewer side effects than the presently used cytostatic or cytotoxic drugs. This has led to emphasis of the studies on effectiveness of various natural products from Chinese traditional herbs. HCC is chosen for presence studies because it is one of the most common malignant neoplasm in China. Its high incidence also reported to cause the first leading death in Taiwan. Unfortunately, HCC is response poorly to chemoprevention and chemotherapy. Therefore, new chemotherapeutic agents with better efficiency and less toxic effects need to be developed, which prompted us to search for potential anticancer agents among natural products of Chinese traditional herbs.

三、Results and Discussion

The cytotoxic activity of JA on Hep3B and HepG2 hepatocellular carcinoma cells was studied by MTT assay. Cells were treated with the JA for up to 6 days. JA inhibited the growth of Hep 3B and HepG2 in a dose-dependent manner ranging from 0.005 to 1 μM. The IC50 (50% cell inhibitory concentration) value for JA on Hep3B and HepG2 cells were 0.038 μM and
0.078 μM, respectively (Table 1). The inhibiting efficacy of Hep3 B and HepG2 cells in soft agar assay was also evaluated. At day 14 post-treatment, approximately 50% of the colonies of both cells were inhibited by JA at concentrations of 0.039 μM and 0.05 μM, respectively (Table 1). Noticeably, the IC_{50} value of JA on Chang liver cells and PBMCs are 0.949 μM and 23 μM, respectively (Table 1).

Based on the cell growth inhibition results, we next investigated whether JA treatment can induce cell death, and if so, which cell death mechanism may be triggered by JA. JA treated- or untreated-tumor cells were stained with Annexin V or PI for various time intervals and analyzed by flow cytometry. DNA fragmentation was determined by gel electrophoresis. As shown in Fig. 2A, exposure of JA (1 μM) resulted in significant increases in the binding of Annexin V-fluorescein to the cytoplasmic membrane (17.76% in Hep3B and 14.56% in HepG2) at 12 h compared to cells exposed to vehicle alone. In addition, the highest numbers of Annexin V-positive cells were observed in Hep3B and HepG2 cells after 72 h treatment with JA (52.2% in Hep3B and 49.66% in HepG2) (Fig. 2A). Furthermore, apoptotic activity was confirmed by staining cells with PI, and the accumulation of cells at sub-G1 phase in both cell lines was measured by Flow cytometry. As shown in Fig. 2B and 2C, JA (2 μM) treatment of Hep3 B and HepG2 cells for 48 h resulted in increase the accumulation of sub-G1 cells 14.72% and 457.24%, respectively and reached their peak levels at 72 h. In addition, JA treatment for 72 h significantly increased the amount of the accumulation of sub-G1 cells in both cell lines in JA dose-dependent manner. To confirm these results, both cell lines were treated with vehicle or JA (0.5-2 μM) for 24, 48, and 72 h, respectively, and a JA time- and dose-related DNA laddering was observed by gel electrophoresis (Fig. 2D and Fig. 2E). Taken together, these data demonstrate that JA treatment of Hep3 B and HepG2 cells results in an changed phosphatidylserine distributions, accumulation of sub-G1 cells, and degradations of DNA, indicating that JA-induced death of both human hepatocellular carcinoma Hep3B and HepG2 cells exhibits typical apoptotic cell death.

Apoptosis involves cascade of proteolytic event, much of which is performed by caspases. To investigate whether caspases are involved JA-induced apoptosis in Hepatocellular carcinoma cells, the expression of activated forms of caspase-8, caspase-9, and caspase-3 following JA treatment in Hep 3 B and Hep G2 cells was assessed by immunoblotting. As shown in Fig. 3A, the expression of both the 43- and 41-kDa active forms of caspase-8 slightly appeared within 6 h in Hep3B cells and within 12 h in HepG2 following JA treatment, reaching a peak at 72 h in both tumor cells. Additionally, both 37- and 35-kDa activated forms of caspase-9 also appeared within 6 h in both tumor cells following JA treatment, reaching as peak at 72 h in both cell lines (Fig. 3B). Furthermore, both the 17- and 12-kDa activated forms of caspase-3 appeared within 12 h of JA treatment in both tumor cells, reaching peak levels at 72 h (Fig. 3C). Moreover, Fig. 3 A, 3B and 3C also show that JA treatment for 48 h in Hep3B and HepG2 cell lines significantly increased the activated forms of caspase-8, -9 and -3 in both tumor cell lines in a JA dose-dependent fashion. No active forms of any of the caspases were observed in untreated cell lines (Fig. 3A, 3B, and 3C). Caspase-2 was not involved in JA-induced apoptosis in either cell (data not shown). Moreover, treatment with either the caspase-8 inhibitor Z-IETD-fmk or caspase-9 inhibitor Z-LEHD-fmk almost completely blocked JA-induced apoptosis in both tumor cells (Fig. 3D). These results indicated that JA-induced apoptosis occurs via activatio of caspase-8 and -9 in human hepatocellular carcinoma Hep 3B and Hep G2 cell lines.

PARP protein is a pivotal substrate for active caspases, and cleavage of PARP is
indicative apoptosis, in which PARP (116-kDa) is cleaved into a 89-kDa product during apoptosis. To investigate the cleavage of PARP, Hep3B cells were treated with JA (1 μM). The 89-kDa cleaved form of PARP appeared within 24 h, reaching peak level at 72 h (Fig. 2F). Moreover, the levels of cleavage form of PARP also slightly increased at 48–72 h in HepG2 cells following JA (1 μM) treatment (Fig. 2F). In addition, treatment with JA ((1 μM) for 48 h in Hep3B cells significantly increased the level of cleaved PARP in a dose-dependent manner. Similarly, no PARP cleavage was detected in untreated-cells.

Caspase-3 can cleave its substrate DFF45 prior to the initiation of apoptotic DNA fragmentation and DFF45 can cleave into fragment s of 30-kDa and 11-kDa. As shown in Fig. 2G, treatment of Hep3B cells with 1 μM of JA for indicating time intervals, DFF45 is slightly decreased within 24 h and was maximal at 72 h. In addition, the 30-kDa cleaved form of DFF45 appeared as early as at 6 h and increased its plateau from 6 to 72 h. Furthermore, the 30-kDa cleaved form of DFF45 also increased at 24–72 h in Hep G2 cells following JA (1 μM) treatment (Fig. 2G). Figure 2G also shows that JA treatment for 48 h in Hep 3B cells significantly increased the amount of cleaved DFF45 in a JA dose-dependent manner. No 30-kDa cleaved form of DFF45 was detected in both untreated cells. These data demonstrated that caspase activation, and cleavage of PARP and DFF45 are involved in JA-induced apoptosis in both hepatocellular carcinoma tumor cells.

Cytochrome c and Smac/DIABLO release from mitochondria into the cytosol is required for the activation of caspases and the fragmentation of DNA. Therefore, the release of mitochondrial cytochrome c and Smac/DIABLO into the cytosol was examined by immunoblot analysis. As shown in Fig. 3A, 3B and 3C, cytosolic levels of cytochrome c and Smac/DIABLO increased significantly in both cells within 24 h and reached peak levels at 48–72 h following treatment with JA (1 μM). Conversely, levels of cytochrome c and Smac/DIABLO decreased in the mitochondria-enriched fractions of both cell lines within 24 h and became undetectable 72 h following JA (1 μM) treatment. The levels of cytochrome c and Smac/DIABLO were significantly increased in the cytosol and decreased in the mitochondria-enriched fraction in a JA dose-related manner after 48 h in Hep3B cells of JA treatment (0.125–1.5 μM) (Fig. 3A and 3B). In contrast, in the absence of JA treatment, cytosolic levels of cytochrome c and Smac/DIABLO remained low (Fig. 5A, 5B, 5C, and 5D). Our data suggest that the release of cytochrome c and Smac/DIABLO from mitochondria into the cytosol plays a role in JA-induced apoptosis in Hep3B and HepG2 cells.

XIAP is an inhibitor of apoptosis protein which can inhibit cell death by interaction with caspases. Smac/DIABLO has been reported to antagonize the antiapoptotic function of XIAP, an inhibitor of apoptosis that acts through caspases. We therefore investigated whether Smac/DIABLO and XIAP are involved in JA-induced apoptosis. The cell lysates of vehicle- or JA-treated Hep 3B and HepG2 cells were analyzed by Western blotting. As shown in Fig. 3D, XIAP levels slightly decreased within 12 h and reached a minimum at 72 h in Hep3B and 48 h in HepG2 cells after JA treatment (1 μM). In addition, XIAP protein levels were significantly reduced in a JA dose-dependent manner (0.125–1.5 μM) (Fig. 3D). In contrast, XIAP levels were not affected in vehicle-treated cells. These results confirm that the activation of caspase-9 and caspase-3 by JA in Hep3B and HepG2 cells may correlate with an increase of Smac/DIASBLO in the cytosol and a decrease in XIAP protein levels.

The Δψm is an early event of apoptosis, and a decrease of Δψm is associated with mitochondrial dysfunction. Changes of Δψm in apoptotic cells were analyzed using the mitochondrial dye rhodamine 123 or
DiOC₆(3) and by staining with PI. As shown in Fig. 4A, 4B and 4C, a significant, time-dependent decrease in rhodamine 123 and DiOC₆(3) intensity in the mitochondria in JA-treated Hep3B and HepG2 cells (1 μM JA) was observed. The loss of Δψₘ was observed as early as 12 h and reached a minimum 72 h after JA treatment in both cell lines (Fig. 4A, 4B and 4C). Furthermore, treatment of HepG2 cells with various concentrations of JA (0.125–1.5 μM) for 48 h resulted in a dose-dependent loss of Δψₘ (Fig. 4A and 4B). This time-dependent loss was confirmed with confocal microscopy using rhodamine 123 staining, which showed a decrease at 12 h and reached its lowest levels 72 h after JA treatment in both cell lines (Fig. 6B and 6C). In addition, a JA dose-dependent (0.25 μM –10 μM) loss of Δψₘ was also confirmed with confocal microscopy (Fig. 6B and 6C). In contrast, there was no change of Δψₘ in vehicle-treated cells as measured by DiOC₆(3) flow cytometry, or rhodamine 123 confocal microscopy staining (Fig. 6A, 6B, and 6C). Moreover, treatment of both cell lines with 20 μM Z-VDVAD-fmk or 10 μM cyclosporin A (a ligand of cyclophilin D) prior to the addition of JA blocked mitochondrial dysfunction as imaged by confocal microscopy and JA-induced apoptosis measured by PI flow cytometry (Fig. 6D and 6E), indicating that mitochondrial dysfunction in JA-induced apoptosis requires the upstream action of caspase-2 as well as changes in mitochondrial membrane permeability. Taken together, our results confirm that a loss of Δψₘ is essential for the JA-induced apoptotic release of cytochrome c and Smac/DIABLO from the mitochondria into cytosol.

To determine whether XIAP protein is involved in the action of JA, whole cell lysates of vehicle- or JA-treated Hep3B and HepG2 cells were analyzed by Western blotting. As shown in Fig. 3D, the amount of XIAP significantly decrease within 12 h, and maximal at 72 h in Hep3B and 48 h in HepG2 cells, respectively, after treatment with 1 μM of JA. In addition, the decrease of XIAP in JA (ranging from 0.125 to 1.5 μM)-treated Hep3B cells was significantly showed a dose-dependent fashion (Fig. 3D). These data indicated that the promotion of caspase-9 and caspase-3 activities by JA in Hep3B and HepG2 cells may correlated with the increase of cytochrome c and Smac/DIABLO in cytosol, and decrease in protein level of XIAP.

The mitochondrial membrane potential (Δψₘ) is an early event of apoptosis and decreasing in Δψₘ is associated with mitochondrial dysfunction. Changes of Δψₘ in apoptotic cell were analyzed by the using the mitochondrial dye rhodamine 123 or DiOC₆(3), and by staining with PI. As shown in Fig. 4A, 4B and 4C, a significant decrease in the accumulation the intensity of rhodamine 123 or DiOC₆(3) on mitochondria in JA- (1 μM) treated Hep3B and HepG2 cells was observed in a time- and dose-dependent manner. The loss of Δψₘ was observed within 6 h, and peaked at 72 h after treatment of JA in both tumor cells. Furthermore, treatment of Hep 3B cells with various concentrations of JA (ranging from 0.5 to 5 μM) for 48 h resulted in loss of Δψₘ in a dose-related manner (Fig. 4A and 4B). In addition, the loss of Δψₘ showed significant time-dependent manner after treatment with JA in both tumor cells using confocal microscopy rhodamine 123 staining image (Fig. 4D). In contrast, there was no change in Δψₘ in vehicle treated-cells measuring by rhodamine 123 and DiOC₆(3) flow cytometry or by rhodamine 123 confocal microscopy staining image (Fig. 4A, 4B, 4E and 4D). Moreover, treatment of both cells with 20 μM of Z-IETD-fmk or 10 μM cyclosporin A (a inhibitor of cyclophilin D) prior to the addition of JA (1 μM) blocked mitochondrial dysfunction imaging by confocal microscopy (Fig. 4E) and JA-induced apoptosis measuring by PI flow cytometry (Fig. 4E), indicating that mitochondrial dysfunction in JA-induced apoptosis required the action of upstream caspase-8 and changes in mitochondrial membrane permeabilization of tumor cells.
Taken together, these results further indicate that the loss of Δψm is essential for the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol, and thereby induces its apoptotic effect in human hepatocellular carcinoma cells by the treatment of JA.

The Bcl-2 family proteins are the best characterized regulators of apoptosis such as Bcl-2 and Bcl-XL (suppress apoptosis), whereas others, such as Bid, Bax and Bak (promote apoptosis) \(^{38-40}\). To examine whether the levels of death-related proteins in these cells are involved in JA-induced apoptosis, we determined the regulation of Bcl-2, Bcl-XL, Bid, Bax and Bak protein levels during JA-induced apoptosis. Both tumor cells were treated with different concentrations of JA at the indicated various time intervals, Western blot analysis was performed to analyze the protein expression. As shown in Fig 5A, Hep3B cells did not show any Bcl-2 protein level in this experiments, whereas the total and mitochondria-enriched fraction protein of HepG2 cells were showed a decrease in the Bcl-2 protein from 24 to 72 h after treatment of JA (1 μM) and these expressions were showed in a time-dependant fashion. Moreover, treatment of Hep3B cells with various concentrations of JA (ranging from 0.125 to 1.5 μM) for indicated time intervals resulted in decrease of Bcl-XL protein expression in whole cell lysates and mitochondria-enriched fraction in a time- and JA dose-dependent manner (Fig 5B). The amount of whole cell lysates and mitochondria-enriched fraction of Bcl-XL significantly decrease within 6 h, and maximal at 72 h in Hep3B after treatment with 1 μM of JA (Fig 5B). A similar time-related decline of Bcl-XL protein levels in mitochondria-enriched fraction were also observed in JA-treated HepG2 cells (Fig 5B). In this study, we also determined the changes in amount of Bax and Bak of total, cytosol, and their mitochondria-enriched fraction in response to JA treatment in both tumor cells. As shown in Fig. 5C and 5E, the mitochondria enriched fraction of Bax and Bak slightly increased within 6 h, peaked its level at 12-72 h in Hep3B tumor cells after treatment of JA (1 μM), but no change of total Bax and Bak in Hep3B cells. In addition, the mitochondria-enriched fraction of Bax dramatically increased in a dose dependent manner in Hep3B after treatment of JA (Fig. 5C). In contrast, the amounts of cytosolic Bax and Bak of both tumor cells were slightly decreased in a time-related manner after treatment of JA (Fig. 5C, 5D and 5E). Moreover, the mitochondria enriched fraction of tBid slightly increased at 6 h, reaching peak levels at 72 h in both tumor cells. Conversely, the amounts of total and cytosolic Bid of both tumor cells were slightly decreased in a time-related manner after treatment of JA. These results indicated that Bcl-XL, Bax, Bak and Bid protein in both tumor cells are involved in the mechanism of JA-induced apoptosis. In addition, JA-induced apoptosis was accompanied with the decrease of Bcl-2 protein levels in HepG2 cells, but this observation was not found in Hep3B cells.

We than ask whether JA is able to prevent the growth of pre-existing Hep3B cells implanted-NOD-SCID mice. Mice were inoculated Hep3B cells s.c. into right flank on day 0 and divided randomly into two groups on day 4. One group (N=6) orally administered daily with JA (32 mg/kg) for 60 consecutive days. The control group (N=7) orally received vehicle. The tumor volume and body weight were recorded from day 9 (treatment started) until day 60 (treatment stop). Tumor-bearing mice treated with 32 mg/kg of JA drastically inhibited tumor growth and tumor weight (P < 0.05) as compared to vehicle-treated mice (Fig 6).

The present study is the first report to show the evidence that JA is able to inhibit the cell growth and the cologeneity of human hepatocellular carcinoma Hep3B and HepG2 cells, and to induce cell death through apoptosis in a time- and dose-dependent manner. The induction of apoptosis by the release of cytochrome c from mitochondria to cytosol, decrease of
XIAP protein contents, activation of caspases, fragmentation of DNA and the apoptosis of hepatocellular carcinoma Hep3B and HepG2 cells. In this study, treatment with JA induced cell death was monitored by MTT assay, colony formation assay, phosphatidylserine translocation to outer leaflet of cytoplasmic membrane and sub-G1 peak formation in flow cytometry analysis, and fragmentation of DNA by agarose electrophoresis. Based on these results, JA-induced human hepatocellular carcinoma Hep3B and HepG2 cell death was proved to be a typical apoptosis processes.

The process of apoptosis can be triggered by multiple ways, including activation of cell surface death receptors of TNFR family, oxidative stress, γ-irradiation, chemotherapeutic and chemopreventive agents. Chemotherapeutic drug-induced apoptotic pathways are complicated, especially involving signal transductions in cells. The mechanism of apoptosis is executed with a cascade of sequential activation of initiator and effector caspases. Caspases, the cysteine protease family, are selectively cleaved at an aspartate residue to produce the active enzyme. Once activated, the effector caspases can cleave various death substrates, which in turn lead to the morphological changes and finally cause apoptosis. Functional caspase-3 is essential for apoptotic processing including chromatin condensation and DNA fragmentation in all cell types. It has been suggested that caspase-9 can be activate down stream of caspase-3. In addition, caspase-3 has more specific activity for PARP cleavage than caspase-9. Activation of caspase-9 and caspase-3 requires proteolytic processing. Their inactive proforms are 47 and 32 kDa. Upon activation, caspase-9 is cleaved into activated form of 37 and 35 kDa. Caspase-3 then cleaved into 17 and 12 kDa. In present study, treatment with JA resulted in a dose- and time-dependent processing of caspase-9 and -3 into 37 and 35, 17 and 12 kDa active fragment, respectively. It is noteworthy that the amounts of these active fragments of caspase-3 were significantly increased after treatment with JA (1 µM) for 24 h. This data is consistent with the time point of activation of caspase-9. Therefore, the activation of caspase-9 occurred prior to activation of caspase-3.

Mitochondria play an important role in the regulation of apoptosis and the mechanism of cytochrome c-dependent apoptosis is well defined. The release of cytochrome c from mitochondria into the cytosol has been suggested as pivotal events in apoptosis. Anti-cancer drugs induced apoptosis has been related to mitochondrial damage, and thereby loss of the mitochondrial membrane potential (ΔΨm) leads to the release of cytochrome c from the mitochondrial intermembrane space. In response to apoptotic stimuli, cytochrome c translocators from mitochondria to the cytosol, the release of cytochrome c binds to Apaf-1 and then induces the aggregation and processing of capase-9, which in turn activates downstream caspases. In our studies, JA-treated cells led to the release of cytochrome c from the mitochondria to cytosol and then to activate downstream caspases. Consist with this finding, the mitochondria in vehicle- or JA-treated cells before 24 h retained much more cytochrome c than those of the cells after 24 h in JA-treated cells. IAPs, inhibitor of apoptosis proteins, regulate intrinsic cellular multifunctions including apoptotic signalling, cell cycle regulation and ubiquitylation, which are the endogenous inhibitors that block both the initiator and effector caspases. Previous in vitro kinetic studies have shown that XIAP is the most potent caspase inhibitor in the IAP family and binds only to processed caspase-9 but not to the inactive procaspase-9. Smac/DIABLO, a mitochondrial protein that negatively regulates the caspase-inhibiting activity of IAPs, by binding to XIAP and results in a conformational change in XIAP leading to the decreased in affinity of XIAP for caspases and relieves the caspase-9 to
promote apoptosis 34,49,60. In this study, treatment of JA resulted in the decrease of XIAP protein contents in a dose- and time-dependent fashion. It is worthy to provide kinetic analysis to demonstrate the time point of activation of caspase-9 prior to the decreases of XIAP content. Taken together with our findings, treatment of JA lead to overwhelm XIAP-caspase-9 interaction, and caspase-3 is then activated by small amounts of active caspase-9. The cleaved of caspase-9 in caspase cascade induces cell apoptosis.

Mitochondria play an important role in the regulation of apoptosis 53 and the mechanism of cytochrome c- and Smac/DIABLO action to induce apoptosis has well been defined 34,56,65. In response to apoptotic stimuli, cytochrome c and Smac/DIABLO translocat from mitochondria to cytosol, then bind to Apaf-1 and induce the aggregation and cleavage of caspase-9, which in turn activates downstream caspases 60. Anti-cancer drugs induced apoptosis could cause the loss of Δψm and release of cytochrome c and Smac/DIABLO from the mitochondrial intermembrane space have been reported 58,66. Similarly, in JA-treated cells above apoptosis-related events were also detected, indicating that similar apoptotic mechanism is functioning, which will trigger the activation of downstream caspases. Furthermore, we clearly showed that in JA treated hepatocellular carcinoma cells (HCC) Smac/DIABLO was released from mitochondria caused decrease expression of XIAP, which further in turn activates caspase-9. The activated caspase-9 then triggers the downstream caspase cascades to induce cell apoptosis. We also confirmed that in JA-treated cells caspase-8 activation triggered the mitochondrial dysfunction of the Hepatocellular carcinoma, which was demonstrated by the inhibition of caspase-8 inhibitor Z-IETD-fmk. Apoptotic mitochondrial permeability transition appears to be mediated by opening of the permeability transition pore complex including cyclophilin D, adenine nucleotide translocator and voltage-dependent anion channel 67,68. Cyclosporin A, an inhibitor of cyclophilin D, directly binds to mitochondrial cyclophilin D and blocks permeability transition 68,69. In this study, cyclosporin A prevented JA-induced apoptosis and reduced Δψm, suggesting that cyclophilin D related permeability transition pore complex is involved in JA induced apoptosis, which causes the release of cytochrome c and Smac/DIABLO. Taken together, in JA treated HCC, caspase-8 is the upstream regulator of mitochondria dysfunction, which triggers mitochondrial alteration including loss of Δψm or release of cytochrome c and Smac/DIABLO related downstream caspase cascades.

The members of Bcl-2 family can either inhibit (Bcl-2 and Bcl-Xl) or induce (Bax and Bad) apoptosis 68,70,71. Bcl-2 members also control the process of mitochondria-mediated apoptosis, including the release of cytochrome c and Smac/DIABLO 72. Our data showed that Bcl-Xl protein levels decreased in both cells after treatment of JA. Bcl-2 was undetectable in Hep3B cells with or without JA treatment. Differently, Bcl-2 protein was substantially expressed in HepG2 cells at a time- and JA dose-dependent manner. Bax and Bak play a pivotal role in to mitochondrial dysfunction in response to various apoptotic stimuli or stress 73,74. The interaction between Bcl-2 family proteins and the mitochondrial membrane can regulate the releases of cytochrome c and Smac/DIABLO from mitochondria to cytosol 66,73,75. Previous studies demonstrated that anticancer agents-induced a caspase-2 activation dependent apoptosis, which needs translocation of Bax and Bak from cytosol to mitochondria and release of cytochrome c and Smac/DIABLO to cytoplasm required 76,77. Consistently, our data also showed that the treatment of JA with HCC resulted in activation of caspase-8 and upregulation of Bax and Bak protein expressions. All together, we interpret that in JA treated HCC caspase-8 triggered translocation of Bax and Bak to mitochondria, and
subsequently, cytochrome c and Smac/DIABLO were released into cytosol to downstream signals of apoptotic death cascades. Most importantly, we demonstrate that the nature product of JA effectively suppressed the tumor growth in mice, which were xenograftly inoculated with Hep3B cells. Based on our present in vitro and in vivo study, JA is a potential anticancer agent because of its lack of toxicity and side effects in tested cells and animals.

In summary, we clearly show that JA could induce apoptosis of two HCC Hep3B and HepG2 cell lines. JA initially induces activation of caspase-8 and inhibits the expressions of Bcl-XL and Bcl-2. JA also upregulates the expression of Bax and Bak, resulting in an altered ratio to Bcl-XL and Bcl-2, which then trigger mitochondria-mediated caspase cascades and cause cell death. Furthermore, dietary oral administration of JA inhibits significantly HCC xenograft in NOD-SCID mice. Taken together, our findings demonstrate that JA is a promising chemopreventive or chemotherapeutic agent against HCC.

References


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Table 1. Effects of JP crude extract and its pure compound JA response for 50% growth inhibition (IC$_{50}$) on human hepatocellular carcinoma by MTT assay.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>(JA) growth inhibition (µM)</th>
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<tr>
<td>Hep 3B</td>
<td>0.038 ± 0.006</td>
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<tr>
<td>Hep G2</td>
<td>0.078 ± 0.008</td>
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<tr>
<td>Chang liver cells</td>
<td>0.049 ± 0.117</td>
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<td>PBMC</td>
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Fig. 1A

Hep3B MC540

HepG2 MC540

Fig. 1B

Hep 3B

Fig. 1C

Hep G2

Fig. 1D

D

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<thead>
<tr>
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