Bitter melon juice activates cellular energy sensor AMP-activated protein kinase causing apoptotic death of human pancreatic carcinoma cells

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Prognosis of pancreatic cancer is extremely poor, suggesting critical needs for additional drugs to improve disease outcome. In this study, we examined efficacy and associated mechanism of a novel agent bitter melon juice (BMJ) against pancreatic carcinoma cells both in culture and nude mice. BMJ anticancer efficacy was analyzed in human pancreatic carcinoma BxPC-3, MiaPaCa-2, AsPC-1 and Capan-2 cells by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, cell death enzyme-linked immunosorbent assay and annexin/propidium iodide assays. BMJ effect on apoptosis regulators was assessed by immunoblotting. In vivo BMJ efficacy was evaluated against MiaPaCa-2 tumors in nude mice, and xenograft was analyzed for biomarkers by immunohistochemistry (IHC). Results showed that BMJ (2–5% v/v) decreases cell viability in all four pancreatic carcinoma cell lines by inducing strong apoptotic death. At molecular level, BMJ caused caspases activation, altered expression of Bel-2 family members and cytochrome-c release into the cytosol. Additionally, BMJ decreased survivin and X-linked inhibitor of apoptosis protein but increased p21, CHOP and phosphorylated mitogen-activated protein kinases (extracellular signal-regulated kinase 1/2 and p38) levels. Importantly, BMJ activated adenosine monophosphate-activated protein kinase (AMPK), a biomarker for cellular energy status, and an AMPK inhibitor (Compound C) reversed BMJ-induced caspase-3 activation suggesting activated AMPK involvement in BMJ-induced apoptosis. In vivo, oral administration of lyophilized BMJ (5 mg in 100 μl water/day/mouse) for 6 weeks inhibited MiaPaCa-2 tumor xenograft growth by 60% (P < 0.01) without noticeable toxicity in nude mice. IHC analyses of MiaPaCa-2 xenografts showed that BMJ also inhibits its proliferation, induces apoptosis and activates AMPK in vivo. Overall, BMJ exerts strong anticancer efficacy against human pancreatic carcinoma cells, both in vitro and in vivo, suggesting its clinical usefulness.

Introduction

Pancreatic cancer is an aggressive malignancy that develops in a relatively symptom-free manner and is usually at advanced stage at the time of diagnosis. Typically, it takes about 1–2 decades for the development of clinically defined ‘pancreatic cancer’, but symptoms are not obvious till the late stage of the disease. Therefore, pancreatic cancer is often termed as a ‘silent killer’. Last year alone, ~44 030 new cases of pancreatic cancer were reported in the USA, with ~37 660 associated deaths (1). Gemcitabine is the frontline chemotherapeutic treatment in pancreatic cancer patients, but the remedial and survival benefits of chemotherapy alone or in combination with other therapies are extremely low as the median life of pancreatic cancer patients postdiagnosis is <6 months and overall 5 year survival is 3–5% (2). These statistical facts clearly show that pancreatic cancer is dreadful and untreatable, and that there is an urgent need to identify additional novel and effective agents to manage pancreatic cancer as well as its progression to aggressive stage.

Bitter melon (Momordica charantia, Family: Cucurbitaceae) is a commonly consumed vegetable in the Asian and African continents (3,4), and there is a growing interest in bitter melon because of its beneficial effects against diabetes, obesity, hyperlipidemia and so on (4,5). Bitter melon has been evaluated in human population in several clinical trials for its antidiabetic effects and has plenty of human safety data (4–6). Besides its antidiabetic effects, bitter melon extract and its bioactive compounds have shown anticancer efficacy against leukemia, breast, prostate and colon cancers (4,7–11); however, there is no published report on bitter melon’s efficacy against pancreatic cancer. In this regard, it is important to emphasize here that a direct correlation has been established in recent studies between diabetes and pancreatic cancer (12), and the use of antidiabetic drug metformin has been associated with reduced risk and improved survival in diabetic patients with pancreatic cancer (13).

Cancer cells gain growth advantage by shifting their metabolism to glycolysis (termed as ‘Warburg effect’), where much of the cellular adenosine triphosphate (ATP) is generated by glycolysis rather than oxidative phosphorylation (14–17). In case of depletion of intracellular energy by energy restriction or energy restriction-mimetic agents, ATP level drops and adenosine monophosphate (AMP) level rises, leading to allosteric activation of AMP-activated protein kinase (AMPK) by redundant AMP (16–18). AMPK is a highly conserved serine/threonine protein kinase, now regarded as a ‘fuel sensor’ of the biological system and is an essential link between cellular metabolism and signaling pathways (16,19). AMPK is also activated by its phosphorylation at Thr172 site by upstream kinases such as LKB1 and TAK1 (16). Activated AMPK phosphorylates a series of substrates including rate-limiting enzymes in fatty acid and cholesterol synthesis and glucose metabolism, thereby curbing cellular ATP consumption (16,17,19,20). Activated AMPK also inhibits mammalian target of rapamycin signaling and protein translation as well as targets several signaling molecules such as p53, p73, cyclin-dependent kinase inhibitors, Sirt1, caspase-3 and so on (16,20). AMPK activation represses cancer cells growth and induces apoptosis by targeting the metabolism and signaling pathways (19,21–24); therefore, AMPK is suggested as a new target for cancer therapy. Importantly, Cucurbite triterpenoids from bitter melon are known to activate AMPK in L6 muscle cells and 3T3L1 adipocytes (25) and using AMPK inhibitor pyrazolopyrimidine, Grossmann et al. (8). have shown in breast cancer cells that the antiproliferative effect of eleostearic acid, which constitutes about 60% of bitter melon seed oil, is partly dependent upon AMPK activation.

Taken together, based on above-described studies showing: (i) strong antidiabetic and anticancer effects of bitter melon, (ii) a direct correlation between diabetes and pancreatic cancer (iii) and that bitter melon constituents activate AMPK, in this study, we examined, for the first time, the anticancer activity of bitter melon juice (BMJ) and the involvement of AMPK activation in its efficacy against human pancreatic carcinoma cells. Our results show that BMJ inhibits the
growth of human pancreatic carcinoma cells both in vitro and in vivo, and that BMJ induces apoptotic cell death by altering the balance between proapoptotic and antiapoptotic molecules and by activating AMPK in pancreatic carcinoma cells.

Materials and methods

Reagents

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent, Harris hematoxylin, β-actin antibody, formic acid and ammonium acetate were purchased from Sigma–Aldrich (St Louis, MO). Antibodies for cleaved caspase-3, cleaved caspase-9, phosphorylated extracellular signal-regulated kinase (ERK) 1/2 and p38, total ERK1/2 and p38, phosphorylated AMPK (Thr172), total AMPK-α, Bak, Bcl-2, X-linked inhibitor of apoptosis protein (XIAP) and antirabbit peroxidase-conjugated secondary antibody were from Cell Signaling (Danvers, MA). Antibody against survivin was from Novus Biologicals (Littleton, CO). Annexin V-Vybrant apoptosis assay kit was from Molecular Probes (Eugene, OR). AMPK inhibitor Compound C was from Calbiochem (La Jolla, CA). Diaminobenzidine kit was from Vector Laboratories (Burlingame, CA). Streptavidin and proliferating cell nuclear antigen (PCNA) antibody were from Dako (Carpinteria, CA). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay kit was from Promega Corporation (Madison, WI). Authentic samples of ‘Momordicine I’, ‘Momordicine II’ and ‘Kuguaglycoside G’ were from Dr Jun Ma (FDA; College Park, MD) and ‘Cucurbitacin I’ was from Sigma–Aldrich; their structures are shown in Figure 1. High-performance liquid chromatography–grade methanol, acetonitrile and water were from Fisher Scientific (Pittsburgh, PA).

BMJ preparation

Bitter melons (Chinese variety) were purchased from a local grocery store. Fruits were washed with water and wiped to dryness. They were then slit horizontally to remove pulp and seeds. After deseeding, fruits were weighed and cut into 2–5% (v/v in medium) of pure BMJ was used for cell culture studies. For in vivo studies, BMJ was lyophilized to give light yellow–green foam, which was grounded into a fine powder and stored protected from light at 4°C. As needed, 2–5% (v/v in medium) of pure BMJ was used for cell culture studies. For in vivo studies, BMJ was lyophilized to give light yellow–green foam, which was grounded into a fine powder and stored protected from light at 4°C.

BMJ analysis

BMJ has several chemical constituents including triterpenes, glycosides, saponins, alkaloids, oils, proteins and steroids (4). We analyzed four triterpenes namely Momordicine I, Momordicine II, Kuguaglycoside G and Cucurbitacin I in BMJ. We established a liquid chromatography/tandem mass spectrometry (MS) method to monitor the stability as well as batch to batch consistency/ reproducibility of BMJ and lyophilized powder, as detailed in Supplementary Method, available at Carcinogenesis Online. The Supplementary Table 1 and Supplementary Figures 1–9, available at Carcinogenesis Online, provide additional information pertaining to these methods and observations.

Cell viability assay

Human pancreatic carcinoma BxPC-3 and MiaPaCa-2 cells were from American Type Culture Collection (Manassas, VA). AsPC-1 and Capan-2 cells were kindly provided by Dr Colin D. Weekees (University of Colorado, Denver). BxPC-3 cells were cultured in RPMI 1640 with 10% fetal bovine serum; AsPC-1 and Capan-2 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum with essential amino acids; and MiaPaCa-2 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 2.5% horse serum under standard culture conditions (37°C, 95% humidified air and 5% CO2). Cells were plated at a density of 2000 cells per well in 96-well plates, and after 24 h, cells were treated with BMJ (0–5% v/v) for 24–72 h. At the end of each treatment time, fresh media containing 20 μl of MTT (5 mg/ml stock) was added, and cells were incubated for another 3 h in CO2 incubator. Thereafter, media was removed from each well, dimethylsulfoxide was added and the color intensity was estimated by measuring absorbance at 570 nm using a plate reader.

Apoptosis assays

BxPC-3 and MiaPaCa-2 cells were collected via brief trypsinization following treatment with BMJ for 24 h and the extent of apoptosis was determined with cell death enzyme-linked immunosorbent assay kit (Roche, Mannheim, Germany). In another apoptosis assay, at the end of BMJ treatment, cells were stained with apoptosis assay kit 2 (Molecular Probes) following the manufacturer’s protocol, and the extent of apoptosis was determined by flow cytometry analysis of annexin V-propidium iodide-stained cells.

Western blot analysis

Human pancreatic carcinoma cells were treated with BMJ and total cell lysates or cytosolic fractions were prepared following published methods (26). The protein concentration of lysates was estimated using Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8–16% Tris–glycine gels and blotted onto nitrocellulose membranes. Membranes were probed with specific primary antibodies overnight at 4°C followed by peroxidase-conjugated appropriate secondary antibody for 1 h at room temperature and visualized by enhanced chemiluminescence detection system from GE Healthcare (Buckinghamshire, UK). For certain proteins, membranes were also probed with appropriate

Fig. 1. Chemical structure for ‘Momordicine I’, ‘Momordicine II’, ‘Kuguaglycoside G’ and ‘Cucurbitacin I’.
secondary IR dye-tagged antibodies and visualized using Odyssey infra-red imager (LI-COR Biosciences, Lincoln, NE). Membranes were also stripped and re-probed again for protein of interest or β-actin antibody to check protein loading; however, only representative β-actin blots are shown.

**Xenograft study**

All the protocols used were approved by the institutional animal care and use committee of the University of Colorado. Athyinic (BALB/c, nude) male nude mice (4 weeks old) were obtained from NCI (Frederick, MD) and fed irradiated AIN76A powdered diet (Dyets, Bethlehem, PA) and water ad libitum. For xenograft study, ~3 million MiaPaCa-2 cells were mixed with matrigel (1:1) and injected subcutaneously into the right flank of each nude mouse. The next day (day 1), mice were randomly distributed into two groups (n = 7 for each group) and were administered via oral gavage either water (100 µl) or hypo-osmolar BMJ powder (5 mg/100 gmouse/day) for 6 weeks. Body weight of each mouse was monitored regularly throughout the study. Once the xenograft started growing, its size was measured in two dimensions using digital vernier calipers. Tumor volume was calculated using the formula 0.5236 L₁(L₂)³, where L₁ and L₂ represent the long and short axis of the tumor measurements, respectively. At the end of the study, each tumor was carefully dissected and weighed, and then fixed in formalin and processed for immunohistochemistry (IHC) analysis.

**IHC analyses**

Tumor samples were processed and immunostained following published methods (27–29). Percentage of PCNA- and TUNEL-positive cells was calculated by counting the number of positive-stained cells (brown stained) and the total number of cells at five arbitrarily selected fields from each tumor at ×400 magnification. AMPKβ1/2 immunoreactivity was analyzed in five random areas for each tumor tissue and was scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining) and 4+ (very strong staining).

**Statistical analyses**

All statistical analyses were carried out with Sigma Stat software version 2.03 (Jandel Scientific, San Rafael, CA). Statistically significant difference between the control and treated groups were determined either by unpaired Student’s t-test or one-way analysis of variance followed by Bonferroni t-test.

**Results**

**Chemical analysis and composition of BMJ**

As depicted in Supplementary Figures 1–4, available at Carcinogenesis Online, the compounds (Momordicine I, Momordicine II, Kuguaglycoside G and Cucurbitacin I, respectively) were observed to ionize as their corresponding hydrate forms. Upon establishing an liquid chromatography/MS–MS method to monitor these authentic samples, we sought to prepare standard curves, which may be subsequently used to quantify these known compounds in different batches of BMJ and/or powder. As depicted in Supplementary Figure 5, available at Carcinogenesis Online, we were able to observe linear standard curves; data were fit to 1/x² weighted linear regression models (IHC) analysis.

**Apoptosis induction**

Apoptosis induction involves a change in balance between anti-apoptotic and proapoptotic molecules toward apoptosis. Accordingly, next we examined the effect of BMJ treatment on several molecular regulators of apoptosis in both BxPC-3 and MiaPaCa-2 cells. Western blot analyses showed that BMJ treatment activated caspase-3 and caspase-9 in both cell lines (Figure 3C). We also found that BMJ had differential effect on the expression of antiapoptotic molecules Bcl-2 and Bcl-XL depending upon the cell type (Figure 3C). Bcl-2 levels were significantly decreased in BxPC-3 cells without an effect on Bcl-XL except at the highest concentration (4% BMJ v/v) and 48h of treatment (Figure 3C). Conversely, Bcl-2 remained largely unaffected in MiaPaCa-2 cells except at the highest concentration (4% BMJ v/v) after 48h of treatment, but Bcl-XL levels decreased strongly with BMJ treatment (Figure 3C). Importantly, BMJ caused the upregulation of proapoptotic Bak in both the cell lines (Figure 3C). BMJ treatment also led to the downregulation of XIAP and survivin levels in both cell lines (Figure 3C). Besides Bcl family members, several other molecules (e.g. p21, CHOP, ERK1/2, p38) have also been directly or indirectly associated with apoptosis (30–34). Accordingly, we also assessed BMJ effect on these molecules, and as shown in Figure 3C, BMJ treatment also enhanced p21, CHOP, phosphorylated ERK1/2 and phosphorylated p38 levels in both BxPC-3 and MiaPaCa-2 cell lines without affecting total ERK1/2 and p38 levels. As above results suggested the involvement of intrinsic pathway in BMJ-caused apoptosis, next we studied the release of cytochrome-c from mitochondria to cytosol, and BMJ treatment of both BxPC-3 and MiaPaCa-2 cell lines resulted in cytochrome-c release into the cytosolic fraction (Figure 3D), suggesting that BMJ-induced apoptotic death of pancreatic cancer cells does involve intrinsic apoptotic mechanism.
**BMJ activates AMPK in human pancreatic carcinoma cells**

As mentioned earlier, AMPK is a sensitive indicator of cellular energy status and is activated by low cellular ATP/AMP ratio and considered a novel cancer drug target (16,18). Notably, Cucurbitane triterpenoids from bitter melon have been shown to activate AMPK in L6 muscle cells and 3T3L1 adipocytes (25). Accordingly, next we examined BMJ effect on AMPK phosphorylation at Thr172 site, which is a measure of its activation. BMJ (2–4%, v/v) treatment caused a significant AMPK activation in both BxPC-3 and MiaPaCa-2 cell lines (Figure 4A). Specifically, in BxPC-3 cells, compared with untreated control cells showing no activated AMPK, the AMPK activation was robust with BMJ at 4% after 24 h and at 3–4% after 48 h of treatment (Figure 4A). MiaPaCa-2 cells also showed a strong increase in activated AMPK by BMJ compared with control cells, which also had substantial basal level (Figure 4A). Because we found a big difference in basal activated AMPK levels in control BxPC-3 and MiaPaCa-2 cell lines (Figure 4A), we further expanded these studies in another human pancreatic carcinoma cell line, namely AsPC-1 cells, which also showed strong BMJ effect on viability in Figure 2C. Similar BMJ effect, as in MiaPaCa-2 cells, was also observed in these cells regarding AMPK activation (Figure 4A). Next, we used a specific inhibitor of AMPK activity, that is, Compound C to assess the role of activated AMPK in BMJ-induced apoptotic death. As shown in Figure 4B, in the presence of Compound C, BMJ effect on caspase-3 activation in BxPC-3 cells was compromised suggesting the important role of activated AMPK in BMJ-mediated apoptotic death in pancreatic carcinoma cells.

**BMJ inhibits the growth of MiaPaCa-2 xenograft in athymic nude mice without noticeable toxicity**

To further translate our cell culture findings to in vivo situation, we next examined the efficacy of BMJ against MiaPaCa-2 xenograft in athymic nude mice. In this study, lyophilized BMJ was mixed with water at a concentration of 5 mg/100 ml (w/v) and was administered in mice via oral gavage. BMJ feeding for 6 weeks caused a significant reduction in MiaPaCa-2 xenograft volume from 1795 ± 215 mm³ (in control group) to 741 ± 172 mm³ (in treated group) (P < 0.01) (Figure 5A). Furthermore, estimation of tumor weight at the end of the study showed a significantly strong reduction in MiaPaCa-2 tumor weight from 2.12 ± 0.27 g (in control) to 0.77 ± 0.23 g (in treated group) accounting for 64% inhibition (P < 0.01) (Figure 5B).

In this study, mice were also observed for general signs of toxicity such as weight profile, where BMJ administration at above-mentioned dose regimen did not cause any weight loss (data not shown) indirectly implicating that BMJ is well tolerated by mice at this dose. Furthermore, the hematoxylin and eosin analyses of pancreas and liver showed no adverse effect of BMJ on the histology of these organs (Figure 5C). Together, these results suggested the strong in vivo efficacy of BMJ against human pancreatic carcinoma MiaPaCa-2 xenograft growth without any apparent side effects.

**BMJ’s in vivo efficacy against MiaPaCa-2 xenograft growth is through inhibiting proliferation, inducing apoptosis and activating AMPK**

To assess whether the observed molecular changes and biological responses observed in cell culture exist in xenografts as well, next we performed IHC analyses on tumor tissues from both control and BMJ-fed mice for the biomarkers of proliferation (PCNA) and apoptosis (TUNEL). As shown in Figure 6A, BMJ treatment moderately but significantly decreased the cell proliferation as PCNA-positive cells decreased from 47.6 ± 2.7 in control group to 36.4 ± 1.9 (P < 0.01) in BMJ-treated group. IHC analyses also revealed that TUNEL-positive cells were markedly increased in the xenografts from BMJ-treated group as compared with control group. The percent TUNEL-positive cells increased from 20.6 ± 2.1 in control group to 37.6 ± 2.5 (P < 0.001) in BMJ-treated group (Figure 6B). Furthermore, as shown in Figure 6C, BMJ feeding also significantly activated the AMPK in the xenografts, where AMPKTh(172) immunoreactivity score increased from 1.1 ± 0.37 in control group to 2 ± 0.13 in BMJ-treated group. These in vivo results further supported the BMJ effects observed in cell culture in terms of proliferation inhibition, apoptosis induction and AMPK activation in pancreatic carcinoma cells.

**Discussion**

Prognosis of pancreatic cancer remains dismal and a late-stage diagnosis and lack of effective therapeutic options further fuel the need for better strategies to intervene this deadly malignancy. The long-standing diabetes, obesity and diets with high fat and meat contents
have been implicated in increasing the risk of pancreatic cancer (35). Other conditions that increase the risk of this malignancy include pancreatitis, cholelithiasis and gastrectomy (36). Current treatment options such as surgery, chemotherapy and so on have not been able to improve the extremely low 5 year survival rate of pancreatic cancer. Curative surgery is considered an option in patients diagnosed at early stages of the disease; however, success is limited even in these cases due to micrometastasis (37). In case of advanced pancreatic cancer, only gemcitabine offers limited benefit in improving an overall survival of the patients. In general, pancreatic cancer exhibits high level of inherent and acquired resistance to chemotherapy, which might be the underlying cause of poor prognosis of this disease (38). Therefore, newer strategies with effective treatment are required to treat pancreatic cancer patients and to improve their overall survival. Results from this study suggest that BMJ could be an effective treatment option against pancreatic cancer.

Bitter melon is traditionally used for its hypoglycemic effects and to regulate weight gain and lipid metabolism (39). In recent years, there are also accumulating reports showing anticancer efficacy of bitter melon (4,7–11). Ru et al. (11) reported that oral administration of BMJ inhibited the prostate cancer progression in TRAMP mice through interfering cell cycle progression and cell proliferation. Bitter melon extract is shown to inhibit DMBA-induced mouse skin tumorigenesis (40). Bitter melon seed oil in diet inhibits azoxymethane-induced rat colon carcinogenesis through elevating the colonic peroxisome proliferator-activated receptor-γ and modulating

Fig. 3. BMJ induces apoptotic death in human pancreatic carcinoma cells. BxPC-3 and MiaPaCa-2 cells were treated with BMJ for 24h and the extent of apoptotic death was estimated by (A) cell death enzyme-linked immunosorbent assay kit and (B) annexin V/propidium iodide staining. (C) BxPC-3 and MiaPaCa-2 cells were treated with 2–4% BMJ (v/v) for 24 and 48h. At the end of each treatment time, total cell lysates were prepared and analyzed by western blotting for cleaved caspase-3, cleaved caspase-9, Bcl-2, Bcl-XL, Bak, XIAP, survivin, p21, CHOP, phosphorylated and total ERK1/2 and p38. (D) BxPC-3 and MiaPaCa-2 cells were treated with 2–4% BMJ (v/v) for 24h and 48h. At the end of each treatment time, cell fractionation was performed and cytosolic fraction was analyzed by cytochrome-c levels by western blotting. Membranes were stripped and re-probed for β-actin to determine protein loading. Bars indicate mean ± SD, n = 3–4. *P < 0.05; *P < 0.001. cc 3, cleaved caspase-3; cc 9, cleaved caspase-9.
the lipid composition in the colon and liver (9). Furthermore, bitter melon extract has been reported to target p-glycoprotein activity and reverse cancer multidrug resistance (41, 42). Results from this study for the first time showed that BMJ possesses strong efficacy against human pancreatic carcinoma cells both in vitro and in vivo without any noticeable side effects.

Evasion of apoptosis is inherent to pancreatic cancer cells and is often encountered during chemoresistance (43). Apoptosis is regulated via a balance between proapoptotic and antiapoptotic molecules. We observed a significant induction in proapoptotic protein Bak but a decrease in antiapoptotic protein Bcl-2 or Bcl-XL in cell line–specific manner by BMJ. We also observed a significant reduction in the levels of cellular inhibitors of apoptosis molecules namely survivin and XIAP by BMJ. Survivin could interact with either Smac or XIAP to inhibit apoptosis (44), and XIAP binds directly and inhibits caspase-3, -7 and -9, thus negatively regulates apoptosis (45). Notably, overexpression of XIAP has been observed in pancreatic cancer and XIAP is considered as a biomarker of chemoresistance (46). In addition to Bcl-2 family members and IAPs, we also observed an increase in CHOP levels, which is a proapoptotic molecule and is activated in response to endoplasmic or genotoxic stress (30). Bcl-2 family proteins are thought to be affected by CHOP by yet unknown mechanisms (31). Therefore, induction of CHOP levels by BMJ might also contribute to apoptosis induction. Stress-activated mitogen-activated protein kinases are also involved in apoptosis induction (32), and we found that BMJ treatment resulted in prolonged and sustained activation of p38 and ERK1/2 without any effect on JNK1/2 (data not shown). Activation of p38 is linked to apoptosis in response to various stress stimuli and can be either cause or consequence of apoptosis (33). Similarly, sustained activation of ERK1/2 by phytochemicals has been previously observed by us and other researchers (34, 47) and is responsible for either cell cycle arrest or apoptosis. Accordingly, it is quite possible that activation of both p38 and ERK1/2 by BMJ also contribute to induction of apoptosis. Overall, BMJ seems to target multiple signaling molecules toward inducing apoptotic death in human pancreatic carcinoma cells.

Bitter melon and several of its constituents have been extensively reported for their efficacy to reduce oxidative stress caused by chemicals or metabolic stress (48–51). However, bitter melon seed oil constituent eleostearic acid was reported to induce apoptotic death in breast cancer cells through oxidation-dependent mechanism (8). These contrasting effects (antioxidant and pro-oxidant) are quite similar to array of earlier experimental results where known antioxidants (quercetin, grape seed extract and so on) have been reported to induce oxidative stress and apoptotic death selectively in cancer cells (52–54). One explanation that partly explains these contrasting effects is the ability of antioxidants to increase oxidative stress and promote apoptosis in cancer cells, whereas antioxidants can also reduce oxidative stress and promote apoptosis in normal cells. This phenomenon is known as the bystander effect and occurs when antioxidants promote apoptosis in cancer cells by increasing oxidative stress and promoting apoptosis, while reducing oxidative stress and promoting apoptosis in normal cells. This phenomenon has been observed in a variety of experimental settings and is thought to be mediated by the activation of stress-activated mitogen-activated protein kinases, which are known to promote apoptosis in response to various stress stimuli. Therefore, it is quite possible that activation of both p38 and ERK1/2 by BMJ also contribute to induction of apoptosis. Overall, BMJ seems to target multiple signaling molecules toward inducing apoptotic death in human pancreatic carcinoma cells.

Fig. 4. BMJ activates AMPK in human pancreatic carcinoma cells. (A) BxPC-3, MiaPaCa-2 and AsPC-1 cells were treated with 2–4% BMJ (v/v) for 24 and 48h. At the end of treatment times, total cell lysates were prepared and western blotting was performed for phosphorylated- and total-AMPK. (B) BxPC-3 cells were treated with BMJ (4% v/v) in the presence or absence of AMPK inhibitor Compound C (10 µM) for 24h. Thereafter, total cell lysates were analyzed for cleaved caspase-3 level by western blotting. Membranes were stripped and re-probed for β-actin. Compd C, Compound C.

Fig. 5. BMJ inhibits the growth of MiaPaCa-2 xenograft in athymic nude mice without noticeable toxicity. Approximately, 3 million MiaPaCa-2 cells were subcutaneously injected on the right flank of each mouse. Mice were oral gavaged either water or BMJ (5 mg lyophilized BMJ/100 µl of water/day) for 6 weeks. (A) Tumor volume (mm³) was measured and plotted as a function of time. (B) At the end of the study, tumors were excised and tumor weight was determined. (C) Histology of pancreas and liver from control and BMJ-fed mice was analyzed by hematoxylin and eosin. Data shown represent mean ± standard error of mean of seven mice in each group. *P < 0.05; **P < 0.01. Con, control.
effects lie in the observation that cancer cells usually generate more reactive oxygen species (ROS) than normal cells due to oncogenic mutations, augmented growth factors production and higher proliferation rate (55). Therefore, any further increase in ROS or oxidative stress by pharmacological agent/s could push the tumor cells beyond the breaking point in terms of DNA damage, lipid peroxidation or protein oxidation; whereas normal cells, because of lower baseline ROS level or oxidant signaling, remain largely unaffected. Therefore, it is quite possible that BMJ-induced apoptotic death could be through an increased oxidative stress in pancreatic cancer cells. However, more studies are needed in future to understand BMJ effect on ROS and cellular redox signaling as well as their connection with apoptosis induction in pancreatic cancer cells.

Another important observation in this study was the BMJ-mediated activation of AMPK in pancreatic carcinoma cells. Activation of AMPK occurs when there is a metabolic stress and ATP/AMP ratio decreases (56). Activation of AMPK in response to metabolic stress switches off intracellular energy consuming anabolic processes and activates energy-producing catabolic processes (57). Chandra et al. (58) have shown that physiological levels of nucleotides including ATP suppress apoptosis via directly binding to cytochrome-c and inhibiting the interaction of cytochrome-c with Apaf-1 and apoptosome formation. Based upon our results, we suggest that BMJ treatment causes metabolic stress through mitochondrial damage or mitochondrial uncoupling leading to cytochrome-c release and disruption of ATP formation. The lower cellular ATP might lead to AMPK activation and apoptosis induction. This suggestion is further supported by results where AMPK inhibition reversed the BMJ-induced caspase-3 activation in BxPC-3 cells. However, further studies are needed to clearly understand the role of AMPK in caspase-3 activation and apoptosis induction by BMJ in pancreatic carcinoma cells.

In conclusion, we have demonstrated that BMJ possess strong efficacy against human pancreatic carcinoma cells without any noticeable side effects. Molecular studies revealed that BMJ activates AMPK in pancreatic carcinoma cells both in vitro and in vivo and induced strong apoptotic death. Considering the short survival and high mortality due to pancreatic cancer, BMJ that is widely consumed as vegetable and for health benefits could have significant translational relevance in managing this deadly malignancy.

Supplementary material

Supplementary Method, Table 1 and Figures 1–9 can be found at http://carcin.oxfordjournals.org/

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