Zanthoxylum avicennae extracts inhibit cell proliferation through protein phosphatase 2A activation in HA22T human hepatocellular carcinoma cells in vitro and in vivo

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Received December 29, 2011; Accepted February 13, 2012

DOI: 10.3892/ijmm.2012.938

Abstract. Hepatocellular carcinoma is a common type of cancer that is usually associated with poor prognosis. In this study, we examined the in vitro and in vivo mechanisms of the traditional Vietnamese herb Zanthoxylum avicennae on the inhibition of HA22T human hepatocellular carcinoma cell proliferation. HA22T cells were treated with different concentrations of Zanthoxylum avicennae extracts (YBBEs) and analyzed with the MTT assay, western blot analysis, flow cytometry, siRNA transfection assays and co-immunoprecipitation assay. Additionally, the HA22T-implanted xenograft nude mouse model was applied to confirm the cellular effects. YBBEs showed a strong inhibition of HA22T cell viability in a dose-dependent manner and significantly reduced cell proliferation-related proteins as well as induced cell cycle arrest in the G2/M phase. Protein phosphatase 2A (PP2A) siRNA or okadaic acid totally blocked YBBE-mediated cell proliferation inhibition. In addition, an HA22T-implanted nude mouse model further confirmed that YBBEs inhibit HA22T tumor cell growth and downregulate the survival and cell cycle regulating proteins, as well as activate the PP2A protein. Our findings indicate that the inhibition of HA22T cell proliferation by YBBEs is mediated through PP2A activation.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the most frequent type of liver cancer, accounting for almost 90% of primary malignant hepatic tumors in adults (1), particularly in parts of the developing world (2). According to the World Health Organization, the burden of HCC is expected to continue to increase. Most HCC cases occur in Asia, with a particularly higher incidence in East Asia, accounting for over 20 cases/100,000 population (3). In spite of the great developments in diagnosis and therapy, the prognosis for patients with HCC remains dismal due to its high rate of metastasis and recurrence. For patients in the advanced stages, the median survival rate is less than 6 months, regardless of the type of the treatment modality used (4).

Alternative treatment options for HCC, along with other types of cancers, are in great demand. Traditional medicinal herbs stand out as promising candidates for cancer therapy due to their low toxicity and side effects. Zanthoxylum avicennae [Ying Bu Bo (YBB)] is a traditional Vietnamese herb with known medicinal applications. It removes rheumatic symptoms, invigorates blood circulation, promotes diuresis and disperses swelling (5-7). The root is used to treat hepatitis and edema due to nephritis as well as rheumatoid arthritis (5-8). In addition, YBB is also used to treat hepatitis B, hepatocirrhosis, colitis and stomatitis in Vietnamese folk medicine (9).

Our previous studies showed that extracts of Zanthoxylum avicennae (YBBEs) induced cell apoptosis through protein phosphatase 2A activation in HA22T human hepatocellular carcinoma cells and blocked tumor growth in xenografted nude mice (9). We further demonstrated the effects of YBBEs on the proliferation of human hepatocellular carcinoma HA22T cells, and evaluated the mechanisms behind its inductive effect on cell proliferation inhibition in vitro and in vivo. Results show that YBBEs can remarkably activate the PP2A.

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Key words: hepatocellular carcinoma, Zanthoxylum avicennae (Ying Bu Bo), protein phosphatase 2A, cell proliferation, nude mouse model
Materials and methods

**Materials.** Antibodies against PCNA, cyclin A, cyclin D1, cyclin E, α-tubulin, p21, p27, p-p53, p53, c-Fos, c-Myc, p-P13K, PP2A-Cα, p-Cdc25C, Cdc25C, p-Cdk1, donkey anti-goat IgG, goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against Cdk1 were purchased from GeneTex, Inc. (Irvine, CA, USA). Antibodies against p-Akt and p-MDM2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Dulbecco’s modified Eagle’s medium (DMEM), phenol red-free DMEM, Basal Medium Eagle (BME), sodium bicarbonate NaHCO₃, thiazolyl blue tetrazolium bromide MTT, propidium iodine and ribonuclease A (RNase A) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s phosphate-buffered saline (PBS) was purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Thermo Scientific HyClone, Logan, UT, USA. Fetal bovine serum (FBS) was purchased from HyClone. Okadaic acid (OA) (PP2A inhibitor, cat.no. 495604) was purchased from Alexis Biochemicals (San Diego, CA, USA). The root bark wood of YBB was collected from Nam Dan, Nghe An, Vietnam.

**Ying Bu Bo extraction.** YBB extraction was performed according to the methods described in our previous study (9). According to the experimental design, the HA22T cell line was incubated with 0, 50, 100, 150, 200 or 250 µg/ml of the extract for 24 h. The dose for the animal experiments was 20 or 40 mg/kg YBBEs.

**Cell culture and treatments.** HA22T cells (BCRC no. 60168) were obtained from the Bioresources Collection and Research Institute, Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were grown as described in our previous study (9). Cells were cultured in serum-free medium for the invasive and metastatic experiments.

**Animals.** About 20 male NU/NU nude mice 20-22 g in weight, 5 weeks of age were obtained from the Biosasco Taiwan Co., Ltd. (Taipei, Taiwan). The mice were given food and water ad libitum. The detailed procedure has been described in a previous study (9). All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan.

**MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldimetha-zolium-bromide] assay.** The YBBEs effect on HA22T cell proliferation was estimated using the MTT assay. HA22T cells were cultured in a 24-well plates (2x10⁵ cells/well) and treated with different concentrations of YBBEs (0, 50, 100, 150, 200 or 250 µg/ml) and then incubated at 37°C for 24 h to determine cell viability. Cell viability was determined using the MTT assay. In the MTT assay, after dissolving formazan crystals, light absorbance was measured at 570 nm using a spectrophotometer (U-2001, Hitachi Instruments, Inc., Tokyo, Japan). The quantity of the formazan product is directly proportional to the number of viable cells in the culture medium.

**Flow cytometric analysis.** The YBBEs effect on the HA22T cell cycle was estimated using flow cytometric analysis. Cells were seeded in 6-well plates (5x10⁵ cells/well) and incubated at different YBBEs concentrations (0, 50, 100, 150, 200 or 250 µg/ml) at 37°C for 24 h. After YBBEs treatment, cells were washed with PBS and treated with trypsin and harvested after trypsin removal. Cells were fixed in 70% ethanol, washed with ice-cold PBS and stained with propidium iodide (PI) buffer, which consisted of 400 µg/ml PI, 1% Triton X-100 and 0.5 mg/ml RNase A in PBS. Cellular PI content was measured on a BD FACSCanto II flow cytometer (BD Biosciences, USA) equipped with an argon ion laser at a wavelength of 488 nm. Data were analyzed using the ModFit LT software.

**Western blot analysis.** HA22T cells were scraped and washed once with PBS. Cell pellets were lysed for 30 min in lysis buffer and spun down at 12,000 x g for 10 min. Tissue samples were homogenized with ice-cold PBS and then subjected to lysis in a solution containing 20 mM Tris, 2 mM EDTA, and 1% glycerol. Supernatants were obtained after centrifugation at 12,000 x g for 40 min. Western blot analyses were performed according to the methods described in our previous study (9).

**Gene knockdown using siRNA.** HA22T cells were seeded into 6-well plates and grown to 80% confluence. siRNA transfection was carried out with DharmaFECT Duo transfection reagent (Dharmacon, Inc., Lafayette, CO, USA). The 100 µl PP2A-Cα siRNA (Santa Cruz Biotechnology, Inc.) and 100 µl negative control transfect Non-Targeting Pool (NT) (Dharmacon, Inc.) were mixed with 100 µl serum-free medium. The detailed procedure has been described in our previous study (9).

**Co-immunoprecipitation assay (Co-IP).** HA22T cells were transfected with PP2A-Cα siRNA first and then treated with YBBEs. Two hundred milliliters Co-IP cell lysis buffer (1.5 mM MgCl₂, 1% Triton X-100, 50 mM HEPES, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM NaVO₃, 10 mM NaF, 10 mM β-glycerolphosphate, and 5 mg/ml protease inhibitor) was added to each plate to lyse cells for 30 min. The cells were then scraped and centrifuged for 10 min at 12,000 x g at 4°C. For each group, 100 µg of the total protein sample was added into each microcentrifuge tube, with a suitable amount of lysis buffer without protease inhibitor added so that the mixture in each of the microcentrifuge tubes had a final volume of 500 µl. Seven milliliters of protein G PLUS-agarose (Santa Cruz Biotechnology, Inc., cat. no. sc-2002) was then added followed by vortexing at 4°C for 1 h with a vortex mixer. Centrifugation at 4°C and 1,300 x g was performed for 30 sec. The supernatant was then removed. For each group, 2.5 µl of each antibody listed as follows was added into each of the microcentrifuge tubes: PP2A-Cα and Akt. Afterward, vortexing at 4°C overnight with a vortex mixer was conducted. Twenty milliliters of protein G PLUS-agarose was added, followed by vortexing at 4°C for 2 h with the vortex mixer. Centrifugation at 4°C and 1,300 x g was conducted for 30 sec. After the supernatant was removed, 1 ml of lysis buffer without protease inhibitor was added to wash the pellet, followed by centrifugation at 4°C and 1,300 x g for 30 sec. Western blotting was performed to detect the PP2A-Cα, p-Akt and Akt proteins.
Liver cancer mice model. The detailed procedure has been described in a previous study (9). Tumors were excised and then examined by western blot analysis.

Statistical analysis. Each sample was analysed based on results that were repeated at least three times using SigmaPlot 10.0 software. The detailed procedure has been described in previous study (9).

Results

The anti-proliferative effect of YBBEs on HA22T cells. No known study has been carried out to investigate the effect of YBBEs on human cancer cell lines. We therefore tested the antiproliferative effect of YBBEs on HA22T human hepatocellular carcinoma cells. We first determined whether YBBEs significantly attenuated cell viability. The MTT assay was used to determine cell viability in cell proliferation and cytotoxicity assays. Our experiments found dose-dependent inhibition of cell viability in the YBBEs-treated groups compared to the control group (Fig. 1A), accompanied with a significant decrease in PCNA, c-Fos and c-Myc markers in a dose-dependent manner (Fig. 1B). These findings provide evidence that YBBEs significantly inhibited the viability and protected against HA22T cell proliferation.

YBBEs inhibit cell cycle progression in HA22T cells. We evaluated the inhibitory effect of YBBEs on the p-MDM2-p53 pathway that regulates cell-cycle related protein expression. YBBEs treatment reduced phosphorylated MDM2 protein levels, accompanied with a significant increase in p53 and p-p53 proteins (Fig. 2A). Therefore, there was no change in the total MDM2 expression levels. We also observed p21 and p27 enhancement at the 24 h time point (Fig. 2B). The cell cycle progression is tightly regulated by a complex network of cell cycle regulatory molecules, such as cyclins. To elucidate how YBBEs modulate the cell cycle in HA22T cells, we measured the cell cycle modulator proteins cyclin D1, cyclin E, and cyclin A expression using western blot analysis. The results showed decreased expression of cyclin D1, cyclin E, and cyclin A proteins in a dose-dependent manner following YBBEs treatment (Fig. 2C). In addition, we treated cells with or without YBBEs and subjected them to flow cytometric analysis to test whether YBBEs induced G2/M phase cell cycle arrest in HA22T cells. We found that YBBEs (0-250 µM) increased the G2 phase population from 15.12 to 45.05% and reduced the G1 phase population from 67.08 to 43.62%, suggesting an accumulation at the G2/M phase in the cell cycle (Fig. 2D and E). To further explore the underlying mechanisms of G2/M phase arrest induced by YBBEs, we examined the regulatory effects of YBBEs on p-Cd25C, Cd25C, p-Cdk1 and Cdk1 expression. As shown in Fig. 2F, cell exposure to YBBEs also resulted in a dose-dependent increase in p-Cd25C and p-Cdk1 protein levels, accompanied with a significant reduction in Cd25C and Cdk1 proteins. According to our results, YBBEs were able to arrest HA22T cells at the G2/M phase.

Okadaic acid (OA) inhibits YBBEs reduced cell cycle progression through the downregulation of PP2A-Cα in HA22T cells. We further determined the role of PP2A on the YBBEs inhibition of HA22T cell proliferation. HA22T cells were pretreated with OA, a pharmacological inhibitor of PP2A, followed by treatment with YBBEs at a concentration of 200 µg/ml for 24 h. OA significantly reversed the YBBEs-induced inhibition of cell proliferation in a dose-dependent manner, as revealed by the MTT assay (Fig. 3A). YBBEs (200 µg/ml) treatment significantly inhibited p-PI3k, p-Akt and p-MDM2 expression and was accompanied with an increase in PP2A-Cα and p53 expression. However, this situation was totally reversed after treatment with OA in the presence of YBBEs (Fig. 3B).

PP2A-Cα siRNA blocks the antiproliferative effect of YBBEs in HA22T cells. To further confirm that YBBEs induce cell prolif-
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Cell cycle progression inhibition by YBBEs in hepatocellular carcinoma HA22T cells. HA22T cells were cultured and incubated with 0, 50, 100, 150, 200 or 250 µg/ml of YBBEs for 24 h. (A) Western blot analysis showing decreased p-MDM2 and increased p53 and p-p53 protein expression. (B and C) Cell cycle controlling protein expression was measured using western blot analysis with antibodies against the proteins indicated. Equal loading was assessed with an anti-α-tubulin antibody. (D) The cell cycle was determined using flow cytometric analysis. (E) Representative histograms clearly showing a significant YBBEs effect on inducing G2 phase cell cycle arrest in HA22T cells. *P<0.05, **P<0.01 vs. the control group. (F) Western blot analysis showing decreased Cdc25C, Cdk1, and increased p-Cdc25C and p-Cdk1 protein expression. β-actin was used as a loading control.

YBBEs-induced Akt dephosphorylation is regulated by PP2A. PP2A regulates the protein kinase signaling pathway activities, including the PI3/Akt pathways. We further assessed the physical interaction between PP2A and Akt. Surprisingly, our results suggested that PP2A is an important mediator in the YBBEs-induced antiproliferative inhibition effect in HA22T cells.

YBBEs inhibits cell proliferation and tumor growth in an in vivo model. To assess the YBBEs antiproliferative effect on tumor growth in the in vivo model, western blot analysis was conducted to observe the cell proliferation and cell cycle modulator protein expression levels in the tumor tissues of mice treated with or without different doses of YBBEs. In our experiments, the p53, p21, p27, p-Cdc25C, and p-Cdk1 expression levels in the positive control group were lower than those in the negative control group (mice without injection of HA22T cells and without YBBEs treatment). However, the expression levels of these proteins gradually decreased as the YBBEs treatment concentration increased (Fig. 5A-D). The in vivo and in vitro experimental results are totally consistent. Thus, YBBEs are able to suppress tumor cell proliferation in vitro. Based on the in vivo tests we also strongly believe that YBBEs effectively inhibit liver cancer cell growth.
Discussion

Uncontrolled tumor cell proliferation plays the most crucial role in hepatocellular carcinoma growth. Therefore, the prevention of cancer proliferation is an important target for improving patient prognosis. There is still no available information addressing the antiproliferative and inhibitory effects of YBBEs in human cancer cells. This study investigated whether...
YBBEs can inhibit cell proliferation in human hepatocellular carcinoma HA22T cells. We found a significant inhibitory effect by YBBEs on HA22T cell viability as revealed by the MTT assay.

PCNA, c-Fos, and c-Myc are associated with tumor cell proliferation. PCNA is a useful marker for proliferative activity, which functions as a cofactor of DNA polymerase and as an important marker for evaluating the proliferation of several cancers, including hepatocellular carcinoma (10). In normal cells, c-Myc is induced upon growth factor stimulation and is constitutively high in transformed cells. c-Myc overexpression is estimated to occur in 70% of human tumors (11). Our results showed that YBBEs significantly inhibited PCNA, c-Fos, and c-Myc levels in HA22T cells.

Chronic cyclin D1 overexpression in transgenic mice is reported to be associated with the rapid development of hepatocellular adenomas and carcinomas (12). Cyclin D1 is a major regulator of cell cycle progression, serving as a rate limiting agent for the G1/S cell-cycle checkpoint (13). In this study, YBBEs administration significantly decreased PCNA, cyclin D1, cyclin E, cyclin A, c-Fos, and c-Myc protein levels. Thus, it can be concluded that YBBEs efficiently suppressed...
the proliferation of human hepatocellular carcinoma HA22T cells by inhibiting PCNA, c-Fos, and c-Myc accumulation and cyclin D1, cyclin E, cyclin A protein expression.

The p53 protein inhibits tumor growth by arresting cell proliferation and inducing apoptosis. The p53 gene alteration is the most frequently identified mutation in human cancers. The loss of p53 function allows cells with damaged DNA to continue to proliferate and, therefore, is associated with tumor progression (14). There is increasing evidence supporting p53 as an attractive target in cancer therapy (15). Our results revealed that YBBEs treatment resulted in a dose-dependent increase in p53 level. It has been shown that p53 may directly facilitate cytochrome c release. p53 has been associated with various biological functions, such as cell cycle arrest (16), metastasis (17).

MDM2 is an oncoprotein that is overexpressed in a range of human cancers (18). Under non-stress conditions, p53 levels are tightly controlled by MDM2 through a well-established auto-regulatory feedback loop (19). It induces MDM2 gene expression, which in turn leads to p53 inactivation and degradation (20). Since MDM2 is reported to be one of the main factors causing p53 degradation via the proteasome-related pathway (21), we therefore examined its involvement in YBBEs-mediated p53 regulation. Western blot analysis assay showed that YBBEs inhibited MDM2 expression in a dose-dependent manner. We also found that p53, p-p53, p21 and p27 were significantly upregulated in the presence of YBBEs. Our research suggested that p53 protein upregulation is involved in MDM2 inhibition by YBBEs in HA22T cells. p53/MDM2 modulation may be one of the important mechanisms behind the YBBEs-mediated anticancer activity.

According to previous studies, to prove that asparanin A causes G2/M arrest in HepG2 cells, it was shown that cyclin A and Cdk1 were downregulated to varying degrees following asparanin A treatment. Exposure to asparanin A also resulted in an increase in p-Cdk1 protein levels (22). In a recent study, HKH40A was used to treat Hep3B cells and was found to increase p-Cd25C and p-Cdk1 expression in a dose-dependent manner (23). Treating HA22T hepatocarcinoma cells with different doses of YBBEs also had similar results, leading to cell cycle arrest at the G2/M phase.

It has previously been reported that sorafenib plus bortezomib significantly suppressed PLC5 hepatocellular carcinoma cell xenograft tumor growth, downregulated p-Akt expression, and upregulated PP2A activity (24). Use of ceramide to treat PC-3 human prostate cancer cells revealed that ceramide-activated PP2A dephosphorylates phospho-Akt (25). In this study, we found that OA reversed YBBEs-mediated downregulation of p-Pi3K, p-Akt and p-MDM2, suggesting that targeting PP2A may be a feasible way to affect the pivotal stage. Silencing PP2A using RNA-interference suppressed the YBBEs mediated antiproliferative effect, confirming that PP2A is indispensable in mediating the effects of YBBEs. These results showed that PP2A has different roles in different cancer cell types in various cellular contexts, which should be further investigated in future studies.

We also found that YBBEs dramatically suppressed HA22T cell proliferation and tumor growth in the nude mouse model. YBBEs showed the strongest effect at the 40 mg/kg concentration.

Based on these findings, YBBEs upregulated PP2A, leading to decreased p-Pi3K, p-Akt, and p-MDM2, and further upregulated cell cycle checkpoint proteins, such as p53, p21, p27, p-Cdc25C and p-Cdk1; and decreased expression of c-Fos, c-Myc, cyclin A, cyclin D, cyclin E, Cdc25C, Cdk1 and PCNA. These results present the molecular mechanisms behind human hepatocellular carcinoma HA22T cell proliferation and tumor growth inhibition by YBBEs. This was confirmed using an in vitro and in vivo model system. The signaling pathways involved are shown in Fig. 6.

In conclusion, our study provides strong evidence that YBBEs significantly inhibit HA22T cell proliferation and tumor growth through PP2A activation. These results are promising enough to consider further investigation of the YBBEs anticancer effect using preclinical studies and clinical trials. Efforts aimed at enhancing the YBBEs function and/or activity may provide an alternative therapy against liver cancer.

Acknowledgements

This study was supported by grant CMU98-CT-19 and in part by the Taiwan Department of Health Clinical Trial and Research Center for Excellence (DOH101-TD-B-111-004) and the Taiwan Department of Health Cancer Research Center for Excellence (DOH101-TD-C-111-005).

References