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Brazilein inhibits survivin protein and mRNA expression and induces apoptosis in hepatocellular carcinoma HepG2 cells

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Hepatocellular carcinomas represent the third leading cause of cancer-related deaths worldwide. Survivin, a structurally unique member of the inhibitor of apoptosis protein (IAP) family, is overexpressed in a wide range of malignancies, including hepatocellular carcinoma. Due to its involvement in cancer progression and treatment resistance, survivin is currently undergoing extensive investigation as a novel intervention target to induce apoptosis in cancer cells by phytochemicals or synthetic agents. Brazilein, a compound obtained in a large amount from the dried heartwood of Caesalpinia sappan Linn., which has long been used in traditional medicine in China, has some pharmacological activities. Human hepatocellular carcinoma HepG2 cells were treated with brazilein and analyzed for survivin protein and mRNA levels by Western blotting and real-time RT-PCR, respectively. Brazilein treatment of cells for 48 h at 5 and 10 μg/ml doses resulted in significantly decrease in survivin protein expression. We also observed that brazilein caused a strong decrease in survivin mRNA expression. In other studies, down-regulation of survivin by brazilein was associated with a strong and prominent caspases-9 and -3 activation as well as PARP cleavage. It was also shown that brazilein induced a strong apoptotic cell death, as shown by DNA ladder assay, and growth inhibition of HepG2 cells. Further studies are needed to investigate in vivo effect of brazilein on survivin expression and associated biological effects in hepatocellular carcinoma that could provide useful information for brazilein efficacy in the prevention/intervention of human hepatocellular carcinoma.

Key words: *Brazilein; HepG2; Survivin, apoptosis*

Hepatocellular carcinoma (HCC) is a major cause of cancer mortality in many parts of the world [1]. The main curative therapies for cancer are surgery and radiation, which, in general, are only successful if the cancer is diagnosed at an early stage. Currently, conventional chemotherapy for the treatment of advanced tumors, although quite effective, has been associated with concerning adverse events, which is a dose limited factor. It is clear that new therapeutic options are necessary. Chinese or herbal medicine provides novel and efficacious agents for the treatment of a variety of disease. It was evidence that Chinese herbs had anti-tumor effects in treatment of HCC [2]. A previous study showed that the extract of Caesalpinia sappan Linn., which has long been used in traditional medicine as a hemostatic and anti-inflammatory agent in China [3, 4], induced cell death in head and neck cancer cells [5]. Brazilein, a compound obtained in a large amount from the dried heartwood of Caesalpinia sappan Linn., has some pharmacological activities such as cardioactive effects as a potential inotropic drug [6]. But its anti-tumor effect has never been demonstrated.

Evading apoptosis is a major contributor to cellular transformation, growth, and development of invasive cancer as well as drug resistance in tumors [7]. In mammalian cells, apoptosis is mainly modulated by Bcl-2 and members of the inhibition of apoptosis (IAP) family of proteins [8]. Survivin is a unique and a small (16.5kD) member of IAP family, which is associated with several subcellular compartments, and is involved in the regulation of cell death, cell cycle progression, and cell division[9–11]. Survivin, as one of the most tumor-specific molecules [12], is overexpressed in a wide range of maligancies [10, 13, 14], suggesting that survivin could be an important and a critical target in cancer prevention and/or therapy [15]. Targeting survivin could also be important because of its differential expression in normal vs. tumor tissue as well as its potential requirement for cancer cell viability. These assumptions are supported by several clinical trials which indicated that survivin expression

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in many tumors is associated with a more aggressive clinical course, an increased rate of recurrence, drug resistance, and lower apoptotic index [13, 14, 16].

With regard to human HCC, several studies in recent years have shown survivin overexpression, implicating its role in increased cell proliferation, progression, local recurrence and disease-free survival [17, 18]. Taken together, these studies clearly suggest that survivin could be an attractive prevention or therapeutic target in HCC control. In this study, we report strong efficacy of brazilein in down-regulating protein and mRNA expression of survivin in human HCC HepG2 cells accompanied by caspases activation, apoptosis induction and cell growth inhibition.

Materials and methods

Extraction of brazilein

Air-dried heartwood of Caesalpinia sappan Linn. (700 g) is extracted with CH_2Cl_2 and acetone successively, for 5 days at room temperature. The crude extracts were evaporated under reduced pressure to afford brownish CH_2Cl_2 (32.1 g) extracts, respectively. The crude CH_2Cl_2 extract was further purified by QCC using hexane as eluent and increasing polarity with EtOAc to give pure brazilein (3.6 g).

Cell culture

The human hepatocellular carcinoma (HCC) cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). HCC cell lines SMMC7721 and QGY7703 were obtained from Shanghai Cell Bank (Shanghai, China). HCC cells were grown in a RPMI1640 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100μg/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. For treatments, exponentially growing HCC cells were collected and re-suspended in fresh culture medium. Stock solutions of brazilein in DMSO were freshly prepared for each experiment. The final concentration of DMSO in all the cultures was less than 0.2%.

Cell proliferation assay

Cell proliferation was assessed using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) staining as described previously [19]. Briefly, $5x10^3$ cells were incubated in 96-well plates with different doses or absence of brazilein for 48h in a final volume of 200 μl. At the end of the treatment, 40 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100μl of DMSO. The activity of the mitochondria, reflecting cellular growth and viability was evaluated by measuring the optical density at 570 nm on micro titer plate reader (Quant Bio-Tek Instruments, Inc.). At each concentration of brazilein three different experiments were carried with three replicates. The viable cells were counted by the trypan blue exclusion assay with a hemocytometer.

DNA ladder assay

After treatment of brazilein with different concentrations for 48hr, 1×10^6 cells were collected and precipitated. Cell pellets were washed, resuspended in cell lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA [pH 8.0], 0.5% Triton X-100), and incubated. RNase A (0.5 mg/ml) and proteinase K (0.5 mg/ml) were added, and the pellets were incubated for 2 h. DNA was precipitated by ethanol, resuspended in distilled water and electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide, and the DNA was visualized using a UV transilluminater.

Western blot analysis

Immunoblotting was carried out following the manufacturer's protocol. Briefly, after treatment of brazilein for 48hr, cells were washed with PBS and lysed (50mM HEPES, 150mM NaCl, 1% Triton X-100, 5mM EGTA, 50mM β-glycerophosphate, 20mM NaF, 1mM Na_3VO_4, 2mM phenyl-methyl sulfonyl fluoride, 10μg/mL leupeptin and 10 μg/mL aprotinin). Cell lysates were centrifuged and the protein content was determined by Bio-Rad DC protein assay kit (Bio-Rad laboratories, Hercules, CA). Equal amounts of protein were separated by SDS–PAGE (10–15%), transferred to nitrocellulose membrane and immunoblotted with antibodies as indicated. Detection was performed using enhanced chemiluminescence (ECL) detection system. Polyclonal rabbit anti-survivin, anti-PARP, and anti-caspase 9 antibodies were purchased from Cell Signal. Polyclonal mouse anti-cleaved caspase 3 antibody was purchased from Santa Cruz, and anti-β-actin antibody was purchased from Sigma.

Quantitative real-time PCR

Total RNA was derived from cells with treatment of different concentrations of brazilein for 48hr. After reverse-transcription, real-time PCR (target gene and GAPDH as reference gene) was carried out in 96-well optical plates using TaqMan technology and analyzed on ABI Prism PE7700 Sequence Detection System according to standard protocols. Data were normalized to internal GAPDH levels and represented as relative expression (E) whereas delta ΔCt indicated the threshold difference between GAPDH and the target gene. The primer sequences were as follows: survivin F 5'-GGCCCAGTGTTTCTTCT-GCTT-3'; survivin R 5'-GCAACCGGACGAATGCTTT-3'. fluorogenic probe (5'-FAM-AGCCAGATGACGACCCCATA-GAGGAACA-3'). PCR reaction were carried out under the following PCR conditions: 50°C for 2min, and then 95°C for 10min, followed by 40 cycles at 95°C 15s and 60°C 60s.

Results

Brazilein down-regulates survivin protein and mRNA expression in HepG2 cells

In order to assess the effect of brazilein on surviving protein levels in HepG2 cells, various concentrations of brazilein treatments were employed, followed by Western

blotting dectection. As shown in Fig. 2A, survivin protein expression was decreased after brazilein treatment for 48 h in a dose dependent manner. The treatment of cells at 1 μg/ml and 2 μg/ml doses did not show any effect on survivin levels, but 5 μg/ml and 10 μg/ml brazilein treatment for 48hr resulted in significant decrease in survivin protein levels in HepG2 cells (Fig. 2A). Two additional times were repeated to validate such a strong effect of brazilein on surviving protein levels. The β-actin blot confirmed equal loading of the samples.

Based on the observed decrease in survivin protein levels by brazilein, we next evaluated whether this decrease was mediated at the level of transcription, through a decrease in survivin mRNA levels. Total RNA was isolated, reverse-transcribed and subjected to real-time PCR for quantitative analysis of survivin mRNA levels under identical treatment conditions. As shown in Fig. 2B, compared to vehicle-treated control, brazilein treatment of cells for 48hr resulted in 27%, 69%, 88% and 95% decrease ($P < 0.01$) in survivin mRNA levels at 1, 2, 5, 10 μg/ml doses, respectively. Though the effect of the 1 and 2 μg/ml brazilein doses on a decrease in survivin mRNA levels were not consistent with the survivin protein results, the effect of 5 and 10 μg/ml doses were comparable to the decrease in protein levels.

Brazilien causes caspase-9, caspase-3, and PARP cleavages in HepG2 cells

The major anti-apoptotic function of survivin, an IAP, has been established through its interaction with caspases, resulting in a block of apoptosis [8]. Base on our findings that brazilein caused significant down-regulation of survivin protein and mRNA expression, we further assessed whether this effect on survivin could result in caspases activation and PARP cleaveage, a hallmark of apoptotic cell death. Cells were treated with different concentrations of brazilein for 48h, and the activation of caspases-9, caspases-3 and PARP was monitored by western blot analysis. As shown in Fig. 3A, brazilein treatment at 1, 2 and 5 μg/ml doses for 48 h resulted in moderate caspase-9 activation, however similar treatment at 10 μg/ml dose caused very strong caspase-9 activation, employing a specific antibody that only recognizes activated caspase-9, the cleaved product of procaspse-9. Next, the effect on caspase-3 activation was assessed. Consistent with caspase-9 observations, brazilein treatment for 48 h at 1, 2, 5 and 10 μg/ml doses resulted in a moderate and very strong caspase-3 activation, as evidenced by western immunoblotting for activated (cleaved) caspase-3 (Fig. 3B). Similar results were also observed in terms of PARP cleavage to its characteristic 85-kD fragment (Fig. 3C). β-actin was used as a loading control after stripped (Fig. 3D).

Brazilein causes strong apoptotic death and growth inhibition of HepG2 cells

The most important aspect of any given mechanistic study is its biological significance. To address this issue, next we

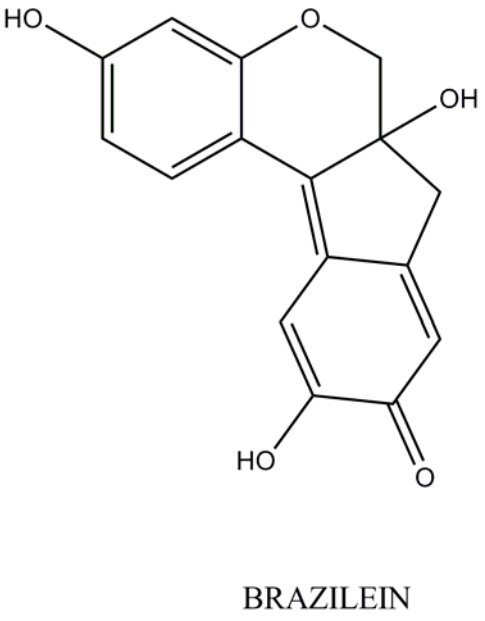

Fig. 1. Chemical structure of brazilein

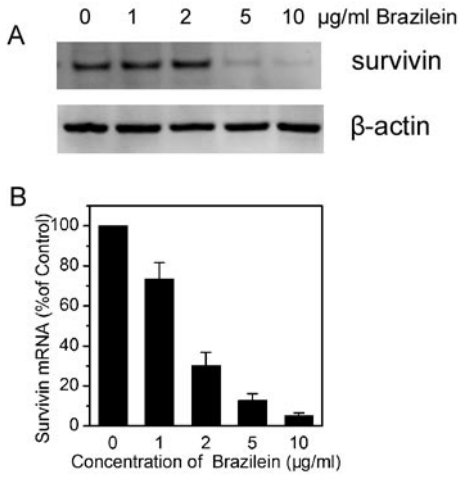

Fig. 2. Inhibitory effect of brazilein on survivin protein (A) and mRNA (B) expression in HepG2 cells. Cells were treated with various concentration of brazilein (0, 1, 2, 5, 10 μg/ml) for 48 h. Cells were collected and lysed in lysis buffer, and equal amount of protein was subjected to SDS-PAGE followed by western blotting with anti-survivin antibody. Signals of proteins were visualized with an ECL detection system. Each membrane was then stripped and re-probed with anti-β-actin antibody to confirm equal protein loading. Data shown are representative of at least three independent experiments. For quantitative real-time PCR, following identical treatments, cells were collected and total RNA was isolated for real-time PCR analysis. The data shown for survivin mRNA levels as percentage of vehicle control are means ± SD of two independent experiments.

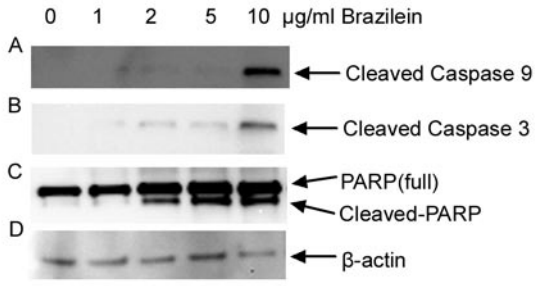

Fig. 3. Brazilein caused caspase-9 (A), caspase-3 (B), and PARP (C) cleavages in HepG2 cells. After treatment of brazilein for 48 h, cells were collected and lysed in lysis buffer, and equal amount of protein were subjected to SDS-PAGE followed by western blotting. Membranes were probed with anti-cleaved-caspase-9, anti-cleaved-caspase-3, or anti-PARP antibody followed by peroxidase-conjugated secondary antibody and visualized with ECL system. Each membrane was then stripped and re-probed with anti-β-actin antibody to confirm equal protein loading.

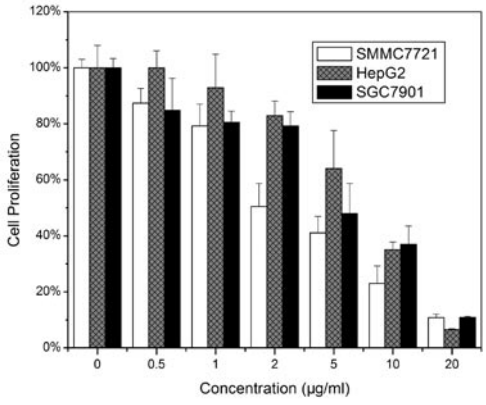

Fig. 4. Brazilein induces apoptotic cell death in HepG2 cells. After cells were treated with brazilein (0, 1, 2, 5, 10 μg/ml) for 48 h, DNA was extracted and loaded on a 1.5% agarose gel, and DNA ladder formation was visualized using a UV transilluminater.

assessed the effect of brazilein on apoptosis of HepG2 cells under identical treatment conditions. After the cells were treated with brazilein for 48 h, DNA was extracted and loaded on a 1.5% agarose gel, and DNA ladder formation was visualized as shown in Fig. 4. Clear ladder formation was visible at doses 5 and 10 μg/ml, indicating that the cells underwent apoptosis. This phenomenon was consistent with the results of caspases and PARP activation. Then the effect of brazilein on cell growth was also examined in HepG2 cells by MTT assay for three times. It was shown that brazilein

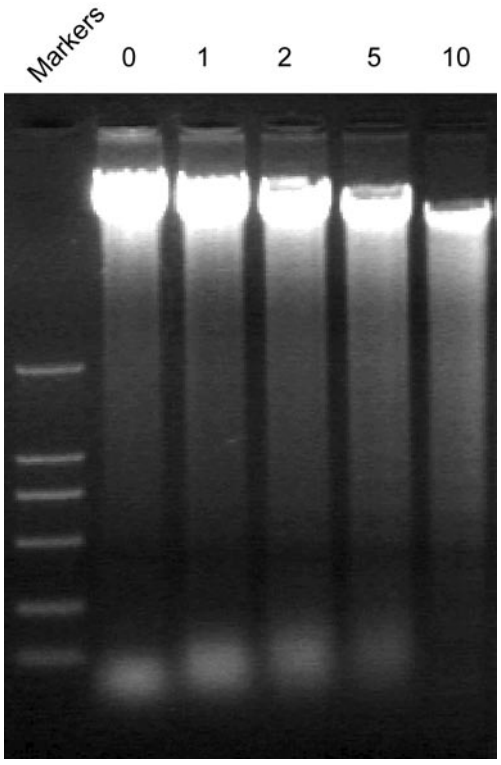

Fig. 5. Brazilein causes strong growth inhibition of HepG2 cells. After 48 h treatment of brazilein, cell survival was determined by MTT assay for three times. The data shown are means ± SD. And two additional hepatocellular carcinoma cell lines SMMC7721 and SGC7901 were employed with identical treatment.

treatment resulted in a moderate to highly significant cell growth inhibition in a dose-dependent manner (Fig. 5). To further confirm this issue, two additional HCC cell lines SMMC7721 and SGC7901 were employed, and the results verified the cell growth inhibition effect of brazilein in HCC cells (Fig. 5).

Discussion

The major finding of the present study is a significant effect of brazilein in down-regulating survivin expression both at protein and mRNA levels, accompanied by caspases activation and PARP cleavage as well as apoptosis induction and growth inhibition of human HCC HepG2 cells. Several molecular epidemiological studies have concluded that there is a strong association between increased survivin levels and progression of human HCC [18]. A wide number of studies in recent years have examined and established the molecular mechanism of apoptosis regulation by survivin via its interaction with caspases pathway [20]. Survivin is

the smallest member in the mammalian IAP family, which is located on chromosome 17q25 and encodes mRNA that is divided into three introns and four exons. In addition to its clear expression at cell cycle-dependent mitosis, survivin is involved in apoptosis resistance where it inhibits caspase-7 and caspase-3 activation [21]. Survivin is also known to regulate the anti-apoptotic activity of v-Rel and NF-kB transcription factor family [22]. Consistent with its anti-apoptotic potential by inhibiting caspases activation, we also found that a strong down-regulation of survivin protein and mRNA expression by brazilein was also associated with very strong caspases activation as evidenced by cleaved caspases products and PARP cleavage, as well as apoptotic death of HepG2 cells. Further studies are needed to define whether survivin down-regulation by brazilein is causative for caspases activation and apoptosis, or if additional pathways also exist in brazilein-mediated apoptotic death of HepG2 cells that are independent of its effect on survivin. And the molecular mechanism of survivin down-regulation by brazilein in HepG2 cells should be defined in future studies.

Caesalpinia sappan Linn., called Su Mu in Chinese, is an indeciduous tree distributed in China, India, Burma and Vietnam. Caesalpinia sappan Linn. has been used as traditional medicine in China for centuries. Its heartwood has long been used in oriental folk medicines to treat infectious diseases and it is also used as emmenagogue, analgesic, and treating for thrombosis or anti-hepatitis B surface antigen (HBsAg) capability [23–26]. In the present study, we showed for the first time that brazilein, a major component of the extract from Caesalpinia sappan Linn., had an anti-tumor effect on HCC cells. A previous report has shown that the extract of Caesalpinia sappan Linn. induced cell death in head and neck cancer cells [5]. Combined with the fact that brazilin, the hydrogenation form of brazilein, has a DNA strand-nicking activity [27], it has great possibility that brazilein in the Caesalpinia sappan Linn. may be one of the major components with anti-tumor effects. Recently, it has become a trend to search crude natural plants for effective ingredients (particularly for some herbal medicines long and widely used clinically). Many achievements have been made through this approach, such as Taxol [28]. In this regard, brazilein would serve a potent anti-tumor drug candidate.

In conclusion, the results of the present study show that brazilein, an important component from Caesalpinia sappan Linn. ethanol extract, causes a strong down-regulation of survivin protein and mRNA expression in human HCC HepG2 cells together with caspases and PARP cleavages, induction of apoptotic death and inhibition of cell growth. Further studies are needed to establish the efficacy of brazilein in pre-clinical HCC models, which would be useful in supporting a rationale for clinical trial in HCC patients.

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