ORIGINAL ARTICLE

Targeted constitutive activation of signal transducer and activator of transcription 3 in human hepatocellular carcinoma cells by cucurbitacin B

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Abstract

Purpose To determine the effect of cucurbitacin B on human hepatocellular carcinoma cell growth and apoptosis, and to explore the potential mechanisms.

Methods In vitro viability of human hepatocellular carcinoma cell line (HepG2) was investigated using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Morphologic changes of cells were evaluated through light microscopy. Cell cycle distribution was evaluated with flow cytometry following PI staining. Apoptosis was evaluated respectively with flow cytometry and fluorescent microscopy following Annexin V-FITC/PI and Hoechst 33258 staining. Western blot assays were performed to determine the expression of pSTAT3 and Bcl-2. Finally, in vivo effect of cucurbitacin B on the growth of HepG2 cells was determined in nude mice.

Results The MTT assay showed that cucurbitacin B inhibited HepG2 cell viability in a dose and time-dependent manner. Cucurbitacin B treatment resulted in accumulation of cells at the S phase of cell cycle as well as apoptosis. Marked morphological changes, including condensation of chromatin, nuclear fragmentation and apoptotic bodies were clearly shown on Hoechst 33258 staining. Western blot showed that cucurbitacin B inhibited STAT3 phosphorylation and down-regulated the expression of Bcl-2. Growth of HepG2 tumor in nude mice was also inhibited by cucurbitacin B.

Conclusion Our results suggest that cucurbitacin B may have a therapeutic value in suppressing the growth of human hepatocellular carcinoma. The mechanism may be attributable to the suppression of STAT3 phosphorylation.

Keywords Cucurbitacin B · Hepatocellular carcinoma · HepG2 · STAT3 · Apoptosis

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Introduction

In association with the dissemination of hepatitis B and C virus infection, hepatocellular carcinoma (HCC) is the fifth most common cancer and causes more than 600,000 deaths annually worldwide [2, 13, 17]. The disease is most prevalent in Eastern and Southeastern Asia and Middle Africa, with more than half of the patients identified in China. Although surgery remains to be the first choice for HCC, tumor size, hepatic functional reserve and/or portal hypertension may all restrict surgical ablation. Chemotherapy and related methods such as trans-arterial embolization (TAE) have been used as palliative therapies for non-resectable patients [12]. Currently, first line drugs used for HCC include fluorouracil, cisplatin, doxorubicin and mitomycin, but most of these are non-selective cytotoxic molecules with significant side effects [15, 21, 23]. Therefore, in many cases no effective therapy can be offered.

The signal transducer and activator of transcription 3 (STAT3) protein, as a member of transcription factor family, has been implicated in signal transduction by various cytokines (e.g., interleukin-6, interferon-α), growth factors (e.g., epidermal growth factor) and oncoproteins. In normal cells, STAT3 protein activation is strictly controlled to prevent unscheduled gene regulation, whereas constitutively activated STAT3 is found to participate in oncogenesis through up-regulation of genes encoding apoptosis inhibitors, e.g., Bcl-2, Bcl-xL and survivin, cell cycle regulators, e.g., cyclins and c-Myc, and inducers of angiogenesis, e.g., vascular endothelial growth factor[1, 5]. Constitutively activated STAT3 has been demonstrated in approximately 50% of HCC, leukemias, lymphomas, multiple myelomas, and cells derived from prostate, breast, lung, head and neck, ovary and gastric cancers [4, 17]. Recent in vitro and in vivo studies also demonstrated that STAT3 antisense oligonucleotide, STAT3-specific siRNA, and anti-cancer agent YC-1 can all induce apoptosis and inhibit tumor growth via suppression of STAT3 activation in HCC cells [9, 17, 19].

Cucurbitacins are compounds isolated from various plants which have been used as folk medicines for centuries in Asian countries such as China and India. A number of compounds from this group have been investigated for their cytotoxic, anti-inflammatory, and/or anti-cancer effects, among which cucurbitacin B has been most widely used [11, 18, 20]. In recent years, several studies have suggested an inhibitory effect of cucurbitacin B on the growth of human cancer cell lines and tumor xenografts including leukemia, lymphoma, multiple myeloma, melanoma and cancers of oral cavity, nasopharynx, breast, prostate, lung and central nervous system in origin [3, 16, 18]. To our knowledge, however, so far there has been no report concerning the anti-tumor effect of cucurbitacin B on human HCC cells, e.g., HepG2.

As constitutive activation of STAT3 was detected in HCC, and blockade of STAT3 signaling has been shown to effectively inhibit cell growth and induce apoptosis in HepG2 cells, we hypothesized that cucurbitacin B would be useful in treating HepG2. To test such a hypothesis, we designed the present study to examine the in vitro and in vivo anti-tumor effect of cucurbitacin B on human HCC cells, including the effects on cell growth, cell-cycle distribution, apoptosis, and the expression of proteins involved in the regulation of cell cycle and apoptosis pathways.

Materials and methods

Reagents and animals

Cucurbitacin B, cucurbitacin B liposome and blank liposome were derived from Department of Pharmacy, Shenyang Pharmaceutical University, China. 5-FU was obtained from Shanghai Xudonghaipu Pharmaceutical Company, China. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was bought from Fluka, USA. Propidium iodide (PI) was from Biosharp, USA. Annexin V-FITC apoptosis detection kit was from Jingmei Biotech Co. Ltd. Shenzhen, China. Hoechst 33258 and DMSO were from Sigma, USA. Phospho-STAT3 (Tyr 705, sc-7993) and actin primary antibodies as well as the second antibodies goat anti-mouse IgG HRP conjugate and goat anti-rabbit IgG HRP conjugate were from Santa Cruz Biotechnology Inc. CA, USA. STAT3 antibody was from Cell Signaling Technology, MA, USA, Bcl-2 antibody was from Beyotime, Jiangsu, China. Enhanced chemiluminescence detection reagents were from Amersham Biosciences, Buckinghamshire, UK. RPMI 1640 and FBS were from GIBCO Life Technologies, Paisley, UK. Other laboratory reagents were from Sigma, USA. Female BALB/c (nu/nu) athymic nude mice, 5–6 weeks of age, weighing 16–18 g, were from Chinese Academy of Medical Sciences and Peking Union Medical College Institute of Laboratory Animal Science, Beijing, China.

Cell cultures

HepG2 cells from human hepatic cancer (provided by Shanghai Institute for Biological Sciences, Chinese Academy of Sciences) were maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (TBD, Tianjin, China), 100 units/ml penicillin G, 100 μg/ml streptomycin. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. Cells were prepared to the same initial density for treatments with cucurbitacin B dissolved in DMSO (Sigma).

Cell viability assay

Effect of cucurbitacin B treatment on the viability of HepG2 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Fluka, USA) assay based on the ability of live cells to cleave the tetrazolium ring to a molecule that absorb at 570 nm. 2.5×10^3 cells (100 μl)/well were grown in 96-well plates for 24 h and the cells were further incubated with different concentrations of cucurbitacin B in RPMI-1640 supplemented with 10% FBS medium. After 24, 48, and 72 h, 10 μl MTT reagent was added to each well, and the cells were further incubated at 37°C for 4 h,100 μl of 10% sodium dodecyl sulfate including 10 mM hydrochloric acid was added to each well for overnight, followed by reading on a scanning multi-well spectrophotometer (Sunrise, RC, Tecan, Switzerland). Untreated cells were used as controls. The final concentration of DMSO (0.25%) showed no interference with the

growth of cells. At least three replicate experiments were performed with three wells per concentration.

Cell morphology

The effect of cucurbitacin B treatment on HepG2 cell morphologic changes was monitored under a light microscope. 1×10^6 cells were seeded in six-well plates and allowed to attach overnight. Thereafter the cells were treated with 10 μM cucurbitacin B or solvent control for 24 h. Effect of cucurbitacin B treatment on cell morphology was monitored under a light microscope and photographed (10× magnifications).

Flow cytometry

Cell cycle analyses were carried out using flow cytometry. HepG2 cells (1×10^6) were seeded in six-well plates and allowed to attach overnight. Growing cells were treated with different concentrations of cucurbitacin B and harvested by 0.25% trypsin (Beyotime Institute of Biotechnology, China) at the indicated time. The cells were then washed twice with PBS, centrifuged at 1,000 rpm for 5 min, fixed in 70% ethanol at 4°C. Before DNA analyses, the cells were washed again with PBS, treated with 50 μg/ml RNase, and stained with 25 μg/ml propidium iodide (Sigma, USA) in the dark. Flow cytometry analyses were carried out on a FACScalibur instrument using the ModFit program (Becton Dickinson, USA).

Apoptosis was detected with an Annexin V-FITC/Propidium iodide (PI) detection kit (Jingmei, China). 5,000 cells per sample were analyzed with a FACScalibur flow cytometer. Annexin V is a Ca^{2+} dependent phospholipids-binding protein that has a high affinity for phosphodylserine (PS), translocated from the inner leaflet of the plasma membrane to the outer leaflet in apoptotic cells. Annexin V-FITC is a sensitive probe for identifying cells undergoing apoptosis, as PS exposure occurs early in the apoptotic process. PI is a nonspecific DNA dye that is excluded from live cells with intact plasma membranes but incorporated into nonviable cells. Single positive populations are considered early apoptotic (Annexin V+/PI−) or necrotic cells (Annexin V−/PI+), whereas double positive (Annexin V+/PI+) cells are thought to be in a late stage of apoptosis.

Hoechst 33258 staining

HepG2 cells (5×10^5) were grown on coverslips placed in six-well plates. Cells were treated with different concentrations of cucurbitacin B, harvested at the suggested time, and then washed in PBS and fixed with methanol and acetic acid (3/1, v/v) at 4°C. Preparations were washed in PBS and stained with Hoechst 33258 (Sigma, 5 μg/ml final con-

centration) for 30 min in the dark, washed again in PBS and finally mounted in a medium containing 80% glycerol in PBS. Processed cells were observed under a fluorescence microscope (Olympus, Japan).

Western blot analysis

Cells (1×10^6/well) were seeded in six-well plates and treated with cucurbitacin B or solvent control. Following removal of the medium, cells were washed with cold PBS, and then scraped and washed twice by centrifugation at $1,000 \times g$ for 5 min at 4°C. The pellet was re-suspended in lysis buffer supplemented with proteases and phosphotase inhibitors and incubated for 1 h at 4°C. The lysate was collected by centrifugation at $14,000 \times g$ for 40 min at 4°C, and the supernatant (total cell lysate) was stored at −20°C. For Western blot analysis, 80 μg proteins were resolved over 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was processed in blocking buffer (5% non-fat dry milk/1‰ Tween 20 in TBS) for 1 h at room temperature, incubated with appropriate primary antibodies in blocking buffer overnight at 4°C, incubated with appropriate horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence and autoradiography using X-ray film. β-actin was detected on the same membrane and used as a loading control.

Anti-tumor activity in the nude mouse tumor xenograft model

All animal studies were carried out in accordance with the "Guide for the Care and Use of Laboratory Animals". HepG2 cells, 5×10^6 cells per 0.2 ml in PBS, were injected subcutaneously into the right flank of animals. Tumor size was measured every other day in two dimensions using a caliper. Volume of the tumors was calculated using the formula: $ab^2/2$, where b is the smaller dimension. When tumors reached about 150 mm^3, animals were randomized in five groups (6 each). In three groups, they were treated with 27.5, 55, and 110 μg/kg per day of cucurbitacin B liposome (*i.v.*), one group was treated with blank liposome in 5% glucose solution as negative control, the other one received 5-FU at 20 mg/kg per every other day as the positive control. At the end of experiment, animals were euthanized and tumor weight was measured. Dosages of cucurbitacin B treatment were chosen based on our previous studies, which are nontoxic and effective, and were well tolerated by the animals in this study.

Statistical analysis

All values are expressed as mean ± SD. One way ANOVA followed by LSD-*t* test and SNK-*q* test was used to
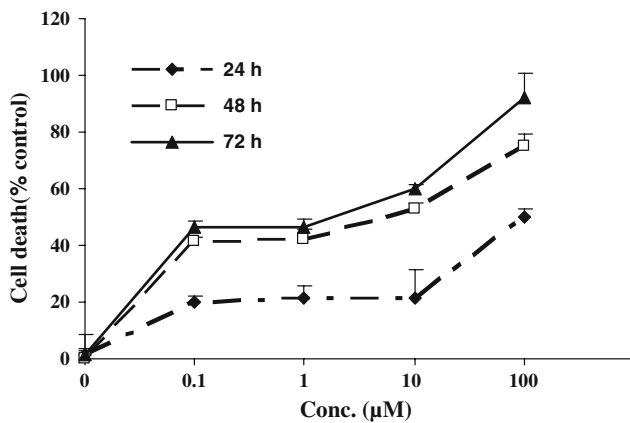
Fig. 1 Dose and time-dependent effect of cucurbitacin B on HepG2 viability. HepG2 cells at 2.5×10^3 cells/well were cultured with cucurbitacin B (0.1, 1, 10, and 100 μM) for 24, 48, and 72 h in RPMI 1640-10% FBS. Daily cell viability was determined by a MTT assay
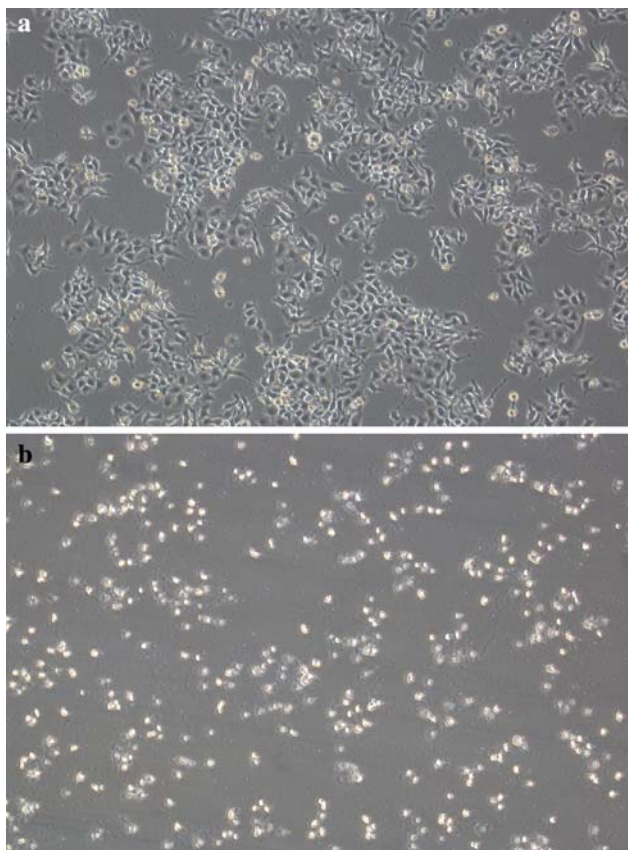

Fig. 2 Effect of cucurbitacin B on HepG2 cell morphologic change. HepG2 cells (1×10^6) were treated with solvent control (**a**) or 10 μM of cucurbitacin B (**b**) and analyzed at 24 h by light microscopy
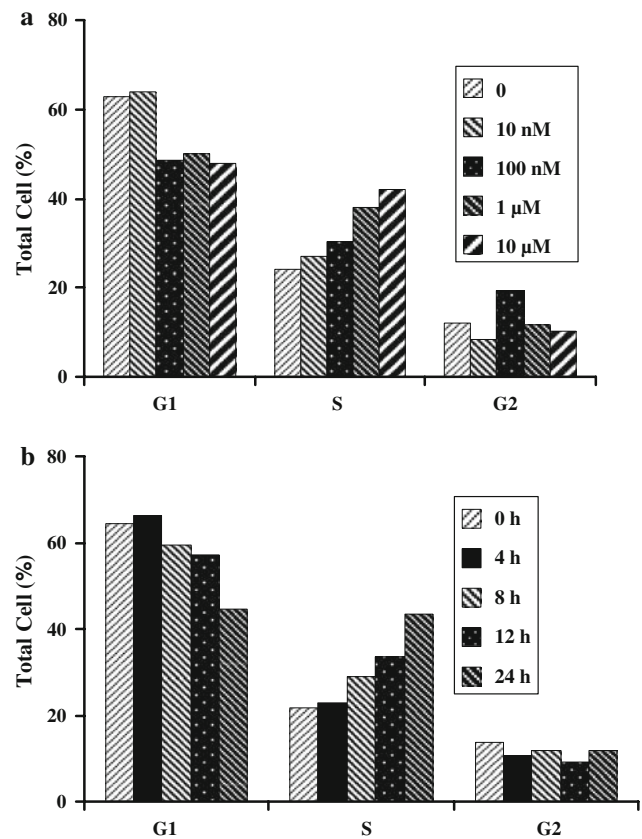

Fig. 3 Effect of cucurbitacin B on HepG2 cell cycle distribution. HepG2 cells (1×10^6) were treated with 0, 0.01, 0.1, 1, and 10 μM of cucurbitacin B and analyzed at 24 h by DNA flow cytometry (**a**). HepG2 cells (1×10^6) were treated with 10 μM of cucurbitacin B and analyzed at 0, 4, 8, 12, and 24 h by DNA flow cytometry (**b**). The values indicate the percentage of cells in the indicated phases of the cell cycle

Results

Effect of cucurbitacin B on HepG2 cell viability

Human HCC cells HepG2 were treated with 0.1~100 μM of cucurbitacin B for 24–72 h, with their viability measured with a MTT assay. Treatment with cucurbitacin B resulted in a dose and time-dependent inhibition of cell viability. The concentration to achieve 50% growth inhibition (IC_{50}) was estimated to be 1.66 μM and 0.52 μM for 48 h and 72 h, respectively (Fig. 1).

Effect of cucurbitacin B on HepG2 cell morphology

To verify whether cucurbitacin B has an effect on the morphology of HepG2 cells, light microscopy was used for checking the cells after treatment with 10 μM cucurbitacin B. Compared with control, processed HepG2 cells clearly showed changes in their general morphology from typical elongated form to round-shaped (Fig. 2).

compare the differences between groups. A two-sided P value less than 0.05 was considered to be statistically significant.

Fig. 4 Apoptosis measured by Annexin V-FITC/PI staining. HepG2 cells were treated with 0 (**a**) 1 μM (**b**) or 10 μM (**c**) cucurbitacin B for 24 h, stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. The horizontal and vertical axes represent labeling with Annexin V-FITC and PI, respectively. LR represents early apoptotic cells (positive for Annexin V only), LL represents live cells. Graphic presentation of data obtained by Annexin V-FITC/PI staining after 24 h treatment with 1 and 10 μM cucurbitacin B was also shown (**d**)

Control

Treated with 1 μM Cucurbitacin B

Treated with 10 μM Cucurbitacin B
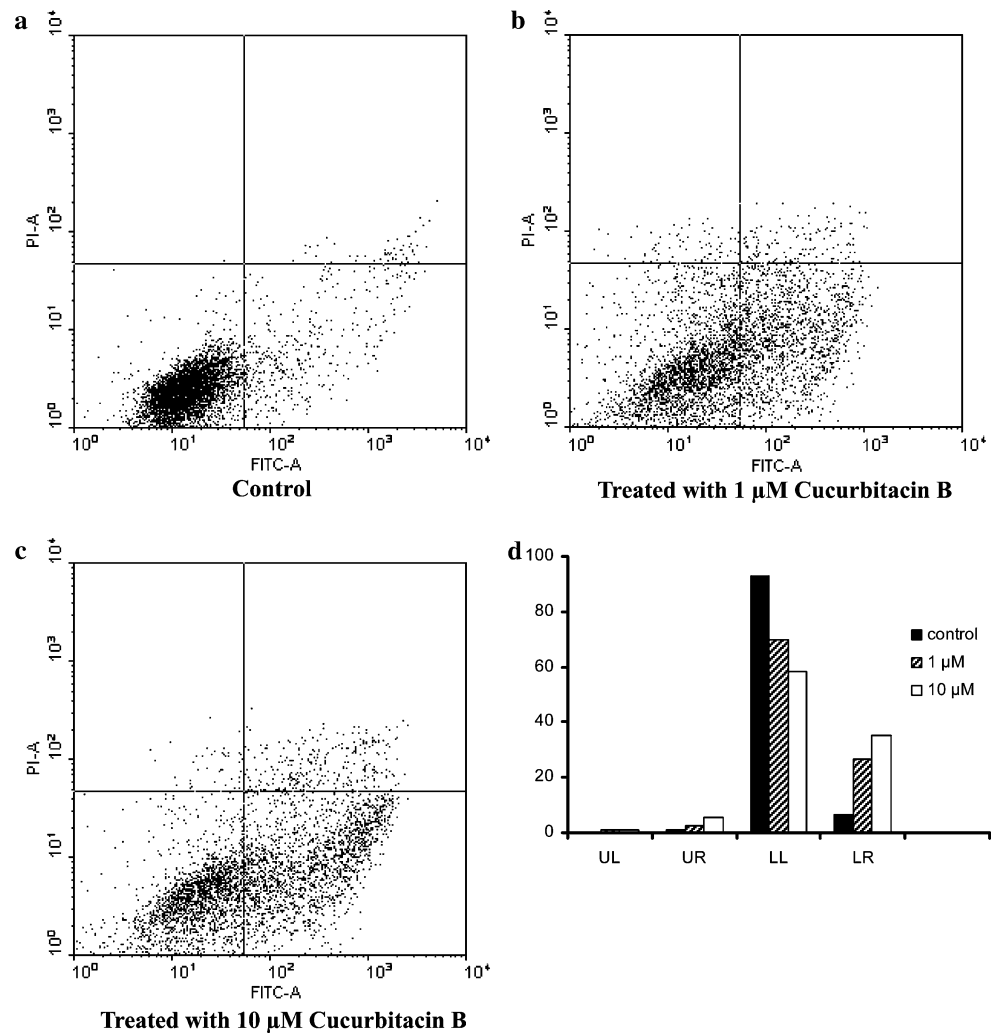
Effect of cucurbitacin B on HepG2 cell cycle and apoptosis

To understand the mechanism by which cell reduction is achieved, we have investigated the effect on cell cycle distribution using flow cytometry. As shown in Fig. 3, the percentage of S phase cells was increased in a dose and time-dependent manner. 24 h exposure of HepG2 cells to 10 μM cucurbitacin B caused a significant increase in the population of S phase cells (from 24.09% to 41.96%) (Fig. 3a). Treating HepG2 cells with 10 μM of cucurbitacin B for 4, 8, 12, and 24 h resulted in similar changes (Fig. 3b).

To examine whether cucurbitacin B induced cytotoxicity involves apoptosis, we evaluated the effect of cucurbitacin B on HepG2 apoptosis with an Annexin V assay and Hoechst 33258 staining. As shown by FACS, there appeared to be a dose-dependent increase in the proportion of apoptotic cells in all samples compared with control (Fig. 4).

After cells were treated with 0, 0.1, 1, and 10 μM cucurbitacin B for 24 h, or with 10 μM cucurbitacin B for 0, 8, 12, and 24 h, marked morphological changes of cell apoptosis including condensation of chromatin, nuclear fragmentations and apoptotic bodies were clearly demonstrated on Hoechst 33258 staining (Fig. 5). Apoptotic HepG2 cells gradually increased in a dose and time-dependent manner.

Effect of cucurbitacin B on HepG2 STAT3 activation

Previous evidence has shown that cucurbitacin B suppresses tumor growth and induces cell apoptosis in A549 cells by inhibiting STAT3 signaling [16]. Here we investigated the relationship between the anti-tumor activity of cucurbitacin B and STAT3 phosphorylation in HepG2 cells. Effect of cucurbitacin B treatment on pSTAT3 expression was determined through Western blot analysis. We observed that cucurbitacin B suppressed STAT3 phosphorylation in a dose-dependent manner. Treatment with 10 μM cucurbitacin B for 24 h suppressed STAT3 phosphorylation almost completely (Fig. 6). Above results suggested that cucurbitacin B may exert its anti-tumor activity on HepG2 cells through inhibition of STAT3 activation.
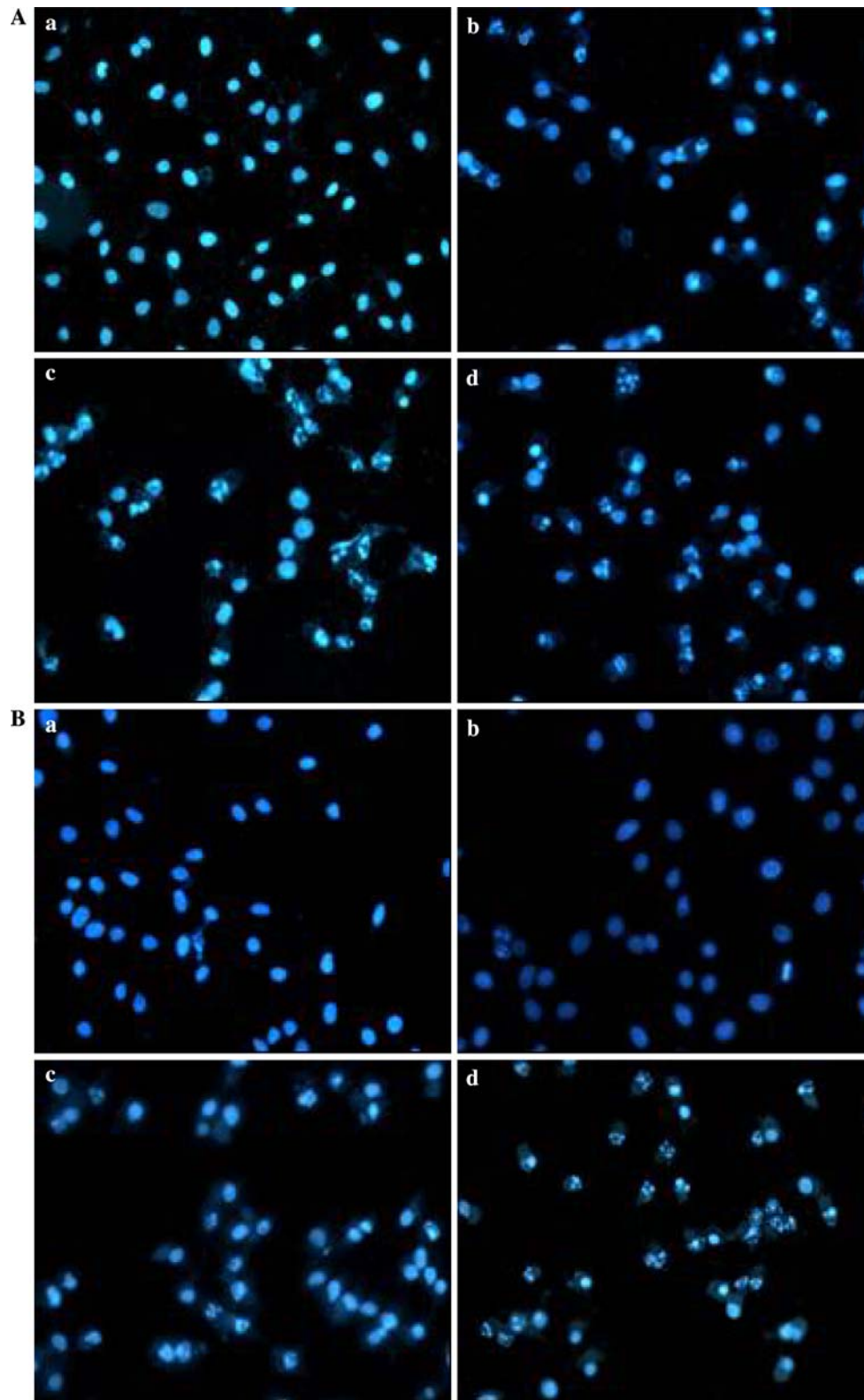
Fig. 5 Apoptosis observed by Hoechst 33258 staining (40×). HepG2 cells treated with 0 (**a**), 0.1 (**b**), 1 (**c**), and 10 μM (**d**) cucurbitacin B for 24 h (**A**). HepG2 cells treated with 10 μM cucurbitacin B for 0 (**a**), 8 (**b**), 12 (**c**), and 24 h (**d**) (**B**)

Cu B (μM)   0   0.1   1   10
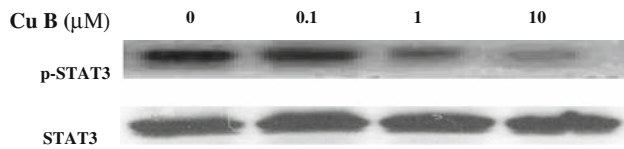
p-STAT3

STAT3

Fig. 6 Western blot analysis of p-STAT3 protein. HepG2 cells were treated with 0, 0.1, 1, and 10 μM cucurbitacin B for 24 h. The cells were harvested and processed for Western blot as described in "Material and Methods". Total STAT3 was used as internal control

Effect of cucurbitacin B on the expression of Bcl-2

Recent evidences have demonstrated that cucurbitacin B regulates anti-apoptotic genes such as Bcl-2 and Bcl-xL by suppressing the activation of STAT3, and consequently inhibits tumor growth and induces cell apoptosis [18]. To determine the expression changes of STAT3 target genes involved in cell apoptosis, we measured the expression of Bcl-2 with Western blot, and found that cucurbitacin B suppressed the Bcl-2 expression in a dose-dependent manner (Fig. 7).

Anti-tumor effect of cucurbitacin B on HepG2 xenograft model

To investigate its effect on tumor growth in vivo, mice with HepG2 xenograft tumors were treated with cucurbitacin B. HepG2 cells were injected into the right flank of 30 mice. Twenty-one days after tumor inoculation, these animals were randomized into five groups (6 each) and received caudal vein injection of 27.5, 55, and 110 μg/kg per day of cucurbitacin B liposome, 20 mg/kg per every other day of 5-FU, or blank liposome in 5% glucose solution. Tumor growth (volume) curves up to day 13 post treatment were drawn. As shown in Fig. 8, cucurbitacin B-treated mice showed a significantly lower growth rate compared to negative controls. At the end of experiment, the average tumor volume of the treated groups were 0.44 ± 0.08 cm^3 ($P < 0.05$), 0.31 ± 0.1 cm^3 ($P < 0.01$), 0.20 ± 0.03 cm^3 ($P < 0.01$) and 0.23 ± 0.02 cm^3 ($P < 0.01$) for 27.5, 55, 110 μg/kg cucurbitacin B or 5-FU treated mice, respectively, which were significantly lower than that of negative controls (0.53 ± 0.09 cm^3), indicating that cucurbitacin B

Cu B (μM)   0   0.1   1   10
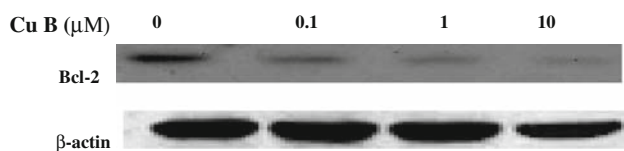
Bcl-2

β-actin

Fig. 7 Western blot analysis of Bcl-2 protein. HepG2 cells were treated with 0, 0.1, 1, and 10 μM cucurbitacin B for 24 h. The cells were harvested and processed for Western blot as described in "Material and Methods". β-actin was used as internal control
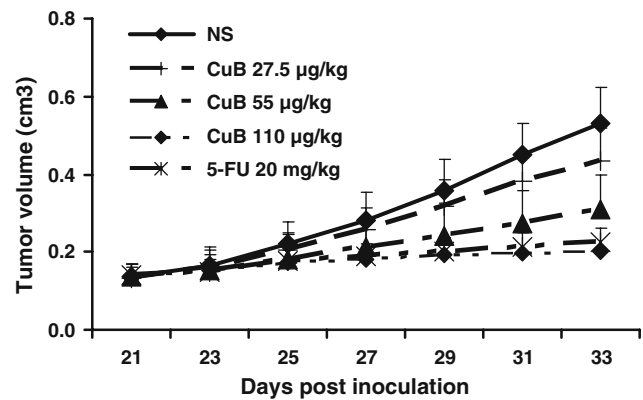
Fig. 8 Anti-tumor effect of cucurbitacin B on a HepG2 xenograft model. Athymic nude mice transplanted with HepG2 cells (6 each) were treated with 27.5, 55, and 110 mg/kg of cucurbitacin B liposome (*i.v.*) daily. Blank liposome in 5% glucose solution was served as negative control, 20 mg/kg per every other day of 5-FU as positive control. Values represent means ± SD

inhibited HepG2 tumor growth in vivo in a dose-dependent fashion.

Discussion

STAT3 is a well-known oncogene that is aberrantly activated in many types of human cancers, including hepatoma, lymphomas, multiple myelomas and cancers of prostate, breast, lung, head and neck, ovary and stomach in origin. The STAT3 signaling pathway has become a target for anti-cancer therapy experiments [1, 7, 17]. Recently, several studies have suggested that cucurbitacin B exerts inhibitory effects against several human cancer cell lines and tumor xenografts via suppression of STAT3 phosphorylation [11, 16, 18]. In present study, we examined the inhibitory effect of cucurbitacin B on HCC cells (HepG2) both in vitro and in vivo, and found that cucurbitacin B exhibited growth inhibitory activity of HepG2 in a dose and time-dependent manner (Figs. 1, 8).

To elucidate the mechanism by which cucurbitacin inhibited cell growth, we performed a series of experiments on cell cycle distribution and apoptosis. Flow cytometry analysis showed that the administration of cucurbitacin B resulted in an accumulation of cells at S phase (Fig. 3). Flow cytometry analysis with Annexin V/PI staining indicated that cucurbitacin B induced HepG2 cell apoptosis in a dose manner (Fig. 4). Fluorescent microscopy observation by Hoechst 33258 staining showed that treatment of HepG2 cells with cucurbitacin B led to the occurrence of typical morphological changes of apoptosis (Fig. 5). These data all suggested that the inhibitory efficacy of cucurbitacin B against HepG2 cells was due to the induction of cell cycle arrest as well as apoptosis.

Sun et al. [17] recently reported that STAT3 was constitutively activated in human HepG2 cells and other human HCC cell lines. In the present study, we also showed that STAT3 was constitutively activated in human HepG2 cells. Treatment with cucurbitacin B decreased the level of p-STAT3 protein in a dose-dependent manner in HepG2 cells (Fig. 6), suggesting that the induction of cell cycle arrest and apoptosis of HepG2 cell line by cucurbitacin B was due to the suppression of STAT3 activation.

Constitutively activated STAT3 is responsible for anti-apoptotic genes such as Bcl-2 family members. On the other hand, over-expression of Bcl-2 has been observed in many cancers including HCC, where it plays a pivotal role in tumor initiation and progression [6, 8, 14]. Recent studies have demonstrated that Bcl-2 silencing can induce apoptosis of human HCC cells and sensitize them for chemotherapeutic drugs [10, 22]. Our results have shown that cucurbitacin B can induce apoptosis of HepG2 cells through down regulation of Bcl-2 expressions via suppression of STAT3 activation.

To determine the potential for cucurbitacin B for inhibiting tumor growth in vivo, we evaluated the anti-tumor activity of cucurbitacin B against HepG2 tumors in a nude mouse xenograft model. The result showed that cucurbitacin B can significantly suppress tumor growth in a dose-dependent manner (Fig. 8). Taken together, our results have demonstrated for the first time that cucurbitacin B can inhibit HepG2 cell viability and induce cell apoptosis through suppression of activated STAT3, suggesting that cucurbitacin B may provide a potential alternative for the treatment of HCC.

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