Cucurbitacin B inhibits growth and induces apoptosis through the JAK2/STAT3 and MAPK pathways in SH-SY5Y human neuroblastoma cells

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Abstract. Cucurbitacin B (CuB) is a tetracyclic triterpene that is contained in extracts from cucurbitaceous plants and has been demonstrated to have anticancer and anti-inflammatory activities. The purpose of the present study was to determine whether CuB exhibits anticancer effects on SH-SY5Y human neuroblastoma cells and to analyze the underlying molecular mechanism. The results demonstrated that CuB not only induced cell cycle arrest at the G2/M phase, but also induced apoptosis as characterized by positive Annexin V staining, downregulation of phospho-Janus kinase 2 (p-JAK2), phospho-signal transducer and activator of transcription 3 (p-STAT3), phospho-extracellular signal-regulated kinases and the activation of c-Jun N-terminal kinase and p38 mitogen activated protein kinase (MAPK). CuB also altered the expression of gene products that mediated cell proliferation (Cyclin B1 and cyclin-dependent kinase 1), cell survival (B-cell lymphoma 2, Bcl2-associated X protein) and increased the expression of p53 and p21. These results provide the evidence that JAK2/STAT3 and MAPKs have crucial roles in CuB-induced growth inhibition and apoptosis in SH-SY5Y human neuroblastoma cells.

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Introduction

Neuroblastoma (NB) is the most common type of extracranial solid tumor among children that arises in the peripheral sympathetic nervous system. NB typically emerges in the adrenal medulla or paraspinal ganglia during embryogenesis. NB accounts for ~10% of pediatric cancer-associated mortalities, with an annual incidence of ~650 novel cases in the USA (1) and it is a malignant tumor with a low degree of differentiation. According to the degree of histopathological differentiation, neuroblastic tumors can be divided into NB, ganglioneuroblastoma and ganglioneuroma (2). The prognosis depends on the patients' age, location, region and biological characteristics of NB, but it is usually poor. In recent years, as methods of early diagnosis have improved, the survival rate of children with NB has significantly increased; however, patients with high-risk cases still have a <40% chance of survival. Therefore, it is urgent to search for a novel adjuvant agent suitable for the treatment of NB with few side effects in order to increase the overall survival rate.

Cucurbitacins are a group of tetracyclic triterpenes isolated from Cucurbitaceae (3). Previous studies have demonstrated that they have a broad range of pharmacological effects, including anti-inflammatory (4,5), anti-fertility (5), anti-viral (6) and anticancer (4,7,8) activities. Chemically, cucurbitacins are highly diverse and are arbitrarily divided into 12 categories (5). Cucurbitacin B (CuB) is one of the most abundant forms of cucurbitacins and the most widely used as an anticancer agent. It has significant anti-inflammatory activity and is used traditionally to treat hepatitis (9). Studies indicate that CuB is capable of inhibiting the growth of a wide spectrum of malignant human cells, including myeloid leukemia, breast cancer, glioblastoma multiforme, pancreatic cancer, laryngeal cancer, melanoma and osteosarcoma cells (10-16). The anticancer mechanism of CuB is activated by different signaling pathways in different cancer cells. Several studies have shown that CuB induces apoptosis by inhibiting the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway (13,14), but others have indicated that CuB is a potent inhibitor of nuclear factor

κ-light-chain-enhancer of activated B cells activation (17), and additionally exhibits anticancer effects through wingless type signaling (11).

In previous years, researchers have searched for novel effective drugs with few side effects suitable for the treatment of NB. Gheeya *et al* (18) used a panel of drugs in order to identify novel effective chemotherapeutics against NB, and they demonstrated that cucurbitacin I inhibits cell growth through inhibition of the STAT3 pathway. Although there is an increase in the amount of evidence indicating that CuB is also an inhibitor of the STAT3 pathway in several tumor cell lines (8,13,14,19), whether CuB exhibits anticancer effects in NB cells and its exact molecular mechanism remain to be elucidated. In the present study, the effects of CuB on human NB SH-SY5Y cells were evaluated, and the molecular mechanisms underlying CuB-induced apoptosis were studied.

Materials and methods

Chemicals and reagents. CuB at 98% purity was purchased from Must Biotechnology Co., Ltd. (Chengdu, China). MTT, dimethyl sulfoxide (DMSO), PD98059, SP600125, SB203580 and interleukin (IL)-6 were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were acquired from HyClone Laboratories (Logan, UT, USA). Propidium iodide (PI), AG490 and bicinchoninic acid (BCA) Protein Assay kit were obtained from Beyotime (Shanghai, China). An Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit was purchased from MultiSciences Biotech Co., Ltd (Hangzhou, China). The primary antibodies specific to p53 (rabbit pAb), p21 (mouse mAb), B-cell lymphoma 2 (Bcl-2; rabbit pAb), Bcl2-associated X protein (Bax; rabbit pAb), phospho-JAK2 (Tyr 1007/1008; rabbit mAb), JAK2 (mouse mAb), phospho-STAT3 (Tyr705; mouse mAb), phospho-extracellular signal-regulated kinases (p-ERK; Thr202/Tyr204; rabbit pAb), phospho-p38 (Thr180/Tyr182; rabbit pAb) and β-actin (mouse mAb) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-c-Jun N-terminal kinase (p-JNK; Thr183/Tyr185; rabbit mAb) primary antibody was purchased from Cell Signaling Technology (Danvers, MA, USA), cyclin-dependent kinase 1 (CDK1; mouse mAb), Cyclin B1 (rabbit pAb), STAT3 (rabbit pAb), ERK (rabbit pAb), JNK1 and 2 (rabbit pAb), and p38 primary antibody (rabbit pAb) and secondary polyclonal antibodies goat anti-mouse immunoglobulin (Ig) G horseradish-peroxidase (HRP)-conjugate and goat anti-rabbit IgG HRP-conjugate were obtained from Boster Biological Technology., Ltd. (Wuhan, China).

Cell line and culture. The human NB cell line (SH-SY5Y) was obtained from the China Center for Type Culture Collection (Wuhan University, Wuhan, China). The cells were cultured in DMEM supplemented with 10% FBS at 37˚C in a humidified atmosphere containing 5% CO_2. All the experiments were performed one day after the cells were seeded.

Cell viability assay. The effect of CuB on the growth and proliferation on cancer cells was assessed by measuring the metabolic activity (MTT assay). The cells were seeded in 96-well plates at a density of $2x10^4$ cells/well with 200 μl culture medium per well for 24 h. On the next day, the medium was replaced with fresh medium containing different concentrations of CuB (0-128 μM). Subsequent to incubation for an additional 24 and 48 h, a total of 20 μl MTT [5 mg/ml in phosphate-buffered saline (PBS)] solution was added to each well and incubated at 37˚C for 4 h to metabolize the MTT into formazan. Next, the supernatant was discarded and 100 μl DMSO was added to each well to terminate the reaction. The absorbance was measured at 490 nm using a microplate reader (Model 680; Bio-Rad, Richmond, VA, USA). Three independent experiments were performed in triplicates. The percentage of proliferation was normalized relative to the control.

Cell cycle analysis. The cell cycle parameters were analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The SH-SY5Y cells were cultured with the indicated concentrations of CuB for 24 h. The cells were harvested by centrifugation, then washed with cold PBS (pH 7.4) and fixed with 70% ice-cold ethanol overnight at -20˚C. Following fixation, the cells were harvested and rinsed once with PBS (pH 7.4) and then incubated with 500 μl PI staining solution (50 μg/ml PI, 100 μg/ml RNase A) for 1 h at room temperature. The relative numbers of cells in the G1, S and G2/M phases of the cell cycle were measured.

Annexin V-FITC/PI staining. The SH-SY5Y cells were exposed to the indicated concentrations of CuB for 24 h. The samples of $1-5x10^5$ cells were harvested and rinsed twice with cold PBS (pH 7.4). The cells were resuspended in 500 μl binding buffer and stained with 5 μl Annexin-FITC and 10 μl of PI for 5 min in the dark at room temperature. The stained apoptotic cells were counted using a FACScan flow cytometer.

Western blot analysis. Following treatment with the indicated concentrations of CuB, the SH-SY5Y cells were lysed and the protein concentration was determined using a BCA protein assay kit. The lysate containing 40 μg of protein was subjected to SDS-PAGE. The protein was transferred to a nitrocellulose membrane, and the membrane was blocked overnight with 1X Tris-Buffered Saline containing 0.1% Tween-20 and 5% skimmed milk at 4˚C. Following blocking, the membrane was washed three times and incubated with the respective primary antibodies for 2 h at room temperature. Next, the membrane was washed three times and incubated with the diluted HRP-conjugated secondary antibody (1:5,000) for 1.5 h at room temperature. Subsequent to the three washes, the membrane was detected using an enhanced chemiluminescence kit (Millipore, Bedford, MA, USA).

Statistical analysis. All the values are expressed as the mean ± standard deviation. Significant differences between the groups were determined using Student's t-test and the statistical significance was expressed as $^{*}P<0.05$ and $^{**}P<0.01$. All the figures shown represent the results from at least three independent experiments.

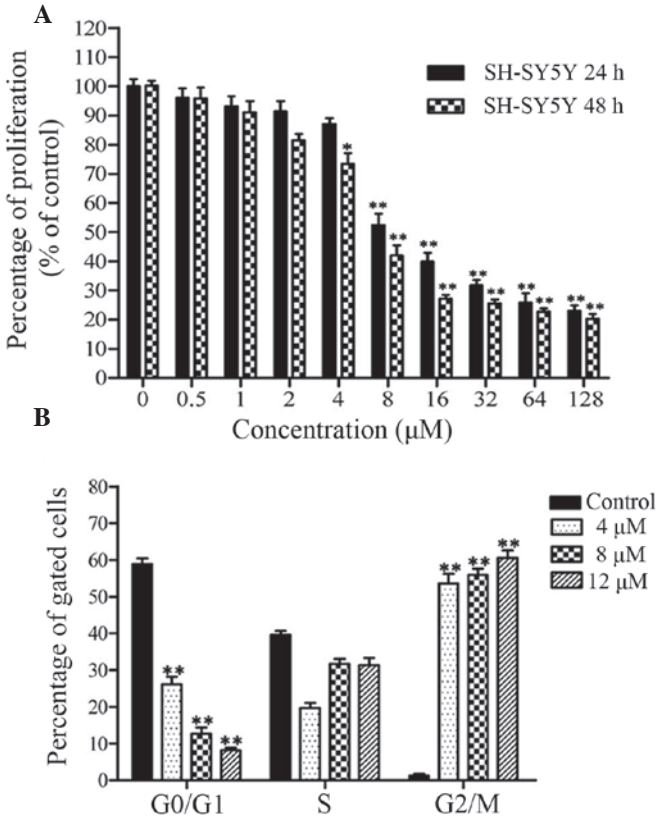

Figure 1. Effects of CuB on the proliferation of SH-SY5Y cells. (A) The SH-SY5Y cells were treated with CuB at the concentrations indicated for 24 and 48 h. The viability of CuB was determined by an MTT assay. (B) The cell cycle distribution was analyzed by PI staining using flow cytometry after CuB treatment for 24 h. The histograms show the cell cycle distributions in the SH-SY5Y cells. All the values are the mean ± SD of three independent experiments. Significant differences from the control are indicated by *P<0.05 and **P<0.01. CuB, cucurbitacin B; PI, propidium iodide; SD, standard deviation.

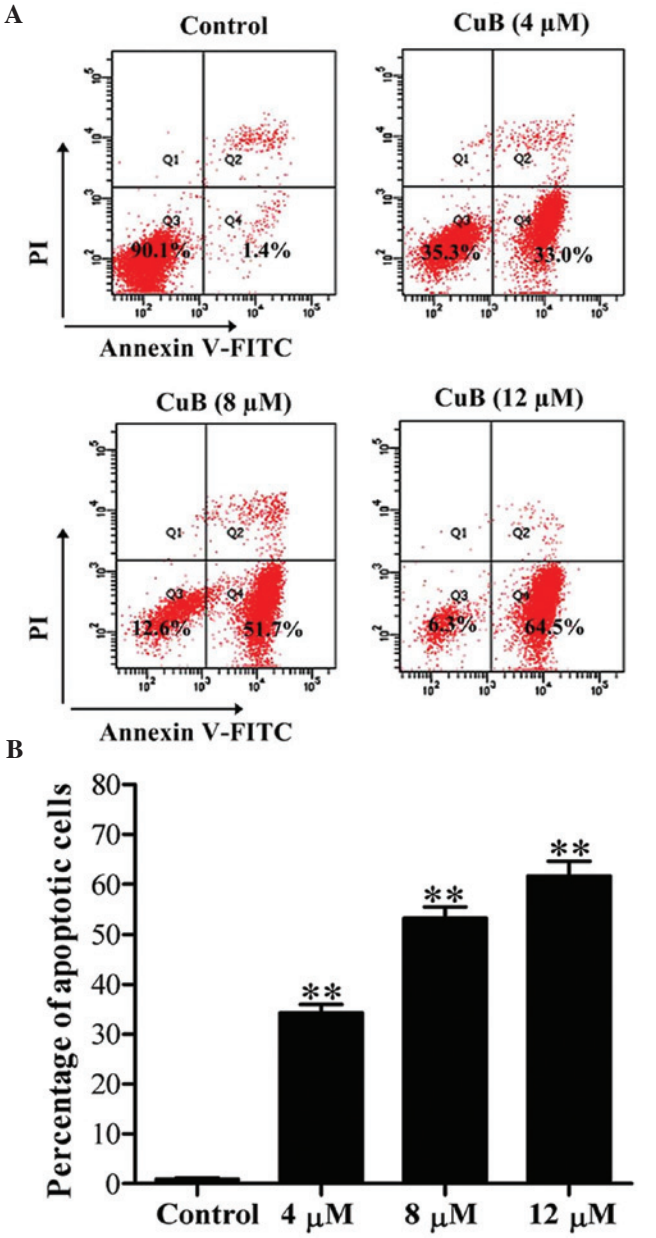

Figure 2. Effects of CuB on the apoptosis of SH-SY5Y cells. (A) The cells were treated with CuB for 24 h, the apoptotic effect of CuB was assessed by flow cytometric analysis after staining with Annexin V-FITC/PI. The right bottom quadrant represents the Annexin V-stained cells (early-phase apoptotic cells). (B) Histograms showing the early apoptotic rate in SH-SY5Y cells. The values are the mean ± SD of three independent experiments. Significant differences from the control are indicated by *P<0.05 and **P<0.01. CuB, cucurbitacin B; FITC, fluorescein isothiocyanate; PI, propidium iodide; SD, standard deviation.

Results

CuB inhibits proliferation of SH-SY5Y cells. In order to investigate the growth inhibition effects of CuB, the MTT assay and PI staining were performed to evaluate the cell viability and cell cycle distribution, respectively.

The viability of cancer cells treated with CuB was investigated by the MTT assay. CuB inhibited cell proliferation in SH-SY5Y cells in a time- and dose-dependent manner (Fig. 1A). The concentrations of 4, 8 and 12 μM CuB were used in subsequent experiments.

Cell cycle arrest caused by CuB for 24 h was investigated by flow cytometry following PI staining. Evident changes were found in the cell cycle distributions when treated with CuB (Fig. 1B). The percentage of cells in the G2/M phase was increased in a dose-dependent manner. These data indicated that CuB treatment suppressed cell proliferation through an increased accumulation of cells in G2/M phase of the cell cycle.

CuB induces early apoptosis in SH-SY5Y cells. Apoptosis in SH-SY5Y cells was detected by flow cytometry following Annexin V-FITC/PI double staining. The results revealed that the early apoptotic rate (Annexin V-FITC-positive and PI-negative cells) was significantly increased following CuB treatment for 24 h and the rate increased in a dose-dependent manner (Fig. 2).

CuB inhibits JAK2/STAT3 signaling cascades. The phosphorylated forms of JAK2 and STAT3 were assessed by western blot analysis in SH-SY5Y cells treated with CuB for 24 h. Constitutive activation of JAK2 and STAT3 were suppressed by CuB in a concentration-dependent manner (Fig. 3A). Next, the role of JAK2/STAT3 in CuB-induced apoptosis by a JAK2 inhibitor (AG490) and an activator of JAK2/STAT3 (IL-6)

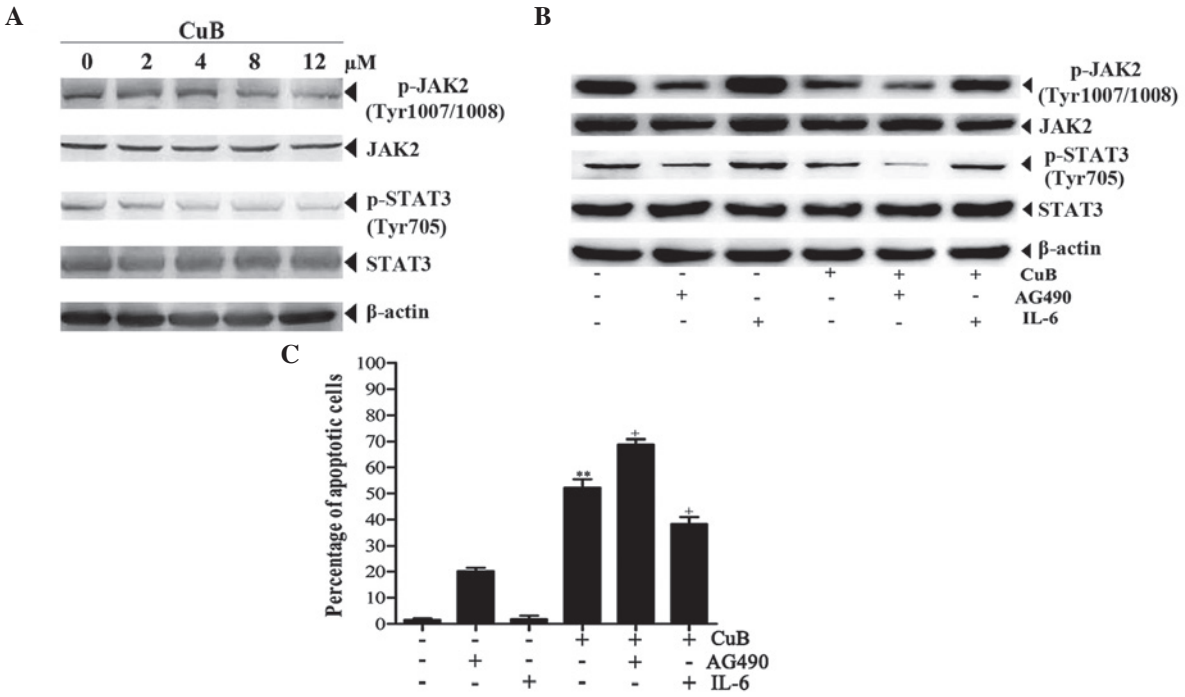

Figure 3. Effect of CuB on JAK2/STAT3 signaling cascades. (A) Cell lysates from the SH-SY5Y cells treated with CuB for 24 h were analyzed by western blot analysis using antibodies against p-JAK2, JAK2, p-STAT3 and STAT3, and β-actin was used as a loading control (bottom panel). (B) The cells were preincubated with or without AG490 or IL-6 for 1 h and further incubated in the presence or absence of 8 μM CuB for 24 h. Next, equal amounts of protein were analyzed by western blot analysis. (C) The apoptotic effects of CuB, AG490 and IL-6 on SH-SY5Y cells were detected by flow cytometry. The values are provided as the mean ± SD of three independent experiments. Significant differences are indicated by **P<0.01 versus control and $^{+}$P<0.05 versus CuB. CuB, cucurbitacin B; p-JAK2, phosphorylated Janus kinase 2; STAT3, signal transducer and activator of transcription 3; IL-6, interleukin 6; SD, standard deviation.

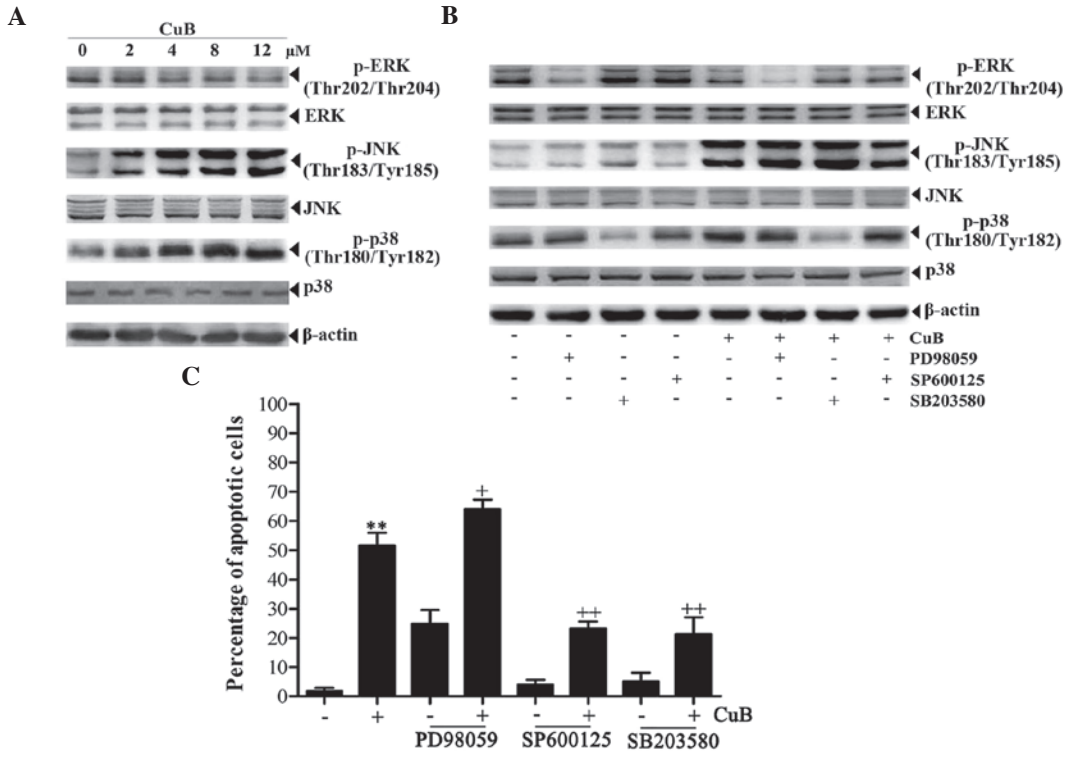

Figure 4. Effects of CuB on the MAPK signaling pathway. (A) The cell lysates from SH-SY5Y cells treated with CuB for 24 h were analyzed by western blot analysis using antibodies against p-ERK, ERK, p-JNK, JNK, p-p38 and p38, and β-actin was used as a loading control (bottom panel). (B) The cells were preincubated with or without inhibitors of signaling molecules for 1 h and further incubated in the presence or absence of 8 μM CuB for 24 h. The concentrations of the inhibitors are as follows: PD98059, 100 μM; SP600125, 20 μM and SB203580, 20 μM. Next, equal amounts of protein were analyzed by western blot analysis. (C) The apoptotic effects of the inhibitors on SH-SY5Y cells were detected by flow cytometry. The values are provided as the mean ± SD of three independent experiments. Significant differences are indicated by **P<0.01 versus control and $^{+}$P<0.05, $^{++}$P<0.01 versus CuB. CuB, cucurbitacin B; MAPKs, mitogen-activated protein kinases; p-ERK, phosphorylated extracellular signal-regulated kinases; p-JNK, phosphorylated c-Jun N-terminal kinases; SD, standard deviation.

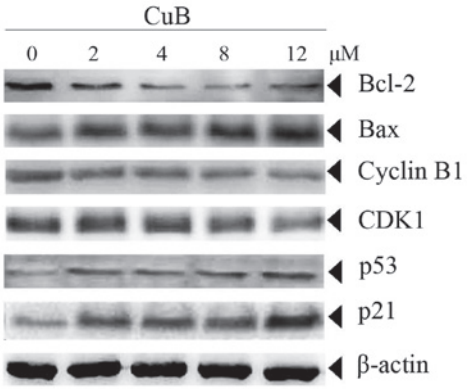

Figure 5. Effects of CuB on the expression of proteins involved in proliferation and apoptosis. The cells were treated with the indicated concentrations of CuB for 24 h. Next, equal amounts of protein were analyzed by western blot analysis using antibodies against Bcl-2, Bax, Cyclin B1, CDK1, p53 and p21. β-actin was used as a loading control (bottom panel). CuB, cucurbitacin B; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; CDK1, cyclin-dependent kinase 1.

was determined. Western blot analysis and flow cytometry were respectively employed to detect protein expression and apoptosis with or without AG490 or IL-6 pretreatment. As expected, the protein levels of p-JAK2 and p-STAT3 were regulated by AG490 and IL-6, while the total protein levels of JAK2 and STAT3 did not exhibit any evident change. Compared with CuB treatment alone, the protein levels of p-JAK2 and p-STAT3 were markedly decreased and the rate of apoptotic cells was markedly increased following the treatment of CuB and AG490. However, the alterations induced by CuB could be attenuated partially by IL-6 (Fig. 3B and C).

CuB induces JNK and p38 MAPK activation and ERK inactivation in SH-SY5Y cells. Western blot analysis was performed to determine whether CuB affects the activation of MAPK cascades, including ERK, JNK and p38 MAPK in NB cells. As a result, CuB upregulated p-JNK and p-p38 MAPK, and downregulated p-ERK in SH-SY5Y cells (Fig. 4A). Therefore, PD98059, SB203580 and SP600125, which are specific inhibitors of ERK, p38 MAPK and JNK, respectively, were used to examine the role of the MAPK signaling pathway in CuB-treated cells. Upon pretreatment with the inhibitors, the protein levels of p-ERK, p-JNK and p-p38 MAPK were all decreased, and the decrease in p-ERK expression was significant compared with CuB treatment alone. ERK, JNK and p38 MAPK did not reveal evident changes (Fig. 4B). Apoptosis analysis by flow cytometry revealed that the ERK inhibitor PD98059 increased the percentage of apoptotic cells induced by CuB, whereas the rates of apoptotic cells induced by CuB were significantly abrogated by the p38 MAPK and JNK inhibitors (Fig. 4C).

CuB alters expression of proteins involved in proliferation and apoptosis. Bcl-2 and Bax have been implicated in apoptosis and mitochondrial dysfunction. For this reason, the effects of CuB on the expression of these two proteins were investigated. The data in Fig. 5 demonstrated that CuB downregulated the anti-apoptotic Bcl-2 and upregulated pro-apoptotic Bax in a concentration-dependent manner in SH-SY5Y cells. In addition, cell cycle proteins linked with the G2/M phase, including cyclin B1 and CDK1, were also downregulated by CuB in a dose-dependent manner. In multi-cellular organisms, p53 is involved in the prevention of cancer. It acts as a tumor suppressor and was reported to regulate the cell cycle through the control of the expression of cyclin-dependent kinase inhibitor p21 (20). The results indicated that p53 and p21 were also upregulated by CuB in a dose-dependent manner in SH-SY5Y cells (Fig. 5).

Discussion

CuB is known for its ability to suppress the proliferation and induce apoptosis in a wide variety of cancer cell lines, and the mechanisms of CuB action differ among different cancer cell lines. Identifying the molecular targets of an agent is very important in the selection of anticancer agents with few side effects on normal cells. The results of the present study indicated that CuB could induce cell growth inhibition by G2/M phase arrest and apoptosis.

JAK/STAT3 is the major anti-apoptotic pathway for the transduction of a multitude of signals which are critical for the development and homeostasis in mammals (21). JAK activation has a significant role in cell proliferation, differentiation, migration and apoptosis (21,22). Constitutive activation of STAT3 has a critical role in cell growth and survival in human solid tumor malignancies (23-25) and the upregulation of the anti-apoptotic proteins in human cancer cells (23,26). JAK2/STAT3 signaling has been extensively validated as a novel molecular target for the agents against human solid tumors (27-29). In the present study, it was observed that CuB inhibited the JAK2/STAT3 signaling pathway by markedly downregulating p-JAK2 and p-STAT3 protein expression. It can be concluded that CuB may act as a JAK2/STAT3 inhibitor in SH-SY5Y cells.

MAPKs are serine-threonine protein kinases and they have a significant role in the regulation of numerous cellular processes, including cell growth and proliferation, differentiation and apoptosis (30,31). MAPKs consist of growth factor-regulated ERKs, JNKs, p38 MAPK and ERK5 (32). ERK, the most widely studied MAPK cascade, has been shown to be a major participant in the regulation of cell growth and differentiation, and the activation of JNK and p38 MAPK signaling cascades generally result in apoptosis (13,30,33). In the present study, CuB was found to activate JNK and p38 MAPK and inactivate ERK in NB cells in a concentration-dependent manner. It can be concluded that CuB may also act as a MAPK regulator in SH-SY5Y cells.

Bcl-2 and Bax belong to the Bcl-2 family and have a significant role in cell apoptosis. Bcl-2 and Bax exhibit anti- and pro-apoptotic activities (34). The results of the present study revealed that Bcl-2 and Bax were downregulated and upregulated, respectively, and further prompted apoptosis. Flow cytometric analysis revealed that CuB induced cell cycle arrest in the G2/M phase. Cyclin B1 and CDK1 are linked to the G2/M phase progress. Consistent with the results of flow cytometry, it was found that CuB inhibited the expression of Cyclin B1 and CDK1 in a dose-dependent manner. p53 and p21 are anticancer proteins that can induce apoptosis, inhibit Bcl-2 and cellular inhibitor of apoptosis proteins and activate

the activities of pro-apoptosis proteins. In addition, the tumor suppressor p53 and its downstream target p21 have been shown to induce cell cycle arrest since they are potent cyclin-CDK inhibitors (35).

The present study demonstrated that human NB SH-SY5Y cells undergo apoptosis in response to treatment with CuB, which occurs through the JAK2/STAT3 and MAPK signaling pathways, and the regulation of gene products that mediate tumor cell survival, proliferation and apoptosis. The present study also reveals that CuB can possibly be used as a novel potent therapeutic agent against NB. However, since all these results were obtained from *in vitro* experiments, *in vivo* studies are required in order to validate these results for the therapeutic use of this agent in humans.

Acknowledgements

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